Jpn. J. Cancer Res. 88, 88–96, January 1997

Establishment by Adriamycin Exposure of Multidrug-resistant Rat Ascites Hepatoma AH130 Cells Showing Low DT-diaphorase Activity and High Cross Resistance to Mitomycins

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A resistant subline (AH130/5A) selected from rat hepatoma AH130 cells after exposure to adriamycin (ADM) showed remarkable resistance to multiple antitumor drugs, including mitomycin C (MMC) and porfiromycin (PFM). PFM, vinblastine (VLB), and ADM accumulated in AH130/5A far less than in the parent AH130 (AH130/P) cells. AH130/5A cells showed overexpression of P-glycoprotein (PGP), an increase in glutathione *S*-transferase activity, and a decrease in DT-diaphorase and glutathione peroxidase activity. The resistance to MMC and VLB of AH130/5A cells was partly reversed by H-87, an inhibitor of PGP. Buthionine sulfoximine, an inhibitor of glutathione synthase, did not affect the action of MMC. *tert*-Butylhydroquinone induced DT-diaphorase activity, increased PFM uptake, and enhanced the growth-inhibitory action of MMC in AH130/5A cells. Dicumarol, an inhibitor of DT-diaphorase, decreased PFM uptake and reduced the growth-inhibitory action of MMC in AH130/P cells. These results indicated that the adriamycin treatment of hepatoma cells caused multifactorial multidrug resistance involving a decrease in DT-diaphorase activity.

Key words: Adriamycin — Mitomycin C — DT-diaphorase — P-glycoprotein — Resistance

Antitumor drug resistance is a major obstacle to effective cancer chemotherapy. One of the mechanisms involved is classical multidrug resistance (MDR), which is dependent on the expression of P-glycoprotein (PGP) in the plasma membrane. PGP is a 180 kDa membrane protein in human multidrug-resistant cell lines, such as myelogenous leukemia cells,[1)] and a 140 kDa protein in mouse cell lines, such as P388/ADR leukemia cells.[2)] It acts as an energy-dependent efflux pump. Overexpression of this protein results in the decreased accumulation of several structurally unrelated antitumor drugs including anthracycline antibiotics, *Vinca* alkaloids, and mitomycin C (MMC).

Although the extent of resistance to MMC in classical multidrug-resistant cells is generally far less than that to adriamycin (ADM), it has been reported that an ADM-induced resistant cell line showed moderate cross resistance to MMC.[3)] The mechanism by which this cross resistance is acquired remains unclear.

ADM has been reported to induce MDR through PGP and also other mechanisms.[4–6)] In addition, it has been reported that resistance to ADM and MMC involves glutathione *S*-transferase (GST), glutathione peroxidase (GSHpx), or NADPH cytochrome *c* reductase activity. Namely, the placental form of GST may affect resistance to ADM,[7)] while GSTs have been implicated in the resistance to alkylating agents.[8)] GSHpx is believed to be involved in the detoxification of ADM through the removal of hydroxy radical.[9)] NADPH cytochrome *c* reductase activates ADM,[10)] and this enzyme also activates MMC by one-electron reduction of the quinone moiety, producing cytotoxic hydroxy radicals, under aerobic conditions.[11, 12)] On the other hand, MMC has been suggested to be activated by DT-diaphorase by two-electron reduction of MMC under aerobic[13)] and anaerobic conditions.[14)] Cells containing high DT-diaphorase activity show high sensitivity to MMC,[15)] while low DT-diaphorase activity was found in doxorubicin-resistant MCF cells.[16)] Although several mechanisms have been suggested to cause the resistance to MMC, it has remained unclear what is the mechanism of cross resistance to MMC in ADM-induced multidrug-resistant cells. In the present study, we established an ADM-induced multidrug-resistant rat hepatoma AH130 cell line that was cross resistant to MMC, and attempted to clarify whether the marked cross resistance to mitomycins is mediated by a mechanism other than PGP. We also tried to modify the resistance *in vitro*.

