Jpn. J. Cancer Res. 86, 460–469, May 1995

Establishment and Characterization of Non-small Cell Lung Cancer Cell Lines Resistant to Mitomycin C under Aerobic Conditions

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To elucidate the mechanisms of acquired resistance to mitomycin C (MMC) in non-small cell lung cancer (NSCLC), we established two MMC-resistant NSCLC sublines by continuous exposure to MMC, using PC-9 as a parent cell line. The sublines, PC-9/MC2 and PC-9/MC4, were 6.4- and 10-fold more resistant to MMC than their parent cell line, respectively, at the IC_{50} value as determined by MTT assay. They exhibited cross-resistance to EO9, but were not resistant to cisplatin, vindesine, etoposide, carboquone, or KW-2149, a novel MMC derivative. They were collaterally sensitive to adriamycin and menadione. Accumulation of the drug was decreased in the resistant sublines to about 60% of that in the parent cells. Cytosolic DT-diaphorase (DTD) activities were decreased to 13.5±3.2 in PC9/MC2 and 1.3±0.6 in PC-9/MC4 from 261.5±92.7 nmol/min/mg protein in the parent PC-9. NADH:cytochrome b_5 reductase activities in both of the resistant cell lines were significantly decreased as compared to that in the parent cell line. Addition of dicumarol resulted in a two-fold increase in IC_{50} value in PC-9, whereas the IC_{50} value showed no change in PC-9/MC4. Moreover, dicumarol did not affect the sensitivities to KW-2149 but decreased the sensitivities to EO9 in both the parent and the resistant cell lines. Formation of an alkylating metabolite was significantly decreased in the resistant cells, in parallel to the degree of resistance. We concluded that deficient drug activation due to decreased DTD activity was important as a mechanism of resistance to MMC in PC-9, a relatively DTD-rich NSCLC cell line.

Key words: Mitomycin C — EO9 — KW-2149 — DT-diaphorase — Dicumarol

MMC[4] is one of the most active drugs against NSCLC among currently available chemotherapeutic agents,[1)] but its efficacy may be limited at least in part due to natural and acquired resistance, as in the case of many other agents. The mechanisms of MMC resistance, investigated in some mammalian cell lines and human breast and colon cancer cell lines *in vitro,* include alteration of drug efflux mediated by P-glycoprotein,[2)] increased DNA repair,[3)] enhanced detoxification of active metabolites by GSH and GST[4,5)] and impaired drug activation.[3,6–8)] MMC, which is known to be a bioreductive alkylating agent,[9)] requires reductive activation in its quinone moiety prior to exerting its cytotoxic activity.[1,10)] Although the mechanisms of MMC bioreduction are not fully understood,[11)] two pathways may be involved in activating the drug[1,10)]; these are the one-electron reduction pathway to generate a semiquinone radical[12,13)] and the obligate two-electron reduction pathway leading to more stable hydroquinone generation[14–17)] mediated by enzymes including NAD(P)H: quinone oxidoreductase [EC 1.6.99.2], also known as DT-diaphorase (DTD).[18,19)] Under anaerobic conditions, NADPH:cytochrome P-450 reductase and xanthine oxidase were demonstrated to be involved in activation via the one-electron reduction pathway.[12,13)] However, findings on the bioreduction of MMC under aerobic conditions has been conflicting.[20–22)] Bligh *et al.* reported involvement of NADPH:cytochrome P-450 reductase in the aerobic reduction in Chinese hamster ovary cell lines and human mammary carcinoma cell lines.[23)] On the other hand, several investigators have reported the importance of the two-electron reduction pathway mediated by DTD in the aerobic activation of MMC in some mammalian and human cell lines.[6,14,17,24,25)] In addition, Gustafson and Pristos demonstrated two-electron reduction of MMC by xanthine dehydrogenase in mouse mammary cell carcinoma EMT6 cells under aerobic conditions.[15)] There is also

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[4] The abbreviations used are: MMC, mitomycin C; NSCLC, non-small cell lung cancer; DTD, DT-diaphorase; PBS, calcium-free and magnesium-free Dulbecco's phosphate-buffered saline; ACNU, 3-[(4-amino-2-methyl-5-pyrimidinyl)-methyl]-1-(2-chloroethyl)-1-nitrosourea; EO9, 3-hydroxymethyl-5-aziridinyl-1-methyl-2-(H-indole-4,7-indione)propenol; DIC, dicumarol; KCN, potassium cyanide; DMSO, dimethyl sulfoxide; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; GSH, glutathione; GST, glutathione *S*-transferase; PNBP, 4-(*p*-nitrobenzyl)pyridine.

some evidence that DTD is closely related to the aerobic cytotoxicity of MMC.[3, 8, 26, 27]

We previously reported the establishment of an MMC-resistant NSCLC cell line with decreased DTD activity.[28] In the present paper, we present the results of experiments using two MMC-resistant cell lines with different degrees of resistance.

