

## DT-diaphorase as a Target Enzyme for Biochemical Modulation of Mitomycin C

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### ABSTRACT

We studied a selective enhancement of the mitomycin C (MMC)-induced antitumor effect focusing on the intracellular metabolism by NAD(P)H:quinone oxidoreductase (DT-diaphorase, DTD). The level of cellular DTD activity related well to the degree of MMC-induced DNA total cross links and cell growth inhibition in human cancer cell lines, KB, PH101, SH101 and K562. A DTD inhibitor, dicoumarol (DIC) or flavin adenine dinucleotide (FAD), inhibited the MMC-induced DNA damage and cytotoxicity at a non-toxic concentration. The DTD-mediated MMC activation was pH-dependent, and highest at pH 6 and lowest at pH 8. Although an inverse relationship appeared to exist between DTD activity and MMC efficacy in human xenografts implanted into nude mice and 9 fresh human tumor specimens, the investigation in 3 culture cells, HEC-46, HCC-48 and HCC-50, established from those xenografts, showed that DTD activated MMC in a pH-dependent manner as well as the other cell lines. Significant tumor pH reduction from 7.1 to 6.7 by continuous glucose infusion also increased the MMC-induced tumor growth inhibition in the human tumor xenografts. Thus, we conclude that bioreductive activation by DTD in a pH-dependent manner may be of key importance in the MMC-induced antitumor effect and that an increased MMC efficacy at a reduced pH caused by hyperglycemia may be applied to clinical use as a new manipulation for a biochemical modulation of MMC.

**Key words:** *DT-diaphorase, Mitomycin C, Biochemical modulation, Microenvironmental pH*

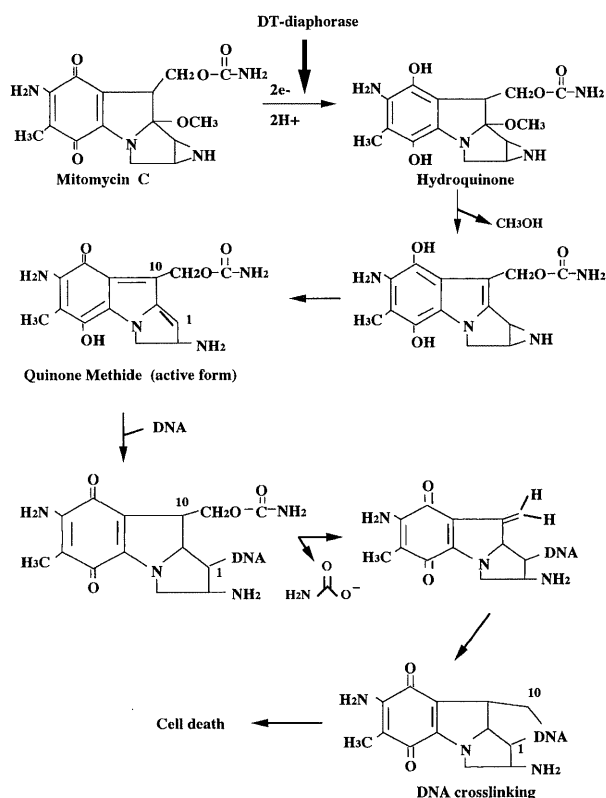
Recent progress in molecular pharmacology has yielded a significant improvement in understanding the complex intracellular biochemistry of anticancer agents such as 5-fluorouracil. The better understanding of the drug action mechanism has made it possible to modulate the therapeutic efficacy of anticancer agents, resulting in a substantial gain in activity without a parallel increase in toxicity<sup>27,28</sup>). The clinical success of biochemical modulation for 5-fluorouracil<sup>1,31</sup>) encouraged us to focus on another key chemotherapeutic agent.

Although Mitomycin C (MMC) has been a mainstay of therapy for patients with gastrointestinal carcinoma<sup>3,6</sup>), the therapeutic index has not been improved remarkably due to a poor understanding of the action mechanism. Recently, there was a striking finding that NAD(P)H:quinone oxidoreductase (DT-diaphorase, DTD) is involved in the bioreductive activation<sup>29,32</sup>). Since a reactive form of MMC has been well known to be capable of crosslinking DNA<sup>8,16</sup>), DTD may be a key enzyme in MMC action and be a target site for the modulation of MMC efficacy. More recent

reports have suggested that MMC is metabolized in a pH-dependent manner by the two-electron reductase, and MMC efficacy may thus be augmented at a reduced microenvironmental pH<sup>2,30</sup>). In conjunction with a finding that hyperglycemia may reduce tumor pH due to the unique glycolysis in tumor<sup>9</sup>), we may develop a new synergistic therapy for MMC.