MATERIALS AND METHODS

Chemicals Daunomycin was generously supplied by Meiji Seika Inc. (Tokyo). ADM, MMC, and 5-fluorouracil were obtained from Kyowa Hakko Kogyo Co., Ltd. (Tokyo). Vinblastine (VLB) and vincristine were purchased from Shionogi & Co., Ltd. (Osaka). Cisplatin was obtained from Nihon Kayaku Co., Ltd. (Tokyo). Chlorambucil and methotrexate were obtained from Sigma Chemical Co. (St. Louis, MO). DL-Buthionine sulf-

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oximine (BSO), 1-chloro-2,4-dinitrobenzene (CDNB), glutathione (reduced form), NADPH, 3,3′-methylene-*bis*-4-hydroxycoumarin (dicumarol), and *t*-butylhydroquinone (*t*-BHQ), were purchased from Wako Pure Chemical Industries, Ltd. (Osaka). [^{3}H]Porfiromycin (350 mCi/mmol) and [^{3}H]VLB (9 Ci/mmol) were obtained from Moravek Biochemicals Inc. (Brea, CA). *N*-[2-(*p*-Bromocinnamylethylamino)ethyl]-5-isoquinolinesulfonamide (H-87) was a kind gift from Dr. Hidaka of Nagoya University School of Medicine, Nagoya.

Cell lines Rat ascites hepatoma cell line AH130, which was established in 1951 from 4-methyldinitrobenzene-fed rats by Yoshida *et al.*,[17] was supplied by Sasaki Institute (Tokyo) and maintained by weekly passage in the abdominal cavity of female Donryu rats in our laboratory. A portion of the cells was kept in continuous culture in RPMI1640 medium with 5% heat-inactivated fetal calf serum and 20 m*M* HEPES (pH 7.2, growth medium) in a humidified atmosphere with 5% CO_2. After continuous culture in the presence of step-wise elevated concentrations of ADM (up to 5 μM) for 6 months, a resistant cell line AH130/5A was obtained. The cells were maintained by subculture twice a week in the growth medium containing 5 μM ADM. Prior to use, AH130/5A cells were cultured in the absence of ADM for 2 to 3 weeks.

Drug sensitivity AH130/P and AH130/5A cells (1.5×10^4 and 3×10^4 cells, respectively) were cultured for 72 h in the presence of antitumor agents in a total volume of 1.0 ml in 24-well culture plates (Falcon 3047). Then, the number of cells was counted under a microscope. At least three separate experiments were performed in duplicate or in triplicate.

Modulation by BSO, H-87, dicumarol, and *t*-BHQ of the sensitivity to antitumor agents (A) Modulation by BSO; Cells were preincubated for 24 h in the presence or absence of 100 μM BSO, which did not affect the cell growth as compared with the control culture. Then antitumor agents were added to the culture, and the cells were successively cultured for 48 h. (B) Modulation by H-87; Cells were cultured in the presence of a non-cytotoxic concentration of H-87 (1 μM) and various concentrations of antitumor agents. (C) Modulation by dicumarol or *t*-BHQ; Cells were preincubated with dicumarol for 30 min or *t*-BHQ for 8 h, then incubated with antitumor agents for 2 h. The cells were washed and resuspended in fresh growth medium, and then cultured for 72 h.

Accumulation of VLB and porfiromycin (PFM) in the cells Cells (1×10^6 cells/ml/tube) were suspended in RPMI1640 medium supplemented with 20 m*M* Hepes (pH 7.4) and 5% fetal calf serum and incubated with or without drugs in the presence of [^{3}H]VLB (0.01 μM) or [^{3}H]PFM (0.2 μM) for a designated time at 0°C and 37°C. The cells were collected by centrifugation at 2°C and washed twice with chilled phosphate-buffered saline (PBS, pH 7.4). Then the cells were solubilized in 1 *N* NaOH solution, neutralized with 1 *N* HCl solution, and added to a scintillation cocktail. Radioactivity was determined with an Aloka LS5000 scintillation counter.

Assay of the activity of GST, GSHpx, DT-diaphorase, and NADPH cytochrome *c* reductase We prepared a centrifuged (100,000*g*) supernatant fraction for the assay of the activity of GST and GSHpx, an S9 fraction for the assay of the activity of DT-diaphorase, and a microsome fraction for the assay of the activity of NADPH cytochrome *c* reductase. For the preparation of these fractions, cells suspended in 40 m*M* Tris-HCl (pH 7.4), 154 m*M* KCl, and 4 m*M* EDTA were disrupted with an ultrasonic homogenizer and centrifuged.

