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Cross-resistance and collateral sensitivity to natural product drugs in cisplatin-sensitive and -resistant rat lymphoma and human ovarian carcinoma cells

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Abstract The cytotoxicity of mitotic spindle poisons, vinca alkaloids and the anthracycline, adriamycin, against cisplatin-sensitive and -resistant rat lymphoma and human ovarian carcinoma cell lines was investigated. Interestingly, it was found that all cell lines were more sensitive to the mitotic spindle poisons, vincristine and vinblastine. Adriamycin was the least effective and taxol had intermediate activity. The Walker rat lymphoma cell line resistant to cisplatin (WR) exhibited the multiple drug resistance phenotype since it showed collateral resistance to all drugs (ranging from twofold to taxol, colcemid and colchicine and sixfold to the vinca alkaloids). Verapamil potentiated the cytotoxic activity of adriamycin and vincristine in a striking fashion with the Walker cells. P-glycoprotein was found to be present in the plasma membranes of the Walker cells with approximately a 2.5-fold increase in the WR as compared to the sensitive (WS) cells. Glutathione levels were elevated in all of the cisplatin-resistant cell lines when compared to the cisplatin-sensitive parental cell lines. A profound effect of buthionine sulfoximine pretreatment on adriamycin cytotoxicity was observed. Glutathione S-transferase (π) was present in all the human cell lines but the WS cells had markedly lower levels (almost negligible) when compared to the WR cells. These observations imply that cisplatin-resistant cells may be more sensitive to mitotic spindle poisons and vinca alkaloids, irrespective of the mechanism of platinum resistance, and that the cytotoxicity of vinca alkaloids could be further modulated by verapamil, irrespective of the presence or absence of P-glycoprotein.

Key words Cisplatin resistance · Mitotic spindle poisons · Hypersensitivity

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Introduction

Cisplatin is a broad-spectrum antitumor drug with proven efficacy against a variety of solid tumors. However, normal organ toxicity and the emergence of a drug-resistant tumor subpopulation limit the clinical potential of cisplatin. Resistance to cisplatin is multifactorial and some of the proposed mechanisms include reduced intracellular drug accumulation, increased drug inactivation, and an increased ability to repair cisplatin-damaged DNA [6, 8, 17, 28, 31, 32].

Although reduced drug accumulation has been observed in cells resistant to the platinum drugs [8, 16], it has been demonstrated that this is not due to the overexpression of the membrane transport pump (P-glycoprotein) [10, 13, 16], which is associated with the multidrug resistance (MDR) phenotype. MDR is characterized by cross-resistance to several structurally unrelated drugs including the vinca alkaloids, anthracycline antibiotics, and other natural product drugs [10].

Increased levels of intracellular thiol and thiol-associated enzymes has also been observed in cisplatin-resistance tumors and is related to the resistance phenotype [4, 9, 12, 13, 18, 22, 24, 28, 29, 31]. Glutathione (GSH), a potent nucleophile, has the potential to react with most xenobiotics and aid in their inactivation. However, in addition to cells resistant to alkylating agents, cells exhibiting the MDR phenotype have also been shown to possess increased levels of intracellular GSH and glutathione salvage enzymes, primarily glutathione S-transferase (π) (GST-π) [12, 13, 29].

In order to successfully treat a cisplatin-resistant tumor, it is important to determine if the neoplastic cells are resistant to other antitumor drugs. Thus, it is of clinical significance to identify the patterns of cross-resistance and the collateral sensitivity of cisplatin-resistant tumor cells. The present report describes and compares the effects of cisplatin, mitotic spindle poisons, and anthracycline antibiotics on the cisplatin-sensitive Walker rat lymphoma cell line (WS) and two human ovarian carcinoma cell lines (2008, 2780), and their cisplatin-resistant variants. The precise mechanism(s) of cisplatin resistance in cisplatin-resistant Walker (WR) cells is unknown. A decrease in intracellular cisplatin accumulation (compared to the cisplatin-sensitive 2008 cells) has been reported to be the basis of cisplatin resistance in the 2008/C13* cells, while an increased thiol metabolizing system (compared to the 2780 cells) has been implicated in the resistance mechanism of the C70 cells [5, 9, 11, 15]. Furthermore, the ability of verapamil, a potent modulator of P-glycoprotein activity and buthionine sulfoximine (BSO), an inhibitor of glutathione metabolism, to enhance the cytotoxic effects of these drugs was also investigated.