MATERIALS AND METHODS

Drugs and chemicals RPMI 1640 medium and PBS were purchased from Nissui Pharmaceutical Co., Tokyo. MMC, adriamycin, 7-N-[2-[[2-(γ-L-glutamylamino)-ethyl]dithio]ethyl]MMC (KW2149) and EO9 were kindly supplied by Kyowa Hakko Kogyo Co., Ltd., Tokyo. [^{3}H]MMC was synthesized by Amersham International (Buckinghamshire, England) by the reported method[29] and was a generous gift from Dr. Kengo Inoue, Kyowa Hakko Kogyo Co., Ltd. CDDP and etoposide were obtained from Nihon Kayaku Co., Ltd., Tokyo; carboquone and ACNU were from Sankyo Co., Ltd., Tokyo. Vindesine was supplied by Lilly Research Laboratories, Indianapolis, IN. 2-Methyl-1,4-naphthoquinone (menadione), DIC, Tween 20, KCN and DMSO were purchased from Wako Pure Chemical Industries, Ltd., Osaka. All other drugs and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise specified.

Cell lines and culture PC-9, a human NSCLC cell line, was used as the parental cell line in this study. This cell line was established by Dr. Y. Hayata, Tokyo Medical College, from human adenocarcinoma of the lung and kindly provided by him. The cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco Laboratories, Grand Island, NY), 100 μg/ml streptomycin, and 100 units/ml penicillin in an incubator under a humidified atmosphere of 5% CO_2 and 95% air at 37°C. PC-9/MC2 was selected by continuous exposure of PC-9 cells to stepwise increasing MMC concentrations from 0.01 μg/ml (0.03 μ*M*) up to 0.02 μg/ml (0.06 μ*M*) for 6 months, and the resistant cell line was isolated by the limiting dilution method. PC-9/MC2 was further exposed to MMC at a concentration of 0.04 μg/ml (0.12 μ*M*) for about 4 months and isolated by the same method, leading to the establishment of the PC-9/MC4 cell line. The resistant cell lines, PC-9/MC2 and PC-9/MC4, were exposed to 0.02 and 0.04 μg/ml of MMC, respectively, for 1 year. Thereafter, the cells were cultured in MMC-free medium for at least 1 month before experimental use under the same conditions as the parent cells. Growth curves for these cell lines were obtained by seeding them in 25 cm^2 plastic culture flasks at a concentration of 3×10^4 cells in 10 ml of medium, followed by incubation under the above-mentioned conditions without medium change. After the incubation period, viable cells were counted by the trypan-blue exclusion method.

Drug sensitivity test Drug sensitivities of cell lines to various antitumor agents were determined by MTT assay[30] with some modification. Exponentially growing cells were harvested and suspended in fresh medium. Cell suspensions adjusted to 2×10^4 cells/ml were seeded into 96-well flat-bottomed culture plates (Falcon 3072, Becton Dickinson and Co., Lincoln Park, NJ) at 2,000 cells/well and incubated for 2 h at 37°C before addition of the drug. After incubation with various concentrations of drugs for 96 h, MTT dissolved in PBS at 5 mg/ml was added to each well at 20 μl/well and the plates were incubated at 37°C for 4 h. After centrifugation at 2,000 rpm for 10 min, the supernatant was carefully aspirated and 200 μl of DMSO was added to each well to dissolve the formazan crystals, followed by shaking for 5 min. Then the absorbance of each well at 560 nm was measured with 660 nm as a reference using a scanning microplate spectrophotometer (EAR 340 AT, SLT, Vienna, Austria). Each experiment was performed in triplicate at least three times independently. The degree of drug sensitivity of each cell line was expressed as the IC_{50} value, defined as the drug concentration inhibiting cell growth by 50% compared to the control wells, determined graphically from the dose-response curve.

Drug accumulation study The cells in a subconfluent state were harvested and trypsinized to afford a single cell suspension. The cells were preincubated at 37°C for 2 h in 15-ml centrifuge tubes. Drug solution was prepared by dilution of [^{3}H]MMC (specific radioactivity 44 Ci/mmol) with the cold drug dissolved in fresh medium and was added to give a final drug concentration of 10 μ*M*. After the intended incubation period, the cells were washed with cold PBS twice. The pellets were lysed with 1 *N* NaOH solution at room temperature overnight and neutralized with an equal volume of 1 *N* HCl, then ACSII scintillator (Amersham) was added and the radioactivity was measured with an Aloka LSC-700 liquid scintillation counter (Arlington Heights, IL).