GST activity was assayed by the CDNB method.[18] GSTpx activity,[19] DT-diaphorase activity[20] and NADPH cytochrome *c* reductase activity[21] were assayed by means of the reported procedures.

Western blotting of PGP and GST isoenzymes Detection of PGP was performed by using C219 monoclonal antibody as follows. Cells were disrupted in 10 m*M* Tris-HCl (pH 7.4) and 10 m*M* $MgCl_2$ with an ultrasonic homogenizer, and the homogenate was centrifuged at 15,000*g* for 10 min. The resulting pellet was solubilized in Tris-HCl buffer containing 1% Triton X-100, and the lysate was centrifuged at 15,000*g* for 10 min. The soluble fraction (150 μg protein per lane) was subjected to SDS-PAGE (7.5% gel) and the separated protein was transferred to a nitrocellulose membrane filter. PGP was detected with C219 antibody (Centocor, Malvern, PA) and horseradish peroxidase-conjugated anti-mouse IgG antibody (MBL, Nagoya).

Detection of the isoenzyme of GST was performed by using rabbit antisera for rat GST-1 and 2, GST-3 and 4, and GST-P (kindly provided by Drs. K. Sato and K. Satoh of Hirosaki University School of Medicine, Hirosaki). The cytosolic fraction (20 μg protein), prepared as described above, was subjected to SDS-PAGE (12.5% gel), and the GSTs were detected by using the above antibodies and an ABC-kit (Vector, Burlingame, CA).

RESULTS

Drug sensitivities We isolated a resistant subline AH130/5A from rat hepatoma AH130 cells after cultivation for 6 months in the presence of stepwise-elevated concentrations of ADM. The doubling times of AH130/5A and parental (AH130/P) cells growing in the logarithmic phase were 22 h and 16 h, respectively.

The sensitivity to antitumor agents of the cells is shown in Table I. AH130/5A cells were significantly resistant to MMC, PFM, ADM, daunomycin, VLB, vincristine, and etoposide, and slightly resistant to chlor-

ambucil, cisplatin, and 5-fluorouracil. These observations indicated that AH130/5A cells have a multidrug-resistant phenotype. The cells showed collateral sensitivity of about 2-fold to methotrexate.

Uptake of VLB and PFM We compared the uptake of VLB and PFM in these cells. Fig. 1 shows that AH130/5A cells accumulated VLB and PFM to a far lesser extent than AH130/P cells. These results indicated that the resistance was partly based on the decreased intracellular accumulation of these drugs.

Assessment of PGP-dependent mechanisms The expression of PGP was examined in the crude membrane fraction (Fig. 2). The results indicated that AH130/5A cells overexpressed PGP compared to AH130/P cells.

To ascertain the contribution of PGP to MDR, we examined the effects of a non-cytotoxic concentration of H-87 (1 μM) on the *in vitro* growth-inhibitory action of VLB, ADM, MMC, cisplatin, and 5-fluorouracil (Table II). H-87 has been shown to reverse MDR by inhibition of PGP.[22] In AH130/5A cells, H-87 potentiated the effect of ADM, VLB, and MMC by 12-, 7-, and 17-fold, respectively, but did not potentiate the effect of cisplatin or 5-fluorouracil. In AH130/P cells, H-87 did not enhance the action of VLB or MMC. Furthermore, the uptake of VLB was entirely restored by verapamil while

Table I. *In vitro* Sensitivities of AH130/P and AH130/5A Cells to Various Antitumor Drugs

Antitumor drug	IC_{50} (μM)		
	AH130/P	AH130/5A	(R. R.)
Adriamycin	0.045±0.005	8.20±0.67	(182)
Daunomycin	0.040±0.004	6.03±0.82	(151)
Vinblastine	0.006±0.001	0.48±0.03	(80)
Vincristine	0.010±0.002	1.43±0.22	(143)
Mitomycin C	0.004±0.001	1.18±0.07	(295)
Porfiromycin	0.009±0.004	1.64±0.45	(182)
Chlorambucil	0.900±0.107	3.60±0.28	(4)
Cisplatin	0.060±0.005	0.53±0.05	(9)
Etoposide	0.056±0.004	7.70±0.11	(138)
5-Fluorouracil	0.735±0.065	3.70±0.20	(5)
Methotrexate	0.0065±0.0009	0.0031±0.0005	(0.5)

Cells were incubated with antitumor agents for 72 h.
R. R. means relative resistance of AH130/5A cells compared to AH130/P cells for the antitumor drug. Data are means± SD of at least three independent experiments.