Materials and methods

Materials

Cisplatin was purchased from Aldrich (Milwaukee, Wis). Taxol (TAX) was provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, NIH (Bethesda, Md.). Adriamycin (ADR), BSO, colchicine (COL), colcemid (COE), verapamil, vincristine (VCR), vinblastine (VBL), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical (St. Louis, Mo.). All drugs were freshly prepared at 100 times the required concentration. If the drugs were dissolved in dimethylsulfoxide, the final concentration of this solvent never exceeded 0.1% in the experimental protocol. The anti-GST-π mouse monoclonal antibody was purchased from Biogenex (San Ramon, Calif.). The anti-P-glycoprotein mouse monoclonal antibody (JSB-1) was from Signet Laboratories (Dedham, Mass.). The alkaline phosphatase conjugated anti-mouse rabbit monoclonal antibody was obtained from Jackson ImmunoResearch Laboratories (West Grove, Pa.).

Cells and culture conditions

Walker rat lymphoma cells sensitive (WS) and resistant (WR) to cisplatin were provided by Dr. J.J. Roberts, Institute of Cancer Research, Sutton, UK. These cells were maintained as described previously [30] except 10% bovine calf serum was employed instead of 10% fetal calf serum. The cisplatin-sensitive (2008) and -resistant (2008/C13*) human ovarian carcinoma cells were kindly supplied by Dr. Paul Andrews, Georgetown University, Md. and the cisplatin-sensitive (2780) and -resistant (C70) human ovarian carcinoma cells were obtained from Dr. T. Hamilton, Fox Chase Cancer Center, Philadelphia, Pa. The human ovarian carcinoma cells were maintained as described previously [1, 2, 9, 14].

Cytotoxicity assay

The *in vitro* cytotoxicity of each drug alone, or in combination, was evaluated by the tetrazolium salt assay [23], utilizing modifications of a previously published procedure [25]. Briefly, cells (1000/well rat lymphoma cells and 5000/well human ovarian carcinoma cells) were seeded in 96-well tissue culture plates (Becton Dickinson, N.J.) and exposed to appropriate concentrations of anticancer drugs for a period of 3 days. In the experiments where pretreatment with verapamil or BSO was being investigated, one-half of the cell number was seeded into the 96-well tissue culture plates and exposed to the indicated concentrations of the modulator for a period of 24 h. Thereafter, appropriate concentrations of the anticancer drug was added to each well and the plates incubated as before for 3 days. A solution (5 mg/ml) of MTT in phosphate-buffered saline was prepared and 20 µl was added to each well 6 h before the end of the exposure period, and 100 µl of 0.1 N HCl-isopropanol was added to dissolve the formazon crystals. The absorbance of each well was measured at 570 nm in a microplate reader (Bio-Tek Instruments VT.). Wells containing no cells were used as blanks and wells containing cells but no drugs were used to determine the control cell survival.

GSH determinations

Cells (5×10^6) were washed with cold phosphate-buffered saline and then lysed by the addition of 5% trichloroacetic acid. After maintaining on ice for 30 min, the samples were centrifuged at $10\,000 \times g$ for 10 min. The supernatant was assayed for GSH content as described previously [26]. Briefly, 0.1–0.2 ml of the supernatant was allowed to react with 0.6 mM DTNB in 0.2 M sodium phosphate buffer, pH 8.0, for 10 min at room temperature. Absorbance of the colored product was estimated at 412 nm. For each experiment, a standard curve of known amounts of reduced GSH was prepared. Protein was determined by the Bradford assay [3] with bovine serum albumin as standard.