Assay of enzyme activities The method of Ernster *et al.*[18] as modified by Benson *et al.*[31] was used in the assay of DTD activity. Sonicates of 5×10^6 cells in 500 μl of 25 m*M* Tris-HCl (pH 7.4) were used as the enzyme source. Aliquots of cell sonicate were added to the reaction mixture consisting of 25 m*M* Tris-HCl, pH 7.4, 0.07% bovine serum albumin, 0.01% Tween 20, 0.2 m*M* NADPH, and 5 μ*M* FAD, with or without 20 μ*M* DIC, in a final volume of 1 ml. The reaction was initiated by the addition of 2,6-dichlorophenolindophenol (DCPIP) at the final concentration of 40 μ*M*, and the decrease of optical density at 600 nm was recorded for 10 min. DTD activities were expressed as the DIC-sensitive part

of the rate of DCPIP reduction, based on an extinction coefficient of 2.1×10^4 liter/mol/cm. In the assay for NADPH:cytochrome P-450 reductase activity or NADH:cytochrome b_5 reductase activity, the method of Phillips and Langdon[32] or Yubisu and Takeshita[33] was followed, respectively.

Western blot hybridization for DTD content The content of DTD was determined by Western blot analysis using mouse anti-human DTD monoclonal antibody, KM1015, raised and purified by T. Tsuruo and Kyowa Hakko Kogyo Co., Ltd. The cell cytosol containing 0.3 to 30 μg protein was applied to 10%/20% gradient polyacrylamide gels containing sodium dodecyl sulfate. Following electrophoresis, the protein on the gels was electrophoretically transferred to PVDF membranes (Millipore Ltd.). The membranes were immersed and incubated with gentle agitation in 0.1% (v/v) Tween 20 in 100 m*M* Tris-buffered saline (TTBS) for 1 h with a buffer change. Then the membranes were transferred to and incubated for 30 min in TTBS containing anti-DTD monoclonal antibody at a concentration of 5 μg/ml, followed by rinsing. All rinsing was done for 45 min with three changes of TTBS buffer. The blots were developed using a Vectastain Elite ABC kit and 3,3′-diaminobenzidine-nickel substrate kit (Vector, Burlingame, CA) following the manufacturer's instructions. The reactions were stopped by rinsing with water.

DIC treatment The effect of DIC treatment on the sensitivity to MMC and other related compounds was examined as follows. DIC was dissolved in 0.5 *N* NaOH and diluted with the medium to the required concentration. The final concentration of NaOH was adjusted to 0.01 *N*. Preliminary data indicated that the final concentration of 500 μM was the maximum non-cytotoxic dose of DIC when the cells were exposed to DIC for 1 h (data not shown). Therefore, this dose was used in the experiments. Sensitivity tests were performed in the 1 h drug exposure design. In summary, 50 μl of DIC solution or vehicle alone was placed in a well of a 96-well V-bottomed plastic plate (Costar, Cambridge, MA), followed by addition of an equal volume of the appropriate concentration of the drug solution. Then, 100 μl of the cell suspension was seeded into each well and mixed by pipetting. After incubation for 1 h under 95% air/5% CO_2, cells were pelleted by centrifugation and the drug-containing medium was aspirated. The cells were washed with the fresh medium and collected by centrifugation. The pellets were resuspended in 200 μl of fresh medium and aliquots of the cell suspension were transferred to the wells of a flat-bottomed plate. After incubation for 96 h, MTT assay was performed. The dose-response curve was plotted from the data of three independent duplicate experiments and the IC_{50} value was obtained graphically.

Measurement of alkylating metabolite The formation of alkylating metabolites of MMC by the cell sonicates was assayed by the method of Kennedy *et al.*[34] with some modification. The subconfluent cells were harvested from a 75 cm^2 plastic culture flask and sonicated in 10 m*M* pottasium phosphate buffer (pH 6.6). The reaction mixture included 1 m*M* NADPH, 1 m*M* NADH, 0.1% PNBP, 0.6 m*M* MMC and an aliquot of cell sonicate in a total volume of 1 ml. The protein content of the cell sonicates in the reaction mixture ranged from 1.1 to 2.0 mg. PNBP and MMC were dissolved in reagent-grade acetone. The reaction was initiated by adding MMC, and the reaction mixture was incubated at 37°C for 1 h. After incubation, the tubes were placed on ice and the reaction was terminated by the addition of 2 ml of acetone followed by the addition of 1 ml of 1 *M* sodium hydroxide for color development. The colored adduct was immediately extracted into 4 ml of HPLC-grade ethyl acetate and after centrifugation at 1000*g* for 2 min, the absorbance at 540 nm of the ethyl acetate phase was determined by spectrophotometer. The amount of the alkylating metabolite was expressed as the absorbance per mg protein.

Protein determination Protein was measured by using the bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, IL).

Statistical analysis Results were expressed as the mean ±SD. Statistical difference was determined by unpaired Student's *t* test, with a *P* value of less than 0.05 as the criterion of significance.