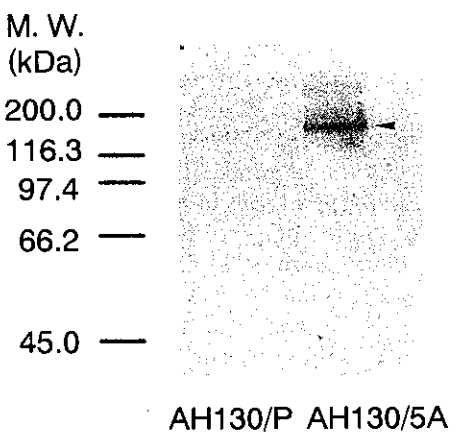

Fig. 2. Western analysis of P-glycoprotein (PGP). The right lane shows AH130/5A cells and the arrowhead indicates the position of PGP. The left lane shows AH130/P cells.

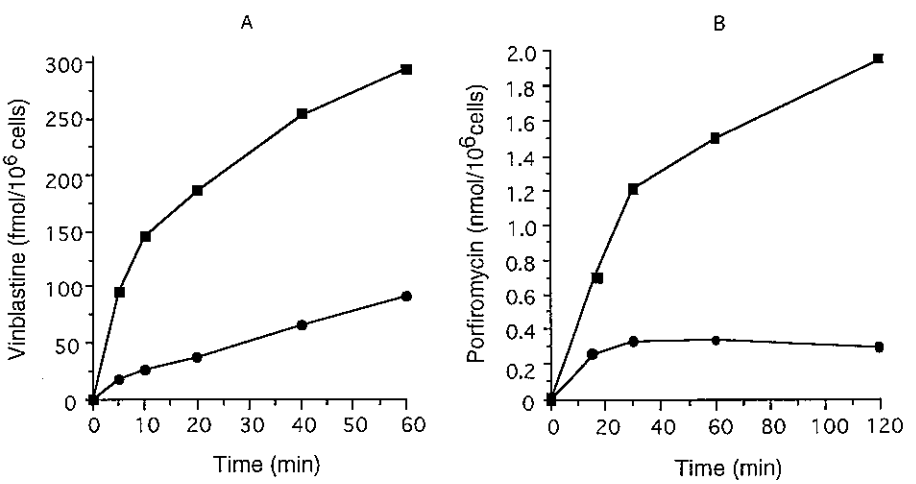

Fig. 1. Time course of the uptake of vinblastine (A) and porfiromycin (B) in AH130/P and AH130/5A cells. Cells (1×10^6 cells/ml) were incubated with 0.01 μM [^{3}H]vinblastine or 0.2 μM [^{3}H]porfiromycin for the indicated times at 37°C. Each point represents the average of triplicate incubations. ■ AH130/P, ● AH130/5A.

Table II. *In vitro* Combination Effect of H-87 with Various Antitumor Agents

	IC_{50} (μM)			
	AH130/P		AH130/5A	
	−	+	−	+
Adriamycin	0.045±0.005	0.013±0.003*	8.20±0.67	0.67±0.04*
Vinblastine	0.006±0.001	0.006±0.001	0.48±0.03	0.07±0.01*
Mitomycin C	0.004±0.001	0.004±0.001	1.18±0.07	0.07±0.03*
Cisplatin	0.060±0.005	0.050±0.004	0.53±0.05	0.59±0.06
5-Fluorouracil	0.735±0.065	0.730±0.068	3.70±0.20	3.40±0.18

Cells were incubated with antitumor drugs in the absence (−) or presence (+) of 1 μM H-87 for 72 h. Data are means±SD of at least three independent experiments. * Significantly different at $P<0.05$.