Immunoblot analysis

For the detection of GST-π, the total cellular homogenate was prepared by lysing cells in 10 mM TRIS-HCl, pH 7.4, 100 mM NaCl, 0.5% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride (PMSF). Lysate (100 µg) from the cell extract was mixed with an equal volume of 2 × SDS buffer (40% glycerol, 6% SDS, 0.25 M TRIS-HCl, pH 6.8, 0.1% bromophenol blue, 0.7 M 2-mercaptoethanol) and placed at 100°C for 3 min. The samples were subjected to electrophoresis in a 15% separating gel according to previously published procedures [20]. A semi-enriched membrane fraction was prepared to detect P-glycoprotein by suspending the cells (5×10^7) in a buffer containing 10 mM TRIS-HCl, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$ and a protease inhibitor cocktail (2 mM PMSF, 2 µg/ml aprotinin, 200 µg/ml EDTA, 1 µg/ml pepstatin). After 10 min on ice, the cells were homogenized with a Dounce homogenizer and adjusted to 250 mM sucrose before centrifugation at $800 \times g$ at 4°C for 20 min. The supernatant was further centrifuged at $100\,000 \times g$ at 4°C for 20 min. The pellet (membrane-enriched fraction) was resuspended in 10 mM TRIS-HCl, pH 7.4, 125 mM sucrose and the protease inhibitor cocktail. The membrane proteins (100 µg) were mixed with solubilization buffer (4 M urea, 0.5% SDS, 50 mM DTT) and subjected to electrophoresis in a 6% separating gel without heating.

For both procedures, the separated proteins were electrophoretically transferred to Immobilon-P membranes. The membranes were preincubated with a blocking solution containing 25 mM TRIS-HCl, pH 7.5, 150 mM NaCl (TBS), 5% (w/v) non-fat dried milk for 60 min at room temperature with agitation. The membranes were

then incubated with the primary antibody in TBS for 2 h. The membranes were washed with TBS containing 0.05% (v/v) Tween-20 (TBS-T), 3 times for 10 min and then treated with alkaline phosphatase-conjugated secondary antibody for 60 min. After thorough washing with TBS-T, the membranes were stained with an alkaline phosphatase detection kit (Bio-Rad, Calif.).

Statistical analysis

P values were determined by paired *t*-test using a SigmaStat Statistical Analysis System, version 1.01.

Results

The relative sensitivity to cisplatin, ADR, COL, COE, VCR, VBL, and TAX was evaluated in three pairs of cisplatin-sensitive (WS, 2008, 2780) and -resistant (WR, 2008/C13*, C70) cell lines (Table 1). The WR, 2008/C13* and C70 cells were 8-fold, 12-fold and 9-fold resistant to cisplatin, respectively, compared to their sensitive counterparts (Table 1). The degree of cisplatin resistance of these cells compares well with previously published values [5, 9, 11, 19, 21]. Relative to WS, the WR cells were resistant to all the drugs tested, clearly indicating the MDR phenotype. Although sensitivity to ADR, COL, and COE was similar in the 2008 and 2008/C13* cells, the 2008/C13* cells were found to be tenfold hypersensitive to VCR, VBL, and TAX compared to the 2008 cells (Table 1). The result with TAX is in agreement with that reported by Christen *et al.* [5]. The C70 cells, besides being resistant to cisplatin, displayed a fourfold resistance to ADR compared to the 2780 cells (Table 1).

The effect of ADR and VCR on WS, WR, 2008, 2008/C13*, 2780, and C70 cell survival was assessed in the presence and absence of 10 μM verapamil (a concentration which had no cytotoxicity with all six cell lines). Pretreatment with verapamil significantly potentiated the cytotoxic effects of ADR and VCR in all the cell lines (Figs. 1, 2). This effect was most pronounced