RESULTS

Characterization of MMC-resistant PC-9 cells Doubling times of PC-9 and its MMC-resistant sublines, PC-9/MC2 and PC-9/MC4, were 20 h, 24 h and 23 h, respectively. The protein contents of the cell lines did not differ significantly (data not shown). The comparative sensitivities of PC-9, PC-9/MC2 and PC-9/MC4 cells to MMC are shown in Fig. 1. The IC_{50} value of PC-9/MC2 was 0.61 μM and that of PC-9/MC4 was 0.96 μM, while that of the parental line was 0.095 μM. PC-9/MC2 and PC-9/MC4 were 6.4- and 10-fold more resistant to MMC than PC-9, respectively. The resistance was stable for at least 4 months. Table I shows the sensitivities to various agents of the three cell lines. KW-2149, a newly synthesized MMC derivative with a disulfide side chain at the 7-N position, exhibited no significant cross-resistance in the MMC-resistant cell lines, though a novel indoloquinone agent, EO9, showed obvious cross-resistance. The degrees of resistance to the latter agent were almost equal to those to MMC. The resistant cell lines, PC-9/MC2 and PC-9/MC4, were 1.5- and 4.0-fold more sensitive to adriamycin, respectively. The resistant cell lines were

more sensitive to the toxicity of menadione than PC-9. PC-9/MC4 was also significantly more susceptible to ACNU, but the ratio of sensitization was small. Cross-resistance was not observed to the other agents examined in our study, including carboquone, an alkylating agent structurally mimicking MMC.

Drug accumulation Incorporation of the drug into the cell was significantly different between the parent cell line and either of the resistant sublines (Fig. 2). The influx rate of MMC was approximately 1.5-fold greater in the sensitive cells than in the resistant cells up to 60 min of incubation time. The rate of drug efflux was slightly but significantly increased in the resistant cell lines (Fig. 2B).

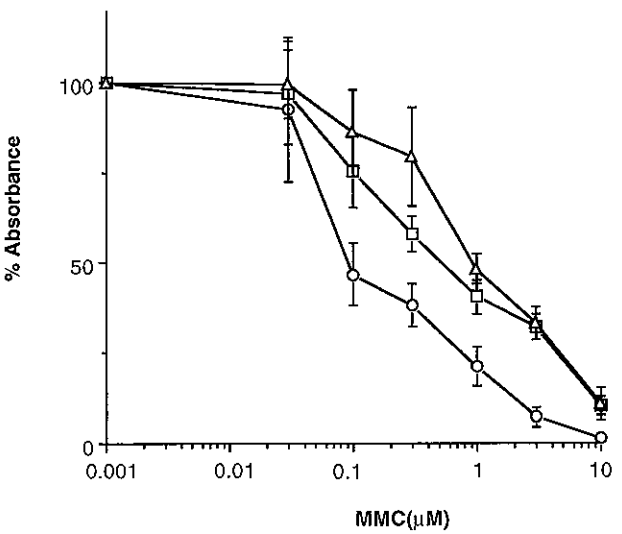


Fig. 1. Growth inhibition curve of PC-9 cell lines upon 96 h exposure to MMC. Surviving fractions were determined by MTT assay as described in "Materials and Methods." Points and bars indicate mean and SD of at least three independent triplicate experiments. ○, PC-9; □, PC-9/MC2; △, PC-9/MC4.

DTD and other flavoenzyme activities Determinations of DTD activity in cell sonicates showed significant differences among the cell lines (Table II). A twenty-fold decrease in the enzyme activity in PC-9/MC2 and a 200-fold decrease in PC-9/MC4 were observed as compared with PC-9. NADH:cytochrome b_5 reductase activity was decreased in both resistant cell lines to one-third of that in the parent cell line. Activities of NADPH:cytochrome P-450 reductase in the cell cytosol up to 5 mg of protein content were below the limit of detection (lower than 0.1 nmol/min/mg protein) in all the cell lines.