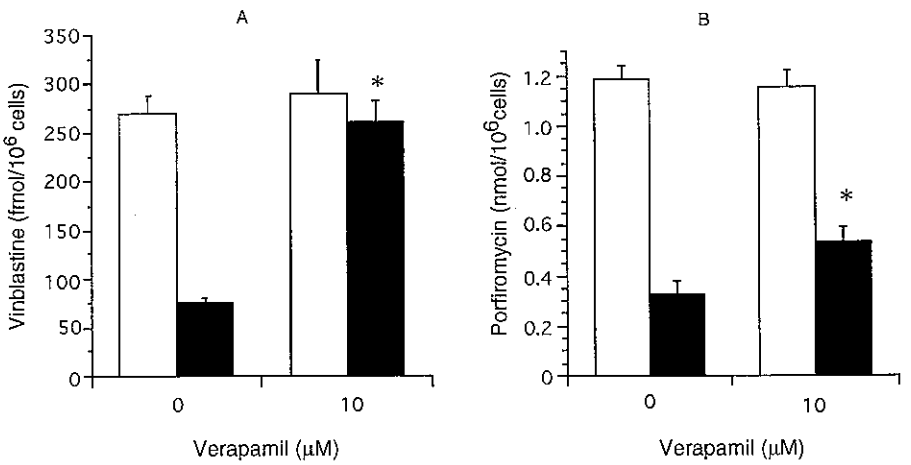

Fig. 3. The effect of verapamil on the intracellular accumulation of vinblastine (VLB) (A) and porfiromycin (PFM) (B) in AH130/P and AH130/5A cells. Cells (1×10^6 cells/ml) were incubated with 0.01 μM [^{3}H]VLB or 0.2 μM [^{3}H]PFM in the absence or presence of 10 μM verapamil for 30 min at 37°C. Each column represents the average of triplicate incubations. * Significantly different at $P<0.05$. □ AH130/P, ■ AH130/5A.

Table III. Comparison of the Activity of GST, GSHpx, DT-diaphorase, and NADPH Cytochrome *c* Reductase

	Enzyme activity (nmol/min/mg protein)	
	AH130/P	AH130/5A
GST	27±4	71±6*
GSHpx	213±14	92±8*
DT-diaphorase	433±101	38±5*
NADPH cytochrome *c* reductase	17±2	16±2

Data are means±SD of at least three independent experiments. * Significantly different at $P<0.05$.

the uptake of PFM was only partly restored by verapamil (Fig. 3). These results suggested that the resistance of VLB and a part of the resistance to MMC were dependent on the overexpression of PGP in AH130/5A cells.

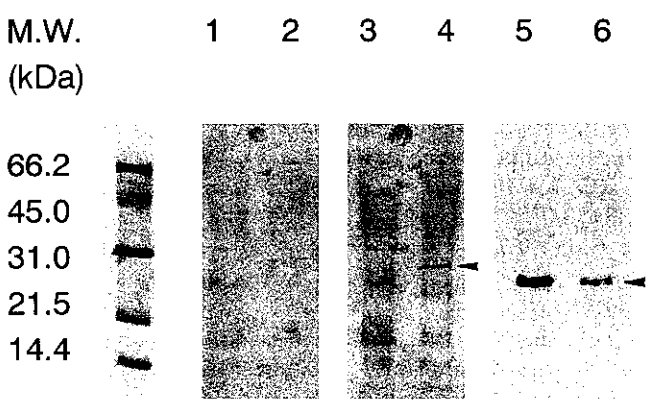

Fig. 4. Western analysis of GST isoenzyme. Lanes 1 and 2, lanes 3 and 4, and lanes 5 and 6 are immunoblots of the cytosol fraction with anti-rat GST-(1, 2), -(3, 4), and -P antibodies, respectively. Odd lanes are AH130/P cells and even lanes are AH130/5A cells. Arrowheads indicate the positions of GST isoenzymes.

