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Tumor-Specific Synergistic Therapy of Mitomycin C: Modulation of Bioreductive Activation

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ABSTRACT

The bioreductive activation of mitomycin C (MMC) has been investigated using 10 human cancer cell lines. Except for 2 lines (COLO201 and COLO320DM), the cellular NAD(P)H: quinone oxidoreductase (DTD) activities correlated well with MMC-induced DNA damage and cytotoxicity. In addition, when the DTD activity was inhibited with 50 mM dicoumarol, the MMC activity decreased significantly. On the other hand, no correlation between the NADPH cytochrome P450 reductase (P450) activity and MMC efficacy was observed. We postulate that two electron reduction by DTD may be more important in MMC activation than one electron reduction by P450. The DTD-mediated metabolism was pH-dependent. In a nude mouse experimental model, the pH in the tumor decreased under hyperglycemic conditions due to unique glycolysis. The administration of m-iodobenzyl-guanidine (MIBG) enhanced the decrease in the pH of the tumor without affecting the pH of normal tissue (liver). It also significantly increased the antitumor activity of MMC. However, this biochemical modulation had no effect in the COLO201 and COLO320DM cells. Other mechanisms may be involved in the regulation of MMC activity in these cells. In conclusion, DTD may be an important target of MMC. Biochemical modulation using MIBG and glucose may selectively enhance the activity of MMC within cancer cells.

***Key words**: DT-diaphorase, NADPH cytochrome P450, Mitomycin C, Biochemical modulation*

Mitomycin C (MMC) remains one of the most active agents for gastrointestinal tumors despite low response rates of about 20%[3,17)]. The limited effectiveness of MMC has stimulated both preclinical and clinical research to enhance its antitumor action[9)] by biochemical modulation, and thus efforts have been directed toward understanding the mechanism of action of MMC.

The cytotoxic activity of MMC is dependent upon its intracellular reductive activation to a reactive form capable of crosslinking DNA[4)]. Recent studies have shown that a two electron reductase, NAD(P)H: quinone oxidoreductase (DT-diaphorase, DTD), plays a significant role in MMC-induced cytotoxicity[22,24)], and our previous studies on DTD using four different types of human cancer cell lines have supported these findings[18,23)]. Furthermore, we have shown that hyperglycemia enhanced the antitumor effect of MMC selectively within the tumors by causing a decrease in the pH of the tumors. This is based on the findings of pH-dependent, DTD-mediated activation of MMC and the unique characteristics of glycolysis in malignant cells[29)].

The role of DTD in MMC-induced cytotoxicity, however, is still controversial[2,14)]. From their studies using cells transfected with the DTD gene, Powis et al have suggested that DTD may not be a clinically useful target for bioreductively activated anticancer drugs such as MMC[21)]. At least five other, different enzymes have been shown to be capable of reductively activating MMC: NADH cytochrome C reductase, NADH: b5 reductase, xanthine dehydrogenase, xanthine oxidase, and most notably, NADPH cytochrome P450 reductase (P450)[1,28)]. Although the majority of studies of the enzymatic activation of MMC focused on DTD, these controversial results have prompted us to determine which enzyme, P450 or DTD, correlates with observed sensitivity or resistance to MMC, and to show whether the other activation mechanisms of MMC exist or not. If DTD is still useful as a target of MMC, the more effective modality to enhance its anti-tumor effect must be studied for its clinical application.

Using 10 different human cancer cell lines, we investigated the activity of cellular NADPH cytochrome P450 reductase, a one electron reductase, to identify the enzyme responsible for MMC activation. To demonstrate the clinical utility of our DTD-targeting biochemical modulation more clearly, we also attempted to use an inhibitor of

mitochondrial respiration, m-iodobenzyl guanidine, as a new modulator of MMC activation[8]. This agent may enhance the anti-tumor activity of MMC through its ability to reduce the pH of the tumor under hyperglycemic conditions.