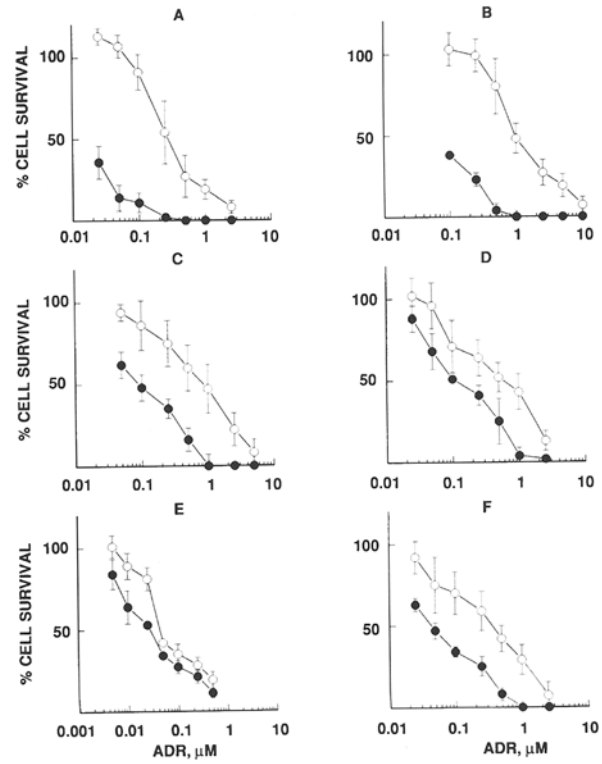

Fig. 1 Effect of 24-h pretreatment with 10 μM verapamil on the cytotoxicity of adriamycin (*ADR*) in cisplatin-sensitive (WS **A**, 2008 **C**, 2780 **E**) and -resistant (WR **B**, 2008/C13* **D**, C70 **F**) tumor cells. Cells were treated with the anticancer drug alone (*open circles*) or in combination with verapamil (*closed circles*). Each point represents the mean of three separate experiments performed with triplicate cultures; *error bars* indicate ± SD

with the rat lymphoma cells. The enhancement in ADR cytotoxicity (expressed as the change in the IC_{50} with and without verapamil) was 57-, 26-, 7-, 3-, 2- and 6-fold for the WS, WR, 2008, 2008/C13*, 2780, and C70 cells, respectively. The enhancement of VCR cytotoxicity produced by verapamil was even more striking (Fig. 2). Concomitant exposure of the tumor cells to verapamil and ADR or VCR also displayed a superior

Table 1 Effect of various anticancer drugs on the cell survival of the Walker rat lymphoma cells and the human ovarian carcinoma cells sensitive and resistant to cisplatin. The error is expressed as ± SD from three separate experiments. IC_{50} values are the concentrations which reduced the cell survival to half the control value and are calculated from a regression analysis of cell survival versus drug concentration. (*WS* Walker rat lymphoma cell line sensitive to cisplatin, *WR* Walker cell line resistant to cisplatin)

	IC_{50} (nM)					
Drugs	WS	WR	2008	2008/C13*	2780	C70
Cisplatin	900 ± 30	7,100 ± 1,000	1,500 ± 500	18,000 ± 4,000	700 ± 200	6,400 ± 1,000
Adriamycin	340 ± 10	1,300 ± 80	680 ± 20	480 ± 30	70 ± 10	290 ± 10
Colchicine	100 ± 20	220 ± 50	20 ± 4	20 ± 2	14 ± 3	16 ± 2
Colcemid	19 ± 1	37 ± 6	36 ± 2	28 ± 1	20 ± 1	23 ± 2
Vincristine	12 ± 4	74 ± 8	22 ± 4	4 ± 1	1.3 ± 0.5	2 ± 1
Vinblastine	6 ± 2	33 ± 4	10 ± 2	4 ± 2	2 ± 0.7	1 ± 0.2
Taxol	190 ± 40	410 ± 110	65 ± 8	4 ± 2	5 ± 1	6 ± 2