Nevertheless, since its discovery, DTD has been considered as a protective enzyme against quinone-induced cytotoxicity<sup>15</sup>). Several reports have supported the hypothesis with proof of an inverse relationship between DTD activity and MMC efficacy<sup>25,26</sup>). The role of DTD in the bioreductive activation of MMC has been the subject of controversy.

The present study was designed to clarify the role of DTD in MMC-induced cytotoxicity and DNA damage, through investigations of human cancer cell lines, human tumor xenografts, and fresh human tumor specimens. We have also attempted to show the possibility of DTD-mediated biochemical modulation of MMC by reducing the microenvironmental pH.



**Fig. 1.** The hypothesis of Mitomycin C (MMC)-induced cytotoxicity.

After MMC is reduced by DT-diaphorase, a rearrangement of MMC occurs with the loss of the methoxy substituent as methanol and the proton assisted aziridine ring opening to generate a reactive quinone methide at the C-1 position. Alkylation of DNA results and subsequent crosslinking occurs after loss of the carbamate generates a reactive imine at the C-10 position of MMC. Cross links result in inhibition of DNA synthesis and cell death.

## MATERIALS AND METHODS

### Drugs

All chemicals were of analytical grade. Mitomycin C (MMC) was provided by Kyowa Hakko Kogyo, Tokyo, Japan. Dicoumarol (DIC), flavin adenine dinucleotide (FAD) and 2, 6-dichlorophenol indophenol (DCPIP) were obtained from Sigma, St. Louis, MO, USA.

### Cells and tumors

The human myelogenous leukemia cell line K562 was kindly provided by Prof. Takashi Tsuruo, Tokyo University, Tokyo, Japan. The human oral epidermoid carcinoma KB was kindly provided by Prof. Shin-ichi Akiyama, Kagoshima University, Japan. They were maintained as previously described<sup>4,17</sup>. The human gastric adenocarcinoma cell line, SH101, and the human

pancreatic adenocarcinoma, PH101, were established from ascites in our laboratory. HEC-46 cell line was established from a xenotransplantable human esophageal cancer line of well-differentiated squamous cell carcinoma, EH-6. HCC-48 and HCC-50 were established from xenotransplantable human colorectal cancer lines of the well differentiated adenocarcinoma, CH-4, CH-5, respectively<sup>35</sup>. These cell lines were incubated in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C and maintained continuously in exponential growth by incubating in RPMI1640 medium with 10% fetal calf serum (FCS), every 3 days<sup>22</sup>. Four human tumor xenografts, SH-6 established from stomach, EH-6 from esophageal, CH-4 and CH-5 from colo-rectal cancers, were sterily transplanted into nude mice. All xenografts were established in our laboratory. The histological types were characterized in SH-6, as moderately differentiated tubular adenocarcinoma, in EH-6, as well differentiated squamous cell carcinoma, and in CH-4 and CH-5, as well differentiated adenocarcinoma<sup>20</sup>. Fresh human tumor and normal tissues were collected from 9 patients at the time of surgery, including 5 gastric, 3 esophageal, 1 breast tumor, and normal adjacent tissues. Each excised tumor specimen was immediately placed in sterile RPMI 1640 medium for cytotoxic assay and in liquid NO<sub>2</sub> for DTD measurement.

### Animals

Male mice of the BALB/c/nunu strain, 4–5 weeks old (CLEA Japan Inc., Tokyo, Japan) were kept under specific pathogen free conditions and used 2 weeks later for experiments.

### Evaluation of MMC efficacy

Exponentially growing culture cells, K562, KB, PH101, SH101, HEC-46, HCC-48, HCC-50, were concentrated to  $5 \times 10^6$  /ml and exposed to indicated concentrations of MMC for 30 min with or without 50  $\mu$ M DIC or 100 mM FAD, an inhibitor of DTD. After two washings in drug-free RPMI1640, the cells were resuspended at a concentration of  $2.5 \times 10^4$  /ml in RPMI1640 medium with 10% FCS and seeded in 24 well plates. After 72 hours incubation in a fresh medium at 37°C, the surviving cells were counted by a Coulter counter and by trypan blue exclusion.