MATERIALS AND METHODS

Drugs

All chemicals were of analytical grade. Mitomycin C (MMC) was kindly provided by Kyowa Hakko Kogyo, Tokyo, Japan. Dicoumarol (DIC) and dichlorophenol indophenol (DCPIP) were obtained from Sigma, St. Louis, MO. MIBG was synthesized from m-iodobenzylamine according to the method of Wieland et al[30]. The product was >98% MIBG-sulfate by HPLC analysis. MIBG was dissolved in a heated (60°C) solution of NaCl (8.67 mg), KCl (0.2 mg), $Na_2HPO_4 \cdot 2H_2O$ (1.15 mg), and KH_2PO_4 (0.2 mg), per ml of water (pH 7.2).

Cells and tumors

The human myelogenous leukemia cell line K562 was kindly provided by Dr. Tsuruo, Tokyo University. The human oral epidermoid carcinoma KB was kindly provided by Dr. Akiyama, Kagoshima University. These were maintained as previously described[5,15]. The human colon adenocarcinoma cell lines COLO201 and COLO320DM were obtained from the Japanese Cancer Research Resources Bank. The human gastric adenocarcinoma cell line, SH101, and pancreatic adenocarcinoma cell line, PH101, were established from ascites in our laboratory[16]. HSC-42, HEC-46, HCC-48, and HCC-50 cell lines were established by Dr. Yanagihara, Hiroshima University, from a xenotransplantable human gastric cancer cell H-111, esophageal cancer cell EH-6, colon cancer line CH-4, and colon cancer line CH-5, respectively[31]. The four parental xenografts and xenoplantable COLO201 and COLO320DM were transplanted serially into nude mice under sterile conditions. All cultured cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and maintained in continuous exponential growth by passage every 3 days.

Animals

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

Evaluation of MMC efficacy

Exponentially growing cell cultures of K562, KB, SH101, PH101, HSC-42, HEC-46, HCC-48, HCC-50, COLO201, and COLO320DM were concentrated to 5×10^6/ml and exposed to the indicated concentrations of MMC for 30 min with and without 50 mM dicoumarol (DIC), an inhibitor of DTD activity. For MMC treatment at different pHs, the buffer medium was prepared by adding 25 mM HEPES and adjusting to the appropriate pH with 1 N HCl or 1 N NaOH. The buffer media were sterilized by filtration, and the pH was measured immediately prior to use and readjusted as necessary. After two washings with drug-free medium, the cells were resuspended at a concentration of 2.5×10^4/ml in RPMI-1640, DMEM, or Eagle's MEM with 10% fetalcalfserum (FCS) and seeded in 24-well plates. After 72 hrs of incubation in fresh media at 37°C, the surviving cells were counted using a Coulter counter and by trypan blue exclusion.

The procedure to evaluate the effect of MMC on human tumor xenografts transplanted into nude mice corresponded to the therapeutic protocol with some modifications[19]. Briefly, tumor specimens were fragmented to approximately 2 mm^3 in RPMI medium. They were implanted s.c. into the flanks of nude mice with trocars, and the resulting tumors were measured across two perpendicular diameters with sliding calipers. Each mouse was weighed every 4 days. When the calculated tumor volume reached 100 to 300 mm^3, the treatment was started. A micro-osmotic pump (Model 1003D, ALZET, Alza Corp., Palo Alto, CA) was transplanted s.c. into the backs of the nude mice. The pump was filled with a 50% glucose solution or saline. MMC and MIBG were administered i.v. daily for 5 days. The doses were 2 mg/kg/ injection for MMC and 20 mg/kg/ injection for MIBG. The sizes of the implanted tumors 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 is the width of the implanted tumor. Next, 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 is the mean initial tumor volume. Tumor-growth inhibition was evaluated by the rate of inhibition in the mean increase of the RV (1-T/C, %) in untreated (C) and treated mice.