Table IV. *In vitro* Combination Effects of BSO, Dicumarol, and *t*-BHQ with Adriamycin and Mitomycin C

Exp.	Antitumor agent	IC_{50} (μM)			
		AH130/P		AH130/5A	
		BSO (100 μM)			
		−	+	−	+
I	Adriamycin	0.045±0.001	0.039±0.004	8.2±1.2	8.9±0.6
	Mitomycin C	0.004±0.001	0.002±0.001	11.8±0.1	11.0±0.1
		dicumarol (100 μM)			
		−	+	−	+
II	Adriamycin	0.405±0.030	0.510±0.041	21.3±0.8	15.2±0.5*
	Mitomycin C	0.032±0.003	0.246±0.023*	23.2±0.7	12.1±0.4*
		t-BHQ (10 μM)			
		−	+	−	+
III	Adriamycin	0.395±0.026	0.415±0.030	19.8±2.2	21.0±0.9
	Mitomycin C	0.056±0.004	0.043±0.010	24.0±4.3	9.0±1.2*

I: Cells were incubated for 24 h in the absence (−) or presence (+) of 100 μM BSO and further incubated with or without antitumor agents for 48 h. II, III: Cells were incubated with or without 100 μM dicumarol for 30 min or 10 μM *t*-BHQ for 8 h and then incubated in the presence of varying concentrations of antitumor agent for 2 h. The cells were washed once and cultured in drug-free medium for 72 h. Data are means±SD of at least three independent experiments. * Significantly different at $P<0.05$.

Assessment of PGP-independent mechanisms To investigate the mechanisms of cross resistance to MMC, we determined the activity of GST, GSHpx, NADPH cytochrome *c* reductase, and DT-diaphorase, as shown in Table III. The microsomal NADPH cytochrome *c* reductase activity in these cells was almost the same. This enzyme does not seem to be involved in the mechanism of drug resistance. The activity of cytosolic GST was 2 times higher in AH130/5A cells than in AH130/P cells. GSHpx and DT-diaphorase activity in AH130/5A cells was decreased to a half and one-eleventh of those in AH130/P cells, respectively.

The expression of GST isoenzyme was investigated by using antibody to alpha (GST-1,2), mu (GST-3,4), and pi (GST-P) class isoenzymes, as shown in Fig. 4. In AH130/P cells, a band of a pi class enzyme was detected. In AH130/5A cells, mu and pi class enzymes were detected, while GST-P levels were significantly lower than in AH130/P cells.

To clarify further the relationship between GST or DT-diaphorase and drug resistance, we investigated the effects of BSO, dicumarol, and *t*-BHQ on the cytotoxicity of ADM and MMC, as shown in Table IV. BSO, which is an irreversible inhibitor of γ-glutamylcysteine synthetase,[23] did not influence the effects of ADM and MMC in AH130/P and AH130/5A cells. Dicumarol, an inhibitor of DT-diaphorase, decreased the action of

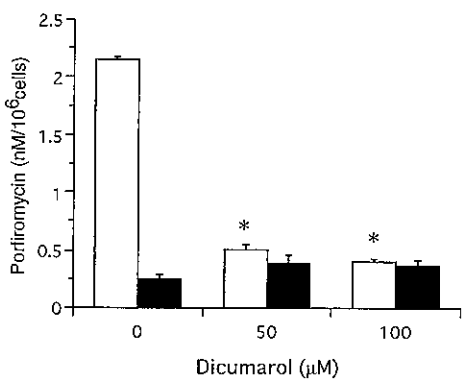

Fig. 5. The effect of dicumarol on the intracellular accumulation of porfiromycin (PFM) in AH130/P and AH130/5A cells. Cells (1×10^5 cells/ml) were incubated in the absence or presence of designated concentrations of dicumarol for 12 h at 37°C. The cells were washed, resuspended in fresh medium (1×10^6 cells/ml), and incubated with 0.2 μM PFM for 30 min at 37°C. Each column represents the average of triplicate incubations. * Significantly different from the corresponding control at $P<0.05$. □ AH130/P, ■ AH130/5A.

MMC in AH130/P cells, while potentiating the action of MMC and ADM in AH130/5A cells. *t*-BHQ, which has been reported to induce the expression of DT-diaphorase, probably through an antioxidant responsive element,[24]