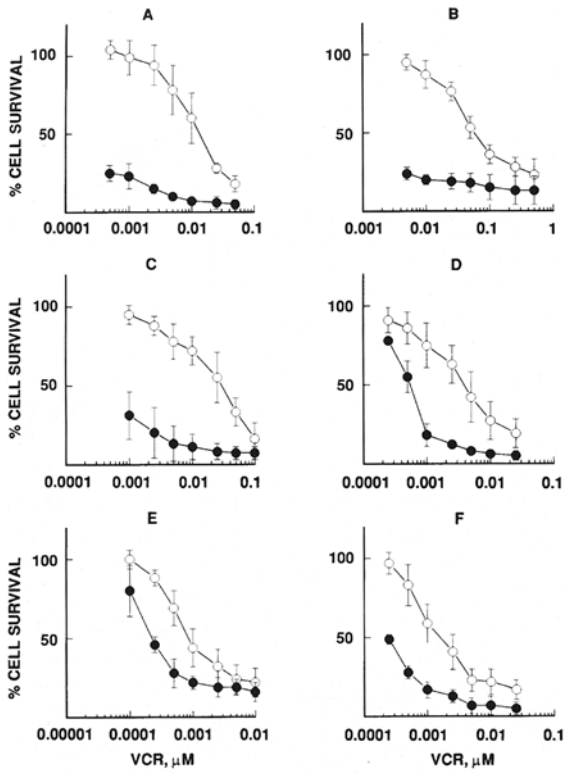

Fig. 2 Effect of 24-h pretreatment with 10 μM verapamil on the cytotoxicity of vincristine (*VCR*) in cisplatin-sensitive (WS **A**, 2008 **C**, 2780 **E**) and -resistant (WR **B**, 2008/C13* **D**, C70 **F**) tumor cells. Cells were treated with the anticancer drug alone (*open circles*) or in combination with verapamil (*closed circles*). Each point represents the mean of three separate experiments performed with triplicate cultures; *error bars* indicate ± SD

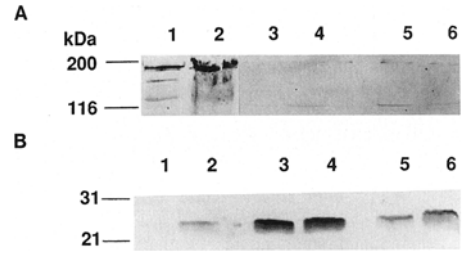

Fig. 3A Analysis of P-glycoprotein content of the Walker rat lymphoma and human ovarian carcinoma cells sensitive (WS, 2008, 2780) and resistant (WR, 2008/C13*, C70) to cisplatin. Lane 1, WS; lane 2, WR; lane 3, 2008; lane 4, 2008/C13*; lane 5, 2780; lane 6, C70. **B.** Expression of glutathione S-transferase (π) in the Walker rat lymphoma and human ovarian carcinoma cells sensitive and resistant to cisplatin. Lane 1, WS; lane 2, WR; lane 3, 2008; lane 4, 2008/C13*; lane 5, 2780; lane 6, C70

Table 2 Glutathione levels in cisplatin-sensitive and -resistant cells. Total glutathione was estimated colorimetrically in the rat lymphoma (WS and WR, respectively) and human ovarian carcinoma (2008, 2008/C13*, 2780, and C70, respectively) cells. The figure in parenthesis is the number of experiments and the error ± SD

Cells	Glutathione, (nmol/mg protein)	Relative increase of resistant/sensitive cells
WS	15.3 ± 8.5 (4)	–
WR	31.7 ± 18 (4)[a]	2.0
2008	11.6 ± 3.6 (4)	–
2008/C13*	17.6 ± 3.0 (4)[a]	1.5
2780	6.4 ± 3.0 (4)	–
C70	30.7 ± 3.4 (4)[b]	5.0

[a] No significant difference between the cisplatin-sensitive and-resistant cells
[b] $P < 0.001$, as compared to cisplatin-sensitive 2780 cells

Table 3 Effect of pretreatment with 10 μM buthionine sulfoximine (*BSO*) on the cytotoxicity of Adriamycin (*ADR*) and vincristine (*VCR*) in cisplatin-sensitive and -resistant rat lymphoma and human ovarian carcinoma cells. The error is expressed as ± SD from at least three separate experiments performed in triplicate. IC_{50} values are the concentration of the drug which reduced the cell survival to half the control value and were calculated from a regression analysis of cell survival versus drug concentration. The relative increase in cytotoxicity is defined as IC_{50} (ADR)/IC_{50} (BSO + ADR)

Cells	IC_{50} (nM)		Relative increase in cytotoxicity
	ADR	BSO + ADR	
WS	340 ± 10	160 ± 9	2.1
WR	1300 ± 80	570 ± 44	2.3
2008	680 ± 20	220 ± 14	3.1
2008/C13*	480 ± 30	180 ± 23	2.7
2780	70 ± 10	9 ± 2	7.8
C70	290 ± 10	60 ± 7	4.8

cytotoxic effect compared to that observed with the anticancer drug alone, however, the degree of potentiation was less when compared to that observed when tumor cells were pretreated with verapamil (data not presented).