Assays of enzyme activity

A cytosol fraction was prepared at 0 to 4°C. Exponentially growing culture cells were washed twice with ice-cold Hank's balanced salt solution (HBSS) and resuspended in 0.25 M sucrose (10^7 cells/ml). Centrifugation at 100000 *g* for 1 hr at 4°C after sonication for 30 sec yielded clear cytosol fractions. The solid tumors were homogenized in 0.25 M sucrose (3.0 ml/g of tissue). After centrifugation at 9000 *g* for 20 min at 4°C, the supernatant was collected, and 0.2 volumes of 0.1 M $CaCl_2$ in 0.25 M sucrose were added. Each sample then was incubated on ice for 30 min. Centrifugation at 27000 *g* for 20 min yielded clear cytosol fractions.

The DTD activity was measured according to the procedure of Ernster[6]. The reaction mixture contained 0.025 M Tris HCl (pH 7.4), 0.7 mg/ml of crystalline BSA, 0.2 mM NAD(P)H, 0.01% Tween 20, 5 mM flavin adenine dinucleotide (FAD), and 40 mM DCPIP. Reactions (3 ml) were performed at 25°C in the presence or absence of 20 mM DIC. DT-diaphorase activity was measured as the DIC-sensitive reduction of DCPIP (aM=21,000M-1 cm-1). The velocity of the reduction of DCPIP was measured by spectrophotometry at 600 nm.

The activity of P450 was measured using cytochrome c as the electron acceptor by the procedure of Strobel and Dignam[25]. The reaction mixture contained 150 μg of cytosol, 100 μM phosphoric acid potassium buffer (pH 7.5), 20 μM cytochrome c, and 100 μM NADPH as an electron donor. The reaction was initiated by the addition of NADPH at 25°C, and the reduction of cytochrome c was followed spectrophotometically at 550 nm for 5 min.

Alkaline elution assay

DNA damage induced by MMC was analyzed using alkaline elution techniques as previously described[11]. In all experiments, the internal standards were (^{14}C)thymidine- labeled cells irradiated with 6 Gy of X-rays under refrigeration. For all cross-linking assays, the control and drug-treated cells also were irradiated with 6 Gy. The MMC treatments lasted for 30 min at 37°C at a concentration of 5 mg/ml/ 1×10^7 cells with or without 50 mM DIC. The total cross-link index was calculated according to the equation: total cross-link index=$(1-R_0)/(1-R)-1$, where R_0 and R are the fractions of DNA retained by the 6 Gy with and without treatment with the drug, respectively.

pH measurements in nude mouse experimental system

Tumor-bearing nude mice were anesthetized with a single i.p. injection of 0.0025 ml/kg of a 1:1, 0.9% NaCl solution of sodium pentobarbital. The tumor surface was exposed without damaging any of the overlying blood vessels. A microelectrode (18 gauge needle, MI-413, Microelectrode, Inc, city, NH) was inserted 5 mm into the tissue, and the pH was measured at six separate sites for each tumor. A laparotomy then was performed, and the pH of the liver, which served as a normal control, was measured by the same procedure.

RESULTS

In cultured human cells, the correlation of the DTD activity to MMC-induced DNA damage and cytotoxicity was investigated (Table 1). The level of the DTD activity (nM/min/mg protein) varied from 105 for COLO320DM to 8260 for KB. Except for COLO320DM and COLO201, the cellular DTD levels correlated well with the MMC-induced cytotoxicity and DNA damage. The higher the cellular DTD activity, the lower the MMC concentration which caused 50% cell growth inhibition (IC_{50}). The total DNA cross-link indices also were closely related to the level of cellular DTD activities in KB, PH101, SH101, K562, HCC-48, HEC-46, HCC-50, and HSC-42 cells. However, these did not correlate in the COLO201 and COLO320DM cells. Although COLO201 and COLO320DM cells had extremely low levels of DTD activity, they were moderately sensitive to MMC. The IC_{50} values were 2.2 mM for COLO201 and 2.5 mM for COLO320DM, which were similar to that for HEC-46. On the other hand, no correlation was observed between the activity of P450 and the MMC-induced DNA damage and cytotoxicity. These results suggest that P450 is not a target for MMC and that DTD plays a more significant role in the bioreductive activation of MMC than does P450, at least in the cells investigated.