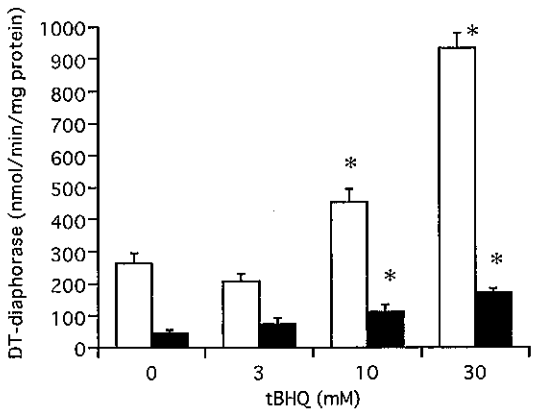

Fig. 6. The effect of *t*-BHQ on DT-diaphorase activity in AH130/P and AH130/5A cells. Cells (2×10^5 cells/ml) were preincubated in the absence or presence of designated concentrations of *t*-BHQ for 18 h and further incubated without *t*-BHQ for 4 h. Then, the cytosolic fractions were prepared and the DT-diaphorase activity was determined. Each column represents the average of triplicate determinations. * Significantly different at $P<0.05$. □ AH130/P, ■ AH130/5A.

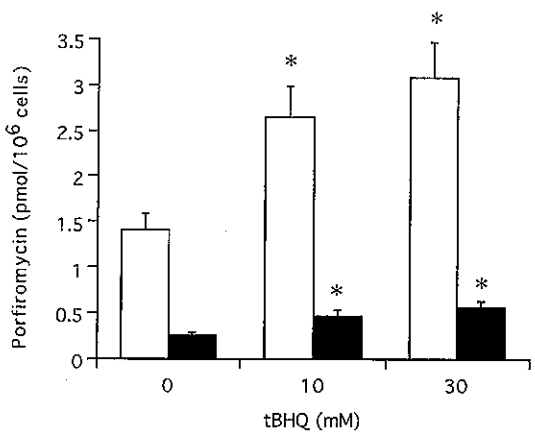

Fig. 7. The effect of *t*-BHQ on the intracellular accumulation of porfiromycin (PFM) in AH130/P and AH130/5A cells. Cells (2×10^5 cells/ml) were preincubated in the absence or presence of designated concentrations of *t*-BHQ for 18 h and further incubated without *t*-BHQ for 4 h. The cells were washed, resuspended in fresh medium (1×10^6 cells/ml), and incubated with 0.2 μM PFM for 30 min at 37°C. Each column represents the average of triplicate incubations. * Significantly different at $P<0.05$. □ AH130/P, ■ AH130/5A.

was found to potentiate the action of MMC in AH130/5A cells.

Fig. 5 shows that 50 μM dicumarol markedly inhibited the accumulation of PFM in AH130/P cells, while slightly increasing the accumulation in AH130/5A cells. This indicates that DT-diaphorase is involved in the accumulation of drugs in AH130/P cells.

In this context, the effect of *t*-BHQ on DT-diaphorase activity was studied in both cell lines. Treatment of cells with *t*-BHQ increased the DT-diaphorase activity in a dose-dependent manner in both cell lines; 30 μM *t*-BHQ increased the activity in AH130/5A cells by up to 50% as compared to that in AH130/P cells (Fig. 6). Furthermore as shown in Fig. 7, we found that PFM accumulation in AH130/5A cells increased in a dose-dependent manner by up to 40% as compared to that in AH130/P cells, after treatment with 30 μM *t*-BHQ.

These results suggested that the accumulation of PFM was affected by intracellular DT-diaphorase activity.

DISCUSSION

We established a multidrug-resistant cell line, AH130/5A, from rat hepatoma AH130 cells by ADM exposure. This cell line showed a quite different pattern of resistance from PGP-mediated multidrug resistant cells in that it exhibited a marked cross resistance to mitomycins.

Several suggestions have been made as to the possible mechanism of MMC-resistance. DNA excision repair has been proposed as a mechanism in Chinese hamster ovary cell mutants.[25] The DNA excision repair mechanism is generally observed in cells resistant to alkylating agents. However, AH130/5A cells were not highly resistant to other alkylating agents such as chlorambucil and cisplatin. Therefore other mechanisms are thought to underly MDR in AH130/5A cells.

Other possible mechanisms of MMC-resistance may involve PGP, GST, GSHpx, DT-diaphorase, and NADPH cytochrome *c* reductase. These enzymes are all involved in either the exclusion, activation, or inactivation of drugs. Among them, GSHpx activity in AH130/5A cells was found to be lower than in AH130/P cells, while the activity of NADPH cytochrome *c* reductase in AH130/5A cells was almost the same as that in AH130/P cells. Thus, these enzymes did not seem to be involved in MDR in AH130/5A cells.