The procedure to evaluate the MMC effect on human tumor xenografts, SH-6, EH-6, CH-4, and CH-5, transplanted into nude mice corresponded to the therapeutic protocol of NCI (Ovejera and Douchens, Contract No. 1-CM-67099-NCI). Briefly, the tumor specimens were fragmented to approximately 2 mm<sup>3</sup> in RPMI1640 medium. They were implanted subcutaneously into the flanks of nude mice with trocars, and the tumors were measured across 2 diameters with sliding calipers. The body weight of each mouse was

taken every 4 days. When the calculated tumor volume had reached 100–300 mm<sup>3</sup>, the treatment was started. MMC was administered daily from day 0 to day 8. The administration doses were 2.8 mg/kg/injection for MMC, 0.1 ml/injection of 100 μM solution for DIC, and 0.1 ml/injection of 100 mM solution for FAD. The implanted tumor sizes were measured every 4 days until day 16. The tumor volume (TV) was calculated according to the equation:  $TV=L \times W^2/2$ , where L is the length and W the width of the implanted tumor. Then the relative tumor volume (RV) was calculated according to the equation:  $RV=V_i/V_0$ , where  $V_i$  is the mean tumor volume at any given time after treatment and  $V_0$  the mean initial tumor volume. Tumor growth inhibition was evaluated by the inhibition rate of the mean increase of RV (1-T/C, %) in the untreated (C) and treated mice (T).

The MMC efficacy in the fresh human tumor specimens was evaluated by original ATP assay with a slight modification<sup>10</sup>). Briefly, 180 μl of dilution, consisting of  $1 \times 10^4$  cells and 20 μl of 10% FCS, was cultivated on a 96-microplate for 72 hours in the presence of 20 μl of the appropriate drug dilution or control medium. One-hundred μl of each sample was transferred to a small tube, then 200 μl of ATP releasing reagent and 200 μl of Luciferin - Luciferase reagent were added for the measurement of the ATP level by a Lumiphotometer TD-400 (Labo Science, Tokyo, Japan). When the ATP level of the controls was higher than 10.0, the assay was defined as being evaluable.

### Alkaline elution assays

Analysis of the DNA damage induced by MMC was performed using alkaline elution techniques as described previously<sup>13</sup>). Briefly,  $3 \times 10^6$ /ml cells were labeled with 0.5 μCi/ml <sup>14</sup>C-thymidine for 20h. In all experiments, internal standards were [<sup>14</sup>C]thymidine-labeled each cell (K562, SH101, PH 101, KB) which was irradiated with 6 Gy of X-rays in the cold. For all cross-linking assays, control and drug-treated cells were also irradiated with 6 Gy. MMC treatments were for 30 min at 37°C at the concentration of 1.5 μg/ml/ $1 \times 10^7$  cells with or without 20 μM DIC or 100 mM/ml FAD. After the irradiation,  $2 \times 10^5$  labeled cells were layered onto polycarbonate membranes of 25mm diameter and 2 μm pore size (Nucleopore, Pleasanton, CA) and lysed with 0.1% SDS, 20mM EDTA and 0.5mg/ml proteinase K at pH10. The DNAs on the filter were eluted with 0.1% SDS, 20mM EDTA(acid form) and tetrapropylammonium hydroxide (Eastman Kodak, Rochester, NY) at pH12.1. Elution was carried out at 0.03ml/min for a total 15h with a highly precise pump with a volume variation of less than 5% (Roller pump RP-MV1, Furue Science Co Ltd, Tokyo). The total cross links index was

calculated according to the equation:  $= \sqrt{(1 - R_0)/(1 - R)} - 1$ , where  $R_0$  and  $R$  are the fractions of DNA retained by 600rad and 600rad with drug treated cells, respectively.

### Measurement of DTD activity

A cytosol fraction was prepared at 0–4°C. Culture cells were grown to confluency under standard conditions. The medium was removed and the cells washed with Hank's balanced salt solution (HBSS) and resuspended with 1 ml of 0.25 M sucrose in ice cold ( $10^7$  cells/ml). The cell suspension was sonicated for 30 sec and then centrifuged at 10,000g for 1hr at 4°C to yield a clear cytosol fraction. Solid tumors were homogenized in 0.25 M sucrose (3.0 ml/g of tissue). After centrifugation at 9,000g for 20 min at 4°C the supernatant fluids were collected, 0.2 vol of 0.1 M CaCl<sub>2</sub> in 0.25 M sucrose was added to each, and the samples were kept on ice for 20 min to yield clear cytosol fractions. Measurement of DTD activity corresponded to Ernster's procedure<sup>5</sup>). Protein determination was made using a Bio-Rad protein assay kit (Bio-Rad Laboratories, CA, USA).