The inhibition of DTD activity by 50 mM DIC caused a significant decrease in the MMC-induced DNA damage and cytotoxicity in KB, PH101, SH101, K562, HCC-48, HEC-46, HCC-50, and HSC-42 cells (Fig. 1), although a single 50 mM DIC treatment inhibited cell growth less than 10%. In contrast, non-toxic doses of DIC did not decrease the MMC efficacy at all in the COLO201 and COLO320DM cell lines. These results suggest that a DTD-mediated bioreduction may be a critical event in MMC-induced cytotoxicity in most cells. Since DNA damage was induced by MMC in COLO201 and COLO320DM cells but both the DTD and P450 activities were extremely low, there may be other mechanisms regulating MMC efficacy in these cells.

As previously suggested, exposure of cells to MMC for 30 min at various pH showed that the MMC-induced cell growth inhibition varied with the microenvironmental pH, which was greatest at pH 6, lower at pH 7, and lowest at pH 8 ex-

Table 1. NAD(P)H: Quinone Oxidoreductase (DT-Diaphorase) Activity, NADPH Cytochrome P450 (P450) Activity, and Mitomycin C (MMC) Efficacy

	DT-Diaphorase activity (nM/min/mg protein)	P450 activity (nM/min/mg protein)	MMC efficacy	
			cytotoxity IC_{50}(μM)	DNA damage DNA total cross-link index
Rat liver	26600			
KB	8260	54	0.4	0.26
PH101	1934	156	1.1	0.23
SH101	1805	185	1.6	0.20
K562	1796	273	1.9	0.18
HCC-48	918	214	2.0	0.19
HEC-46	837	206	2.2	0.12
HCC-50	862	199	4.8	0.06
HSC-42	194	202	8.9	0.03
COLO201	124	175	2.2	0.14
COLO320DM	105	71	2.5	0.13

Each value represents the mean of nine samples, which do not differ by more than 10%.

cept for the COLO201 and COLO320DM cells (Fig. 2). At pH 6, the IC_{50} values (mM) were 0.26 for KB, 0.38 for PH101, 0.42 for SH101, 0.44 for K562, 0.51 for HCC-48, 0.58 for HEC-46, 0.75 for HCC-50, and 2.1 for HSC-42 cells. These values were less than one-fourth of those at pH 8 and correlated well with the cellular DTD levels. A reduction in the microenvironmental pH therefore increased the efficacy of MMC in most of the cancer cells investigated.

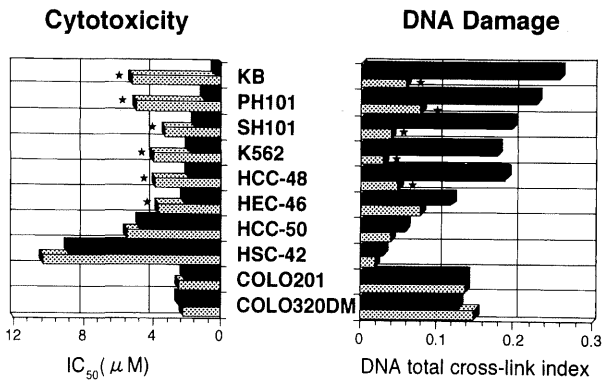

Fig. 1. Effect of a DTD inhibitor on MMC efficacy Exponentially growing cells (5×10^6/ml) were exposed to MMC for 30 min in the absence (■) or presence (▒) of a non-toxic DTD inhibitor, 50 mM DIC. The cytotoxicity and DNA cross-link index were assessed as described in Materials and Methods. MMC-induced cytotoxicity and DNA damage were suppressed in the presence of DIC (*, $p<.05$) except in the COLO201 and COLO320DM cells. Each value represents the mean of nine samples, which did not differ by more than 10%.