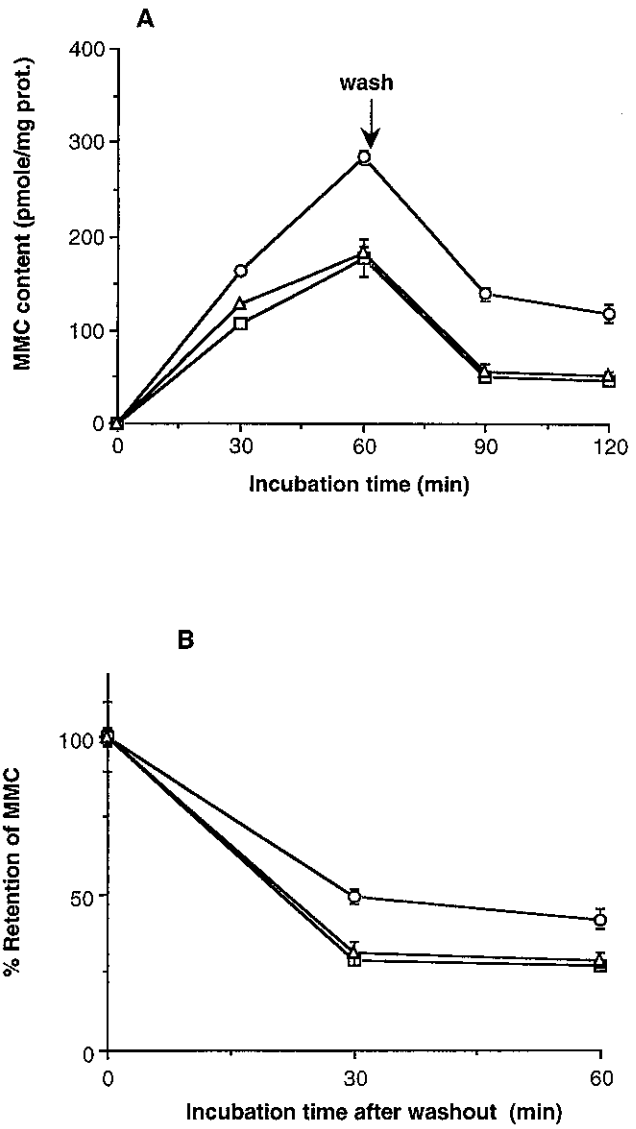
DTD content estimated by Western blot analysis To determine whether the reduced activity of DTD was caused by a reduction in its cellular content, the amount of DTD in the cell sonicate was measured by immunoblotting using monoclonal anti-human DTD antibody. When 1.5 μg of protein was loaded onto each lane, an immunoreactive 33-kD protein was visualized only on the lane of PC-9 as shown in Fig. 3. We could consistently recognize the band when the sample was diluted to 0.3 μg protein per lane, while the protein contents needed for visualization of the bands were 5 μg and 30 μg per lane for PC-9/MC2 and PC-9/MC4, respectively (data not shown). These results indicated that the reduction in DTD protein content was responsible for the reduction of the activity in the resistant cells.

Effect of DIC treatment on MMC sensitivity When the cells were exposed to MMC for 1 h, PC-9/MC4 was 4.6-fold more resistant to the drug than PC-9. Co-incubation of the cells with 500 μM DIC lowered the

Table I. Sensitivities to Various Anticancer Agents of PC-9 Cell Lines

	IC_{50} values (μM)[a]				
	PC-9	PC-9/MC2	(RR)[c]	PC-9/MC4	(RR)
MMC	0.095±0.003[b]	0.61±0.04[d]	(6.4)	0.96±0.08[d, e]	(10)
EO9	0.032±0.015	0.22±0.06[d]	(6.8)	0.31±0.08[d]	(10)
KW2149	0.10±0.05	0.11±0.04	(1.1)	0.14±0.04	(1.4)
Carboquone	0.017±0.009	0.024±0.010	(1.4)	0.026±0.010	(1.5)
Adriamycin	0.40±0.03	0.26±0.03[d]	(0.65)	0.10±0.02[d, e]	(0.25)
Menadione	17±3	9.8±3.8[d]	(0.57)	7.3±1.1[d]	(0.42)
CDDP	1.3±0.4	1.9±0.7	(1.5)	1.7±0.6	(1.3)
ACNU	420±50	320±120	(0.76)	240±60[d]	(0.57)
Vindesine	0.020±0.010	0.021±0.010	(1.0)	0.016±0.007	(0.80)
Etoposide	47±28	67±25	(1.4)	34±16	(0.74)

a) Drug concentration that inhibits cell growth by 50% upon continuous drug exposure for 96 h.
b) Each value is the mean±SD of at least three independent experiments.
c) Relative resistance value: IC_{50} value of resistant cells/IC_{50} value of parental cells.
d) $P<0.05$ as compared to IC_{50} value of PC-9.
e) $P<0.05$ as compared to IC_{50} value of PC-9/MC2.



sensitivity to MMC in PC-9, but did not alter that in PC-9/MC4 (Table III, Fig 4A). The IC_{50} value for 1 h exposure to MMC of the parent cells was elevated from 12 μM without DIC to 24 μM with DIC. The sensitivity to EO9 was more drastically affected by DIC: 37- and 5.6-fold increases in the IC_{50} values of PC-9 and PC-9/MC4 were observed, respectively. The dose-response curves of PC-9 and PC-9/MC4 with DIC were rather similar (Fig. 4B). In contrast, cytotoxicity of KW-2149 was unaffected by DIC in PC-9 and was slightly enhanced in PC-9/MC4, as shown in our previous report.[28]

Formation of alkylating metabolite The rate of metabolite formation was significantly decreased in the resistant cell lines as compared to the parent cells (Fig. 5). There was also a significant difference between the formation of alkylating metabolites by PC-9/MC2 and by PC-9/MC4. The level of the metabolite production showed a good correlation with the susceptibility to MMC and the cytosolic DTD activity.