The *mdr1* gene coding PGP has been shown to be overexpressed in colon, adrenal, kidney, and liver cancer. The tumor cells generally express *mdr1* at lower levels than the surrounding normal tissues, but expression in tumor cells is increased by chemotherapy.[26] In the present study, exposure of AH130/5A hepatoma cells to ADM was shown to induce overexpression of PGP, while H-87, a modifier of VLB-resistance that inhibits PGP,[22] potently reversed this resistance to ADM and MMC. In addition, PFM uptake in AH130/5A cells recovered to half of the level of the parent cells. Thus, mitomycins seemed to be effectively transported by PGP, and a part of the overall resistance to mitomycins in AH130/5A cells appears to be due to PGP.

GST-Pi is believed to be involved in detoxification, and probably resistance, by conjugating to alkylating agents including MMC and cisplatin.[27] It has been reported that a GST inhibitor modulates MMC-resistance.[28] Although AH130/5A cells showed higher GST activity than AH130/P cells, they expressed lower levels of GST-P isoenzyme. This higher GST activity seemed to depend on the overexpression of GST-mu. Our results also showed that BSO did not affect the actions of ADM or MMC. Thus, GSTs and GSH did not seem to contribute to resistance in AH130/5A cells. Recently MRP and GS-X-mediated MDR has been observed in a variety of tumors. As this resistance can be reversed by treatment with BSO, they are thought to be transporters of GSH-conjugates.[29, 30] In the present study, as BSO was ineffective against ADM and MMC, it was speculated that this type of mechanism is not involved in AH130/5A cells.

As for ADM, there are reports indicating that anthracyclines either are[31, 32] or are not substrates of DT-diaphorase.[32] It was also reported that the treatment of cells with ADM could induce the overexpression of DT-diaphorase.[33] Thus, it has been unclear whether the action of ADM might be modified by intracellular DT-diaphorase activity. In the present study, although AH130/5A cells showed significantly lower DT-diaphorase activity than AH130/P cells, the sensitivity to ADM of AH130/P and AH130/5A cells was not affected by either dicumarol or *t*-BHQ. Our results imply that ADM is not a substrate of DT-diaphorase. Further investigation is needed to elucidate the mechanism of low DT-diaphorase activity.

As for mitomycins, we found that DT-diaphorase could be induced by treatment with *t*-BHQ in both parent and AH130/5A cells, and that the uptake of PFM in AH130/5A cells was increased by up to 50% compared to the parent cells, by treatment with 30 μM *t*-BHQ, which similarly increased DT-diaphorase activity by up to 40%. In addition, 10 μM *t*-BHQ slightly potentiated the growth-inhibitory effect of MMC against AH130/5A cells. Moreover, dicumarol, an inhibitor of DT-diaphorase, reduced PFM uptake in the parent cells. These results suggested that the activity of DT-diaphorase affected the accumulation of PFM, probably through reductive activation and binding to target molecules. Thus, in AH130/5A cells, a part of the overall resistance to mitomycins seems to depend on the low activity of DT-diaphorase.

Recently it was reported that transfection of the DT-diaphorase gene did not affect sensitivity to mitomycins.[35] In contrast, another report found a relationship between DT-diaphorase and sensitivity to mitomycins, in DT-diaphorase-transfected cells.[36] Our results support the latter finding, as well as that of another study that showed low incorporation of PFM in resistant cells with low DT-diaphorase activity.[37]

In conclusion, our findings indicated that ADM exposure induced a clone with marked cross resistance to MMC, and that this resistance is related not only to decreased DT-diaphorase activity, but also to overexpression of PGP. In addition, *t*-BHQ treatment increased MMC uptake and partially reversed MMC-resistance, through the induction of DT-diaphorase. Further investigation is needed to elucidate whether this type of multifactorial resistance frequently appears after ADM exposure and whether combined therapy with an agent such as *t*-BHQ would be effective *in vivo*.

ACKNOWLEDGMENTS

The authors thank Miss. Y. Kawakami for her skillful assistance in cell cultures.

(Received August 9, 1996/Accepted October 21, 1996)

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