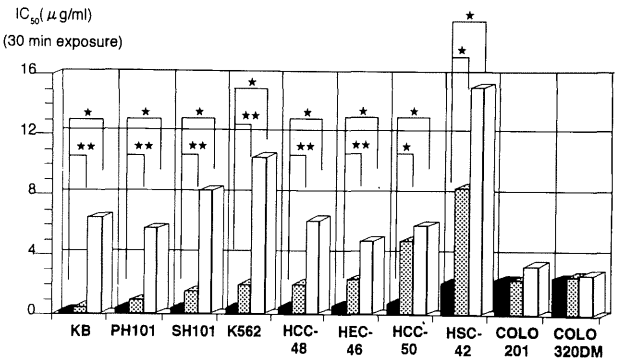

Fig. 2. Microenvironmental pH and MMC efficacy Exponentially growing cells (5×10^6/ml) were exposed to MMC for 30 min in a pH 6 (■), pH 7 (▒), or pH 8 buffer (□). Cytotoxicity then was assessed by the trypan blue dye exclusion test as described in Materials and Methods. MMC-induced cytotoxicity significantly increased at pH 6 but decreased at pH 8 (*, $p<.01$; **, $p<.05$) except for the COLO201 and COLO320DM cells. Each value represents the mean of nine samples, which did not differ by more than 10%.

In solid tumors, a continuous glucose infusion combined with the administration of MIBG can reduce the pH within the tumors specifically. A micro-osmotic pump implanted into the nude mice delivered a 50% glucose solution for 90 hrs at a pumping rate of 1 ml/hr, and 20 mg/kg of MIBG was administered daily for 4 days. This manipulation significantly increased the mean blood glucose concentration of tumor-bearing nude mice from 71.4 mg/dl to 132.4 mg/dl (Fig. 3). Under these conditions, the mean tumor pH were reduced from 7.16 to 6.71 in CH-4, from 7.09 to 6.63 in EH-6, from 7.07 to 6.76 in CH-5, from 7.12 to 6.53 in H-111, from 7.11 to 6.68 in the COLO201 xenograft, and from 7.06 to 6.61 in the COLO320DM xenograft. On the other hand, the pH of liver did not vary significantly. As compared to glucose administration alone, the combination of glucose and MIBG did not increase the blood glucose concentration but did reduce the tumor pH more significantly and selectively (Fig. 4).

Glucose and MIBG treatment increased the efficacy of MMC significantly except for the COLO201and COLO320DM xenografts (Fig. 5), although no positive relationship between the tumor DTD level and MMC activity was observed. DTD-mediated MMC activation may be present in most solid tumors, and its metabolism is pH-dependent, as it is in cultured cells. Interestingly, MIBG and a glucose infusion with MMC did not induce toxicity or pH reduction in the liver tissues. Thus, we suggest that this augmentation of MMC anti-tumor activity may be tumor-specific.

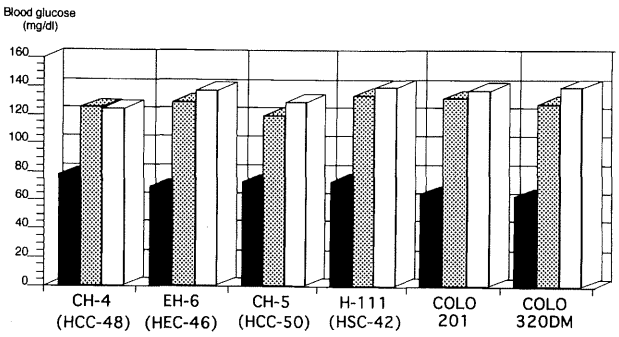

Fig. 3. Blood glucose levels in nude mice
The blood glucose levels in nude mice were measured after the administration of saline (■), glucose (▒) or MIBG and glucose (□). The MIBG was administered intravenously for 5 consecutive days along with a continuous infusion of a 50% glucose solution using a micro-osmotic pump as described in Materials and Methods. The mean blood glucose concentration in the tumor-bearing mice increased from 71.4 mg/dl to 128.3 mg/dl with the glucose alone and to 132.4 mg/dl with the administration of both MIBG and glucose. Each value represents the mean of nine samples, which did not differ by more than 10%.