DISCUSSION

In the previous paper, we reported the establishment of an MMC-resistant NSCLC cell line under aerobic condi-

Fig. 2. A. Uptake and efflux of MMC in PC-9 cell lines. The cells (5×10^6) were cultured with 10 μM [^{3}H]MMC for the indicated time. Efflux was evaluated by washing the cells with warm fresh medium after 60-min drug exposure; they were then resuspended and incubated in drug-free medium for up to 120 min after addition of the drug. The radioactivity of the cells was measured and mean values±SD of three determinations are shown. ○, PC-9; □, PC-9/MC2; △, PC-9/MC4. B. Retention of MMC after washout was expressed as the percentage of retained radioactivity, with the value at 60-min drug exposure taken as 100%.

Table II. Activating Enzymes of PC-9 Cell Lines

	PC-9	PC-9/MC2	PC-9/MC4
Relative resistance	1	6.4	10
DT-diaphorase activity (nmol/min/mg protein)	261.5±92.7	13.5±3.2[a]	1.3±0.6[a, b]
NADPH:cytochrome P-450 reductase activity (nmol/min/mg protein)	ND[c]	ND	ND
NADH-cytochrome b_5 reductase activity (μmol/min/mg protein)	2.23±0.30	0.68±0.17[a]	0.73±0.08[a]

a) $P<0.001$ as compared to the value of PC-9.
b) $P<0.001$ as compared to the value of PC-9/MC2.
c) ND denotes not detected.

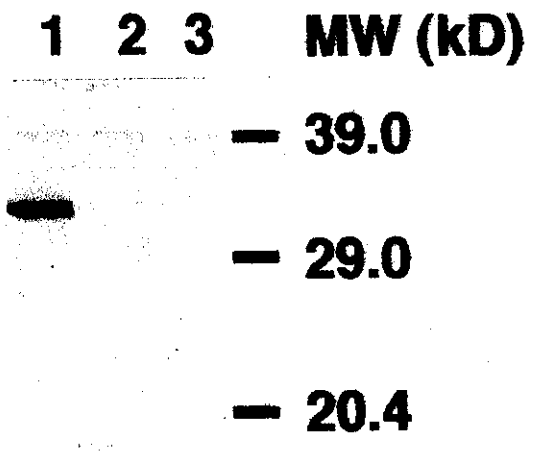


Fig. 3. Western blot analysis of DT-diaphorase content of cell sonicates from PC-9 (lane 1), PC-9/MC2 (lane 2) and PC-9/MC4 (lane 3) cells. A 1.5 μg aliquot of protein was loaded onto each lane.

Table III. Effect of DIC on the Sensitivity to MMC and Related Compounds

	IC_{50} (μM)		Relative resistance[a]
	PC-9	PC-9/MC4	
MMC			
1 h exposure	12	55	4.6
+ 500 μM DIC	24	48	2.0
Protection ratio[b]	2.0	0.87	
KW-2149			
1 h exposure	4.8	5.4	1.1
+ 500 μM DIC	4.8	3.0	0.63
Protection ratio	1.0	0.56	
EO9			
1 h exposure	1.1	11.6	11
+ 500 μM DIC	41	65	1.6
Protection ratio	37	5.6	

a) (IC_{50} of PC-9/MC4)/(IC_{50} of PC-9).
b) (IC_{50} with DIC)/(IC_{50} without DIC).