The reaction mixture contained 0.025 M Tris HCl (pH 7.4), 0.7 mg/ml crystalline bovine serum albumin, 0.2 mM NADPH, 0.01% Tween 20, 5 μM FAD, and 40 μM DCPIP. Reactions (3 ml) were performed at 25°C in the presence and absence of 20 μM DIC. DT-diaphorase activity was measured as the DIC sensitivity reduction of DCPIP at 600nm ( $\epsilon=21000M^{-1}cm^{-1}$ ). The velocity of the reduction of DCPIP was measured by spectrophotometry.

### Modulation of microenvironmental pH and measurement

For the cultured cells, a buffer medium was prepared by adding 25 mM HEPES and adjusting it to the appropriate pH with 1N HCl or 1N NaOH. The buffer media were sterilized by filtration and the pH was measured immediately before use to readjust as necessary. The pH of solid tumor was modulated by continuous administration of 50% glucose using a micro-osmotic pump (Alzet model 1003D, ALTA Corporation, Palo Alto, CA) at the rate of 1.0 μl/hr for 72 hrs. The pump was implanted into the back of the neck of nude mice 1 day prior to the drug treatment. The tumor bearing mice were anesthetized with sodium pentobarbital (Nembutal, 20 μg/g body weight; Abbott, Bad Segeberg, Germany), then the skin and fibrous tissue overlying the tumor surface were carefully removed in order not to damage the blood vessels. The pH of the tumors were measured by 18 gauge needle type of microelectrodes (MI-413, Microelectrode, INC, NH, USA).

**Table 1.** DT-diaphorase Activity and Cytotoxicity Induced by Antitumor Quinones

	DT-diaphorase activity (nM/min/mg protein)	Cytotoxic activity (IC <sub>50</sub> , $\mu$ g/ml)			
		MMC		ADR	
		MMC	MMC+DIC	ADR	ADR+DIC
Rat liver	26600*				
KB	8260	0.4	4.9	0.5	0.6
PH101	1934	1.1	3.4	1.8	1.9
SH101	1805	1.6	3.9	2.5	2.5
K562	1796	1.9	4.2	0.3	0.3

\* The number represents the mean of 9 samples which do not differ more than 15%.  
DIC, dicoumarol ; MMC, mitomycin C ; ADR, adriamycin

### Statistical analysis

Differences between the various test groups were determined by Student's t test. The data of pH values in the various groups were presented as a box plot.  $p < 0.05$  was judged to be of statistical significance.

## RESULTS

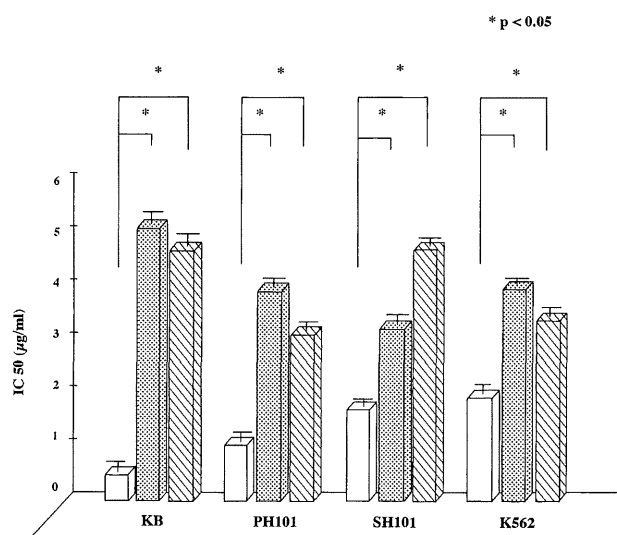
### DTD activity and MMC efficacy in culture cells

In the human culture cell lines, KB cells had a significantly higher DTD activity than PH101, SH101, K562, and were the most sensitive to 30 min treatment with a benzoquinone, MMC. In the presence of a DTD inhibitor, 50  $\mu$ M of DIC, the cellular sensitivity of KB to MMC was decreased to be on a level with the other cells. On the other hand, K562 cells were the most sensitive to the anthraquinone, adriamycin(ADR). DIC treatment did not decrease ADR activity at all in any of the cells (Table 1). These data suggest that DTD may work well in the bioreductive activation of MMC but not of ADR.