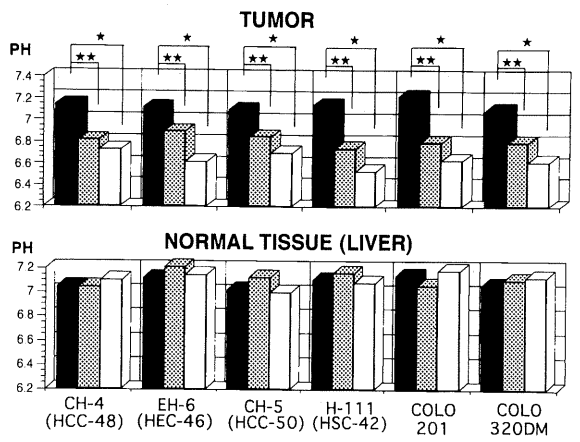

Fig. 4. pH of tumor xenografts and liver tissues
Using a microelectrode, the pH was measured at a 5 mm depth at six points in each tumor and the liver tissue of untreated mice (■), or mice treated with glucose alone (▒), or with both MIBG and glucose (□). MIBG was administered intravenously for 5 consecutive days along with a continuous infusion of a 50% glucose solution using a micro-osmotic pump as described in Materials and Methods. Glucose alone or MIBG plus glucose treatment reduced the tumor pH significantly (*, $p<.01$; **, $p<.05$), but did not change the pH in the liver tissues. Each group consisted of six mice. Each value represents the mean of the results from the six mice, which did not differ by more than 10%.

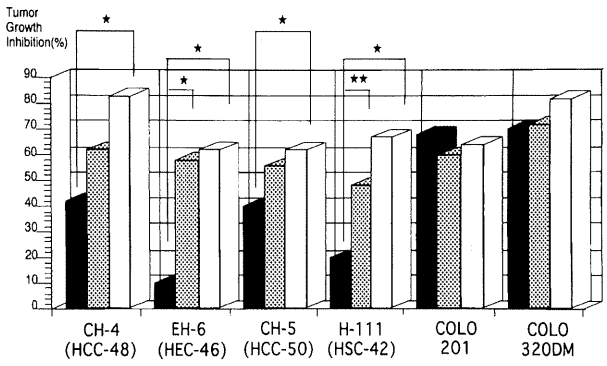

Fig. 5. Tumor growth inhibition induced by mitomycin C (MMC) and biochemical modulation
When the implanted tumor reached a volume of 100 to 300 mm^3, the nude mice were treated with MMC for 5 consecutive days. Each group of six mice either received no further treatment (■), intravenous glucose (▒), or MIBG plus glucose administration (□). MIBG was administered intravenously for 5 consecutive days along with a continuous infusion of a 50% glucose solution using a micro-osmotic pump as described in Materials and Methods. Tumor growth inhibition was enhanced by the administration of glucose, and even more significantly, by the combined administration of MIBG and glucose, except for the COLO201 and COLO320DM xenografts (*, $p<.01$; **, $p<.05$). Each value represents the (1-T/C)%, the inhibition rate of the mean increase in the relative tumor volume.