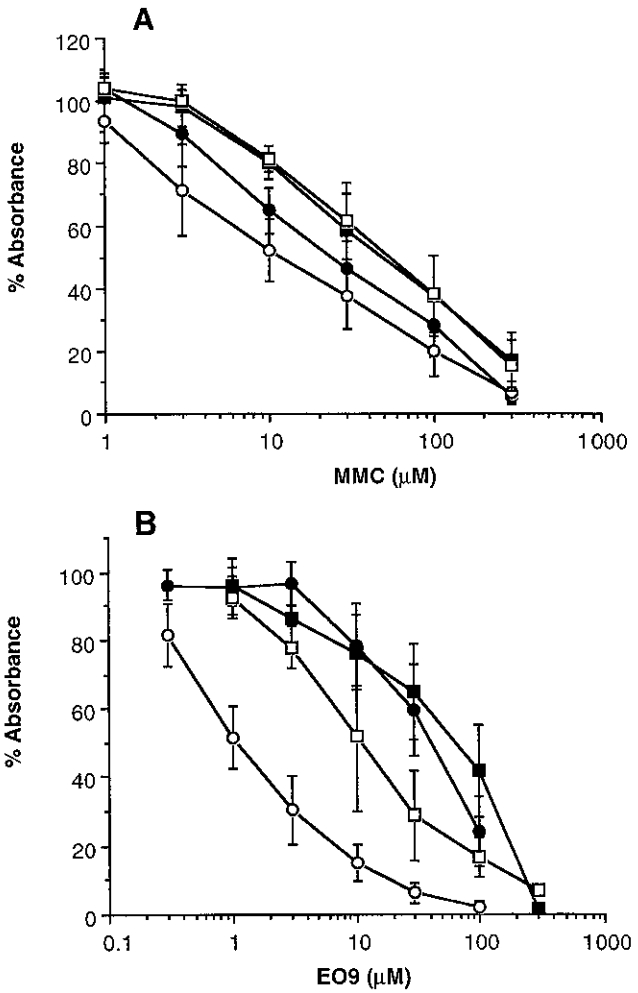


Fig. 4. Effect of DIC treatment on the sensitivity to MMC (A) and EO9 (B). PC-9 (circles) and PC-9/MC4 (squares) were exposed to each drug for 1 h with (closed symbols) or without (open symbols) 500 μM DIC and growth inhibition were measured by MTT assay following 96-h incubation. Points and bars indicate mean and SD of three independent duplicate experiments.

tions and suggested that decreased DTD activity was an important factor inducing MMC-resistance.[28] In the present study, we included the less-resistant cell line, PC-9/MC-2, in the experiments and further investigated the relationship between the enzyme activity and the drug sensitivity.

We observed a marked decrease in cytosolic DTD activity due to decreased protein content of the enzyme in the two resistant cell lines. There was a clear relationship between the drug sensitivity and the enzyme activity among the three cell lines with different sensitivity to MMC. Furthermore, the formation of the alkylating metabolite of MMC in the cell-free system was closely related to the DTD activity and the drug sensitivity. NADH:cytochrome b_5 reductase activity was decreased in the resistant cell lines as compared with the parent cell line, but there was no difference in this enzyme activity between the two cell lines with different degrees of resistance.

Drug accumulation was significantly decreased in the resistant cells to about two-thirds of that in the parent cells. Although this decrease in the drug accumulation may explain some part of the resistance, the difference was too small to explain the several-fold increase of

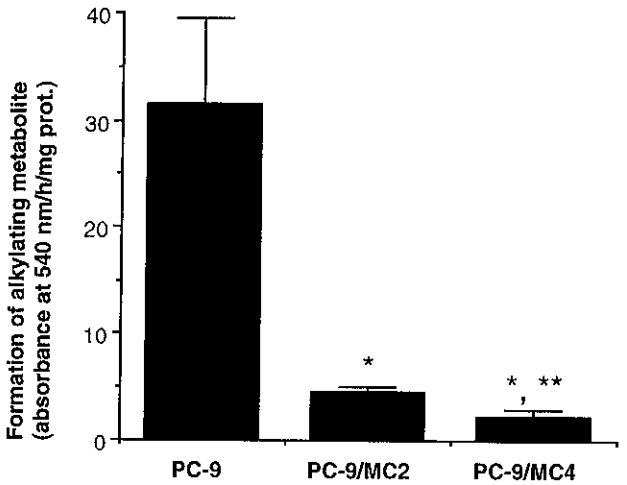


Fig. 5. The amounts of alkylating metabolites after 1 h incubation were 31.5±7.9 by PC-9, 4.5±0.6 by PC-9/MC2 and 2.3±0.6 by PC-9/MC4. Columns and bars indicate mean and SD of four determinations. *; $P<0.005$ in comparison with PC-9. **; $P<0.05$ in comparison with PC-9/MC2.

resistance[35] and decreased metabolite formation by the cytosol of the resistant cells from a given concentration of MMC indicated that deficient intracellular activation, independent of drug incorporation into the cell, is an important mechanism of resistance. The lack of cross-resistance to other natural products excluded association of a multidrug resistance phenotype. As for detoxification of alkylating species, its involvement in the resistance is unlikely because GSH content or GST activity was not significantly different in the highly resistant subline as compared to the parental cells, as we reported previously.[28]

From these observations, decreased DTD activity and consequent deficient drug activation was the most important factor contributing to the resistance to MMC in PC-9 human NSCLC cell lines. Previous reports by several investigators also referred to decrease in DTD activity as a mechanism of resistance to MMC. Siegel *et al.* reported deficiency of DTD in a naturally MMC-resistant colon carcinoma cell line,[6] and Traver *et al.* showed decreased DTD activity in a MMC-resistant subline of HCT116 colon carcinoma cells.[8] In addition, Dulhanty and Whitmore reported MMC-resistant sublines of Chinese hamster ovary cells with decreased activity of DTD.[25] In our present study, the decrease in DTD activities in the resistant sublines was correlated with the degree of resistance, supporting the importance of DTD as a mechanism of resistance. Furthermore, the resistant cells showed cross-resistance to EO9, a new indoloquinone derivative.[36] This seems natural, because the cytotoxicity of this drug was demonstrated to be DTD-dependent *in vitro* and *in vivo*.[36–39]