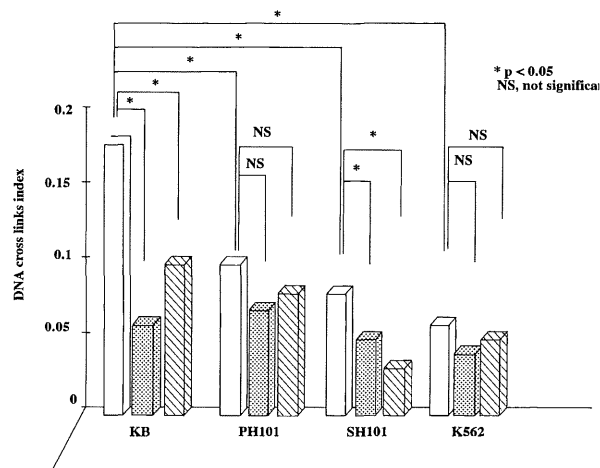
To confirm the functional significance of DTD on MMC activation, we used another DTD inhibitor, flavin adenine dinucleotide (FAD), to suppress DTD activity. As shown in Fig. 2, 100 mM FAD inhibited MMC-induced cytotoxicity as well as 50  $\mu$ M DIC. Concentrations causing 50% inhibition of cell growth (IC<sub>50</sub>) for KB were 0.4  $\mu$ g/ml to MMC alone, 4.9  $\mu$ g/ml to MMC with DIC, and 4.4  $\mu$ g/ml to MMC with FAD. For PH101, SH101 and K562, IC<sub>50</sub> values were 1.1, 1.6, 1.9  $\mu$ g/ml to MMC alone, 3.4, 3.9, 4.3  $\mu$ g/ml to MMC with DIC, and 3.0, 3.7, 3.5  $\mu$ g/ml to MMC with FAD, respectively.

Alkaline elution analysis showed that the total DNA cross links induced by 1.5  $\mu$ g/ml of MMC for 30 min in KB cells was greater than those in PH 101, SH101, and K562 cells. A DTD inhibitor, 20  $\mu$ M DIC and 100 mM FAD, reduced this MMC-

induced DNA damage. The total cross links index for KB were 0.18 to MMC alone, 0.06 to MMC with DIC, and 0.10 to MMC with FAD. For PH101, SH101 and K562, the indices were 0.10, 0.07, 0.08 to MMC alone, 0.08, 0.05, 0.03 to MMC with DIC, and 0.08, 0.04, 0.05 to MMC with FAD, respectively (Fig. 3). These data can be interpreted that a reactive form of MMC metabolized by DTD induces cytotoxicity through the production of DNA cross links.



**Fig. 2.** Effect of a DT-diaphorase inhibitor, dicoumarol(DIC), flavin adenine dinucleotide (FAD), on cytotoxic activities induced by MMC in KB, PH 101, SH 101, K562. Exponentially growing cells ( $5 \times 10^6$ /ml) were exposed to MMC at the various concentrations for 30 min. After 2 washes, cells were incubated for 72hr in drug-free medium and cell growth inhibition was evaluated by Trypan-blue dye exclusion test. Then the concentration to inhibit 50% cell growth (IC<sub>50</sub>) was calculated. IC<sub>50</sub> values obtained by MMC treatment (□) was significantly increased in the presence of 50 $\mu$ M DIC (▨) or 100mM FAD (▤) ( $p < 0.05$ ).



**Fig. 3.** Effect of a DT-diaphorase inhibitor, dicoumarol (DIC) and flavin adenine dinucleotide (FAD), on DNA total cross links induced by Mitomycin C (MMC) in KB, PH101, SH101, K562.

DNA total cross links index in KB were significantly higher than that in PH101, SH101, K562 ( $p < 0.05$ ). MMC-induced DNA total cross links (□) was suppressed in the presence of 50µM DIC (▨) or 100mM FAD (▩). In KB, SH101, the suppression was significant ( $p < 0.05$ ), while it was not in PH101, K562.