DISCUSSION

The enzyme responsible for MMC activation is still not well understood. Despite intensive study, the details of DTD action are also controversial. The NQO1 gene has been characterized as a dioxin-inducible form of DTD, although gene expression and transfection studies have been unable to clarify the role of the two electron reductase on MMC metabolism. Traver et al have shown that DTD may play a significant role in the activation of MMC and that NQO1gene expression is correlated with the enzymatic activity[24,26]. Using NIH3T3 cells transfected with the human NQO1 cDNA, Powis et al. have shown that an elevated DTD activity does not enhance the anti-tumor activity of quinones, including MMC[21]. In an attempt to explain these contradictory results, the characterization of the human DTD isozymes is in progress. Therefore, an understanding of the biologic behavior of DTD in various types of cancers is extremely important.

We have shown here that DTD is a clinically useful target of MMC, and its pH-dependent bioreductive activation can be utilized for tumor-specific biochemical modulation of MMC. Our previous study showed that there were differences in the role of DTD activity on MMC activation between in vitro and in vivo experimental systems[18]. Since the contradictory results may be attributed to its pH-dependent metabolism and heterogeneity of the pH in tumors, we have attempted to modify the pH in the tumors in order to clarify the significance of the activity of DTD. In 8 of 10 cell lines, the cellular DTD activities correlated well with their sensitivities to MMC, and the anti-tumor activity was enhanced at reduced pH. MMC may be activated by DTD in a pH-dependent manner[29]. Since we included 4 lines established from xenoplantable tumors, it is apparent that the heterogeneity of tumor pH influences DTD-mediated MMC activation in vivo.

Nevertheless, in the COLO201 and COLO320DM cells, the role of DTD in MMC activation was not clear. Contrary to the findings in the other cell lines, the cytotoxicity of MMC was independent of their cellular DTD and P450 activities. Tumor pH reduction by MIBG and glucose administration also did not affect the MMC activity. These findings suggest that DTD-mediated metabolism is not always the main pathway of MMC activation. As some investigators have suggested, only when cells have high DTD activity, DTD works on MMC activation[20]. In some cells, there may be other mechanisms which can regulate MMC efficacy. A novel analogue of MMC, 7–N–{{2–{[2–(–L-glutamylamino)ethyl]dithio}ethyl} mitomycin C (KW-2149), has been reported to be activated in the presence of thiol molecules[13]. Since this analogue can circumvent MMC resistance[27], cel-

lular glutathione or one of its activation enzymes may be involved in MMC activation or its detoxification. Thus, we have focused on the function of thiol molecules.

In addition to clarifying the probable mechanisms of cytotoxic activity, the present study demonstrates the possibility of DTD as a clinically useful target for the biochemical modulation of MMC. Tumor selectivity has been the key to all chemotherapies. Tumor selectivity may be achieved by targeting DTD-mediated MMC activation. DTD-mediated MMC metabolism and its pH-dependency was first reported by Kennedy et al[10]. A variety of studies, however, have failed to develop a modality able to reduce tumor pH without inducing toxicity. We have shown that MIBG and glucose administration can reduce pH selectively in tumors. The glucose-mediated pH reduction in malignant tissue is unlikely to be limited to tumors of a specific origin or histologic type[7,12]. We suggest, therefore, that synergistic combination therapy using MMC may be clinically applicable as an effective treatment with low toxicity for various types of cancers. However, one would expect that this approach would be efficacious only for tumors having DTD-mediated MMC activation. For enhancing its efficacy in all tumor cells, the other mechanisms of MMC activation or detoxification must be clarified. We currently are analyzing the glutathione levels, GST π expression, and the effect of γ-glutamyl synthetase cDNA transfection on MMC activity.

In summary, we have shown that DTD is a key bioreductive enzyme for MMC activation and that its combination with MIBG and glucose infusion can enhance the anti-tumor activity of MMC for most malignant tumors. This form of biochemical modulation may enhance the limited effectiveness of MMC, although it may be ineffective against some tumors. To enhance the effectiveness of MMC in these tumors, the other mechanisms of MMC activation and deactivation must be investigated.

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