In contrast to EO9, a newly synthesized MMC derivative, KW2149,[40,41] circumvented the resistance to MMC in our resistant cell lines. This analogue possesses a long disulfide side chain at the 7-N position of the quinone ring of the original compound. This modification may result in extensive activation under DTD-deficient circumstances,[40] leading to circumvention of MMC-resistance. Lack of protection by DIC from cytotoxicity of KW-2149 is compatible with the DTD-independent activation mechanisms of this analogue, which was recently demonstrated in a cell-free system.[42,43] Another MMC analogue was also reported to overcome resistance to MMC by extensive activation under DTD deficiency.[44,45] These observations suggested that new chemicals synthesized by structural modification of MMC may overcome resistance to MMC.[37] Carboquone is also a bioreductive agent,[46] like MMC, and circumvented resistance to MMC in our study; this might also indicate that the mode of activation of carboquone differs from that of MMC.

The importance of DTD in determining sensitivity to MMC was supported by the results of the experiments using DIC as an inhibitor of DTD. In this study, co-incubation of 500 μM DIC with MMC resulted in an approximately two-fold increase in the IC_{50} value to MMC in PC-9, while no significant change was observed in sensitivity to MMC in PC-9/MC4. Protection from the cytotoxicity of MMC by treatment with DIC under aerobic conditions was reported in human colon cancer cells[6] and in murine lymphoblastic cell lines.[26] Good correlations between DTD activity and sensitivity to MMC were also found in human skin fibroblasts[27,47] and colon cancer cell lines.[8] These data strongly suggested that DTD activity is one of the major determinants of sensitivity to MMC. Moreover, the recent report by Malkinson *et al.* demonstrated that NSCLC cells possess elevated DTD activity as compared to normal lung or small cell lung cancer cells, and a close relationship was observed between DTD level and susceptibility to the cytotoxicity of MMC.[48] Our data further support the importance of DTD in the sensitivity to MMC of NSCLC cells.

However, it is noteworthy that the protective effect of DIC against MMC toxicity was at most partial. DIC has protective effect against MMC cytotoxicity through an unclarified mechanism other than inhibition of DTD in DTD-deficient cells or under anaerobic circumstances.[49] The observed change in the sensitivity may be a sum of this protective effect and the effect of inhibition of DTD activity. Another possible explanation is that the mechanism of resistance to MMC in PC-9/MC4 was multifactorial and that decreased DTD activity was only a

partial contributor. The factors inducing resistance to MMC other than decreased DTD activity might include decreased drug accumulation, decreased NADH:cytochrome b_5 reductase activity, and probably enhanced repair of DNA damage. In the case of EO9, DIC almost completely cancelled the difference of sensitivity between PC-9 and PC-9/MC4, which suggested that the DIC-inhibitable factor was the exclusive mechanism of resistance to this compound. Loss of protective effect of DIC against the toxicity of MMC in PC-9/MC4 may mean that DTD activity is not the major determinant of MMC sensitivity in this cell line with suppressed DTD activity. DTD-dependency of MMC toxicity may be influenced by, at least in part, the abundance of this enzyme in the tumor cytoplasm. In the literature, decrease in either DTD or NADPH:cytochrome P-450 reductase activity resulted in resistance to MMC in Chinese hamster ovary (CHO) cell lines.[7,25] This could be attributable to the relatively low activity of DTD, or weak dependency of MMC toxicity on DTD, in the parent CHO cells. The relationship between DTD activity and DTD-dependency of MMC toxicity needs further investigation.

Menadione, a simple quinone, exhibited an enhanced cytotoxic effect on the resistant cell lines. Physiologically, DTD was shown to protect cells against toxicity to menadione by preventing production of the reactive intermediate.[50] A quinone-resistant lymphoblast cell line had elevated DTD activity and was more sensitive to MMC than its parental cell line.[26] Collateral sensitivity to menadione in MMC-resistant cells can be attributed to defective protection mechanisms against quinone toxicity due to decreased activity of DTD. Mechanisms of sensitization to adriamycin, also containing a quinone moiety, in our MMC-resistant cell lines may be similar to those of collateral sensitivity to menadione.[51]

We have established two MMC-resistant lung cancer cell lines exhibiting decreased activity of DTD. We conclude that DTD activity is the important factor responsible for the acquisition of resistance to MMC in our resistant cell lines, although other factors may also be involved. These cell lines should be useful not only for investigation of the action of MMC in NSCLC cells but also to clarify the role of DTD in the cytotoxicity of MMC and other related compounds, especially bioreductive agents, in tumor cells.

ACKNOWLEDGMENTS

We thank Dr. Nagahiro Saijo, Pharmacology Division of the National Cancer Center Research Institute, Tokyo, Japan, for his advice on the establishment of MMC-resistant cell lines, and Drs. Kengo Inoue and Nobuo Hanai, Kyowa Hakko Co., Ltd., Tokyo, for providing the drugs and the monoclonal antibodies.

(Received October 24, 1994/Accepted March 1, 1995)

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