**DTD activities and MMC efficacy in solid tumors**

The relevance of DTD activity to MMC efficacy was investigated using human tumor xenografts implanted into nude mice. In contrast to the observations in the experiments using culture cells, the higher the DTD activity the xenografts had, the lower was their sensitivity to MMC (Table 2). Moreover, MMC efficacy was enhanced by co-treatment with 0.1 ml of 100 mM DIC or 100 µM FAD at a schedule of q4d×3. However, at the dose, DIC or FAD alone did not inhibit tumor growth more than 20%. For EH-6 and CH-4, the combined effect of MMC with a non-

toxic DTD inhibitor was defined as synergistic.

The inverse relationship between DTD activity and sensitivity to MMC was observed more clearly in 9 fresh tumor specimens collected at surgery (Table 3). The levels of DTD activity, which varied from 2935 to 63492 nmol/min/mg of protein, correlated with IC<sub>50</sub> values evaluated by ATP assay ( $r = 0.777$ ,  $p < 0.01$ ). In 3 of the 9 patients, the clinical response to MMC was defined as progressive disease (PD). All of them were gastric cancer patients and had tumors with significant high DTD levels. DTD levels in solid human tumors appear to correlate with the degree of resistance to MMC.

The DTD activity in normal adjacent tissues varied from 662 to 39021 nmol/min/mg of protein and was independent of that of the tumors. In 3 patients treated with MMC, no gastrointestinal toxicity was observed despite the fact that their tumors had particularly high DTD activity. Although the role of DTD in normal tissue is unclear, it can be expected that the levels may predict MMC-induced gastrointestinal toxicity.

These result suggest that DTD-mediated MMC action in solid tumors may differ completely from that in culture cells. Since cellular metabolism is influenced profoundly by the microenvironment, we investigated the effect of extracellular pH on DTD-mediated MMC metabolism to explain the discrepant results in solid tumors versus culture cells.

**Influence of microenvironmental pH on DTD activity and MMC efficacy**

Cytotoxicity for culture cells was measured after 30 min exposure to MMC or ADR followed by 72 hrs cultivation in drug-free medium. MMC-induced cytotoxicity increased when the pH varied from 8 to 6. IC<sub>50</sub> values at pH 6 for KB, PH101, SH101, K562 were less than 1/10 of those at pH 8. Contrary to MMC, ADR efficacy increased with increasing pH (Table 4). Since ADR-induced cytotoxicity is independent of cellular DTD activity, these data can be interpreted

**Table 2.** DT-diaphorase Activities and MMC Efficacies in Human Tumor Xenografts Implanted into Nude Mice

Xenograft	DT-diaphorase activity (nM/min/mg protein)	Tumor growth inhibition (1-T/C, %)		
		MMC	MMC+DIC	MMC+FAD
EH-6	1704	10	56	39
CH-4	1586	42	69	74
CH-5	825	56	61	61
SH-6	299	65	66	78

DT-diaphorase activity was a mean value of 10 samples. Tumor growth inhibition was calculated according to the formula described in "Materials and Methods". Groups of 10 mice were used.

MMC, mitomycin C ; DIC, dicoumarol ; FAD, flavin adenin dinucleotide.

**Table 3.** DT-diaphorase Activities and MMC Efficacies in Fresh Human Tumor Specimens

Tumor	DT-diaphorase activity (nM/min/mg of protein)		Response to MMC	
	Tumor	Normal adjacent tissue	ATP assay (IC <sub>50</sub> , μg/ml)	Clinic
Esophagus	2935	662	1.7	
Stomach	3800	22800	1.8	
Stomach	4500	10350	1.8	
Esophagus	5500	660	0.5	
Breast	25852	4162	6.0	
Stomach	30753	39021	47.1	PD
Stomach	34400	25000	42.0	PD
Esophagus	49584	25297	13.0	
Stomach	63492	12416	45.0	PD

MMC, mitomycin C ; PD, progressive disease

**Table 4.** pH-dependent Cytotoxicities Induced by Quinones in Cancer Cells

	Cytotoxicity (IC <sub>50</sub> , μg/ml)					
	MMC			ADR		
	pH6	pH7	pH8	pH6	pH7	pH8
KB	0.3	0.5	6.3	1.34	0.62	0.41
PH101	0.5	1.1	5.8	3.52	1.9	1.83
SH101	0.5	1.6	8.2	3.44	2.72	1.19
K562	0.5	1.9	>10	0.3	0.24	0.22

The number represents the mean of 9 samples which do not differ more than 15%.

MMC, mitomycin C ; ADR, adriamycin

**Table 5.** pH dependent DT-diaphorase Activities and MMC Efficacies in Cancer Cell Lines Established from Human Tumor Xenografts Implanted into Nude Mice

	DT-diaphorase activity (nM/min/mg protein)			MMC efficacy (IC <sub>50</sub> , μg/ml)		
	pH6	pH7	pH8	pH6	pH7	pH8
HCC-48	1212	918	913	1.6	2	2.4
HCC-50	955	837	626	2	4.8	>10
HEC-46	1315	862	833	1.3	2.2	10

The number represents the mean of 9 samples which do not differ more than 15%.

MMC, mitomycin C

that the DTD-mediated metabolism of MMC may be pH-dependent, which is greater at pH 6 but less at pH 8. To clarify the influence of pH-dependent metabolism on MMC activity in solid tumors, we examined 3 culture cell lines, HCC-48 established from tumor xenograft CH-4, HCC-50

from CH-5, and HEC-46 from EH-6. DTD activity and sensitivity to MMC were higher in HCC-48 and HEC-46, and lower in HCC-50, which is similar to the findings in their parent xenografts. As expected, the enzymatic activities and MMC efficacy were pH-dependent. Although the statistical

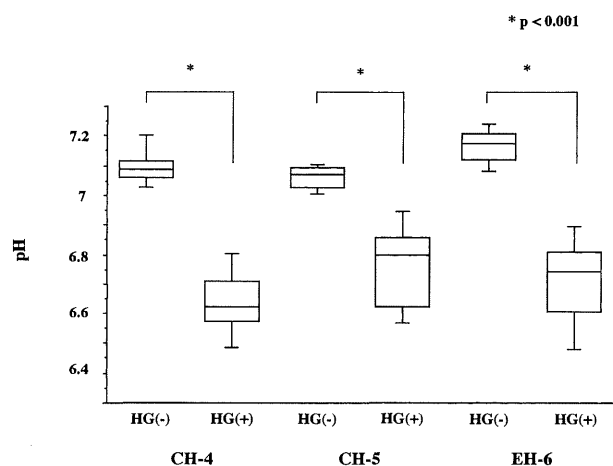
significance was not observed, the cellular DTD activities of HCC-48, HCC-50, and HEC-46 were highest at pH 6. Accompanied by an increase in DTD activity, MMC-induced cytotoxicity was increased (Table 5). This may be a proof that micro-environmental pH complicates any simple relationship between DTD activity and sensitivity to MMC in solid tumors and that DTD activates MMC in a pH-dependent manner.

### Enhancement of MMC efficacy by modulating tumor pH with a continuous glucose infusion

Hyperglycemia has been reported to result in a pH reduction of solid tumors<sup>9</sup>). Using a micro-osmotic pump, we developed a nude mouse experimental system for continuous infusion of 50% glucose. The mean blood glucose concentration was 72.7 mg/dl in the 13 control mice while it was 131.4 mg/dl in the 13 mice treated with continuous infusion for 4 days. The blood glucose concentration had reached a plateau at 24hrs by continuous infusion at the rate of 1  $\mu$ l/hr. The pH values were significantly reduced from pH 7.09 to 6.63 in CH-4, from 7.07 to 6.76 in CH-5, and from 7.16 to 6.71 in EH-6 by means of hyperglycemia (Fig. 4). As expected, in line with the pH reduction, MMC-induced tumor growth inhibition was significantly enhanced in all the xenografts (Table 6).

### DISCUSSION

We have demonstrated in this study that DT-diaphorase (DTD) is a critical determinant of mitomycin C (MMC) bioactivation and that MMC metabolism is pH-dependent. Based on the meaning, we have found that MMC efficacy can be



**Fig. 4.** Effect of hyperglycemia on tumor pH in CH-4, CH-5 and EH-6.

Hyperglycemia (HG) reduced tumor pH of CH-4, CH-5 and EH-6 significantly ( $p < 0.001$ ). Data are presented as box plot ( $n = 24$ ).

**Table 6.** MMC Efficacy and Hyperglycemia

	Tumor growth inhibition (1-T/C %)	
	MMC	MMC+HG
CH-4	41.5	82.3
CH-5	38.4	61
EH-6	4.8	61

Tumor growth inhibition was calculated according to the formula described in "Materials and Methods". Groups of 10 mice were used.

MMC, mitomycin C ; HG, hyperglycemia

augmented by continuous glucose infusion, since hyperglycemia results in tumor-specific pH reduction.

DT-diaphorase (DTD) is a 2-electron reductase, which has been considered to be involved in electron transfer and oxidative phosphorylation in the mitochondrial respiratory chain, in vitamin K-dependent protein carboxylation and the detoxification of quinones<sup>5</sup>). Of these functions, there has been increasing interest in its action on anti-tumor quinones, especially on MMC<sup>12</sup>). MMC hydroquinone derivatives may be conjugated and excreted from cells, therefore 2-electron reduction by DTD has been considered a detoxification reaction<sup>15</sup>). Contrary to the classical understanding, however, recent studies have shown that DTD activates MMC<sup>18,19</sup>).

Despite of the meaning that MMC requires bio-reduction for activation, the enzyme responsible for the reduction has not been well understood. MMC bioactivation has been attributed to a one-electron reduction to a short-lived semiquinone radical by several enzymes, including xanthine oxidase and cytochrome P450 reductase or 2-electron reduction to hydroquinone by DTD<sup>24</sup>). We have shown here evidence of the participation of DTD in the activation of MMC. The level of cellular DTD activity was well related to MMC-induced DNA cross links and cytotoxicity in 7 culture cell lines. The close relationship of DTD activity to cytotoxicity suggests that DTD-mediated 2 electron reduction could be the main pathway in MMC activation. However, for the anthraquinone, adriamycin (ADR), this relationship could not be observed. This is reasonable because ADR has been well known to induce mainly DNA single or double strand breaks through the semiquinone radical formation<sup>21</sup>). DTD-mediated quinone activation apparently depends on the drug structure. This may account for discrepant findings in the role of DTD, concerning whether the enzyme activates or detoxifies antitumor quinones.

The DTD-mediated MMC activation in solid

tumor has been the subject of much controversy. An inverse relationship between DTD activity and MMC efficacy appeared also to exist in solid tumor in this study. We previously suggested that the discrepant results in solid tumors versus culture cells might be accounted for by the heterogeneous pH-distribution caused by the different extracellular pH in solid tumors<sup>33</sup>). It has been suggested that the DTD-mediated metabolism of MMC is pH-dependent<sup>30</sup>). The heterogeneous pH distribution of solid tumors due to their aerobic and hypoxic fractions has also been well documented<sup>33</sup>). In solid tumors, therefore, MMC-induced DNA damage and cytotoxicity may be regulated by both the pH and DTD level. To know the effect of microenvironmental pH on DTD-mediated MMC activation in this study, we examined both nude mouse xenografts and culture cells established from xenografts. In the established culture cells, as well as in the other cell lines, MMC efficacy related to DTD activity. In addition, when tumor pH was reduced by hyperglycemia, MMC inhibited tumor growth more greatly. Thus, we can conclude that MMC is activated by DTD but pH-dependent DTD action may complicate any simple relationship between DTD activity and MMC efficacy in solid tumor.

The pH-dependent metabolism of MMC by DTD was first reported by Kennedy et al<sup>11,23</sup>), and Siegel et al have shown that the catalytic activity of DTD is lost at pH 7.8, but maintained at pH 5.8<sup>29</sup>). Our results support the finding with greater evidence, and show the possibility of applying its unique action to the biochemical modulation of MMC. Although it has been well known that microenvironmental pH varies cellular metabolism and function<sup>34</sup>), a variety of studies have failed to develop a modality for tumor pH modification without inducing any toxicity. Recently, the stagnation in progress was broken through by a finding that hyperglycemia might result in tumor pH reduction due to the different glycolysis from normal tissue<sup>7</sup>). We obtained significant reduction of tumor pH from approximately 7.1 to 6.7 by hyperglycemia at the blood concentration of 131.4mg/dl, which was 1.8 fold higher than for normal control. The pH reduction yielded an increase of MMC-induced tumor growth inhibition from 22.6 to 56.2%. This finding of an increased MMC efficacy at a reduced pH caused by hyperglycemia is of importance in the development of synergistic therapy using MMC. Since glucose is safely administered at high doses in clinical trials<sup>14</sup>), MMC administration under hyperglycemia may be a potent therapy as a tumor-selective modality. The clinical importance of MMC leads us to suggest that the acquaintance may result in a significant progress in medical oncology.

In summary, we have shown that MMC is activated by a DTD-mediated bioreduction in a pH-dependent manner and that hyperglycemia reduces tumor pH, which results in a tumor-specific enhancement of MMC efficacy. Although the pH response to hyperglycemia may be limited and DTD activity must be tumor-specific, our findings are of importance in the improvement of the MMC therapeutic index.

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