Cell membranes were partially purified and analyzed for P-glycoprotein expression. The protein was only detectable in the rat lymphoma cells (Fig. 3A) and there was a 2.5-fold greater expression (analyzed by densitometric scanning; data not shown) in the resistant (WR) as compared to the sensitive (WS) cells.

Next, the intracellular levels of GSH in all the cell lines were determined (Table 2). WR and C70 cells had a twofold and fivefold increase, respectively, in cellular GSH levels, when compared to the sensitive parental cell. In contrast, the cellular GSH levels in the 2008 and 2008/C13* cells were almost the same.

The effects of pretreatment with 10 μM BSO, an inhibitor of GSH metabolism, on the cytotoxicity of ADR, the mitotic spindle poisons, and TAX were studied. The concentration of BSO employed in these experiments produced no toxicity with any cell line. The cytotoxicity of ADR was markedly enhanced by BSO pretreatment (Table 3). However, BSO did not affect the cytotoxicity of COL, VCR, and TAX (data not shown).

Western blot analysis was performed to ascertain the intracellular levels of the enzyme, GST-π, in each cell line. There was a fivefold higher concentration of the enzyme in the WR as compared to WS cells (Fig. 3B). However, no difference was observed between both human ovarian cell lines (2008, 2780) and their cisplatin-resistant counterparts (2008/C13*, C70).

Discussion

A distinct pattern of cross-resistance and collateral sensitivity in the cisplatin-resistant cell lines has been demonstrated even though the resistant subpopulations were selected after chronic exposure to cisplatin alone. Thus, a cisplatin-resistant rat lymphoma (WR) cell line displayed the MDR phenotype, the cisplatin-resistant human ovarian carcinoma cell line (2008/C13*) was hypersensitive to VCR, VBL, and TAX, and the cisplatin-resistant human ovarian carcinoma cell line (C70) was cross-resistant to ADR, when compared to their parental cisplatin-sensitive counterparts (WS, 2008, 2780, respectively). All the cell lines studied were more sensitive to the mitotic spindle poisons, COE, VCR, and VBL when compared to their sensitivity to other drugs. Furthermore, irrespective of the presence or absence of the MDR-mediating P-glycoprotein, verapamil potentiated the cell growth inhibition caused by VCR. This observation may prove to have some clinical relevance. ADR was the least effective and TAX, which has been reported to be markedly cytotoxic with 2008/C13* cells [5], showed an intermediate effect with striking sensitivity exhibited by the 2780 and C70 cells, but a far less dramatic effect with the rat lymphoma cells.

Cisplatin resistance in the Walker cells is poorly understood, but the cisplatin-resistant variant, WR, displayed collateral resistance to all the six drugs tested, ranging from a twofold resistance for TAX, COE and COL to a sixfold effect for the vinca alkaloids, VCR and VBL. These results suggested an MDR phenotype for the WR cells. The MDR phenotype is characterized by overexpression of the P-glycoprotein [10]. This has been correlated with high degrees of resistance to the natural product anticancer drugs. Inhibition of the P-glycoprotein function with verapamil has been reported to potentiate the cytotoxic activity of the natural product anticancer drugs [10,27]. The effect of verapamil on the cytotoxicity of ADR and VCR was therefore investigated. The effect of this calcium channel blocker on the cytotoxicity of both drugs was striking with the Walker cells. Analysis of the P-glycoprotein content of the cell membranes employing western blot analysis showed that the transport protein was detectable only in the Walker cell membrane fraction and not in the membrane fractions from the human ovarian cell lines. A 2.5-fold increase in the level of P-glycoprotein was observed in the membranes from WR cells, a result which correlates with an increased resistance of this cell line to vinca alkaloids, mitotic spindle poisons, and ADR relative to the WS cell line.

Alterations in the cellular thiols have been observed in cells resistant to cisplatin and ADR [4, 9, 12, 13, 18, 22, 24, 28, 29, 31]. Therefore, it was of interest to investigate if thiols were involved in the observed cross-resistance and collateral sensitivity. GSH levels in all three cisplatin-resistant cell lines were increased compared to the cisplatin-sensitive cells but the most striking change was observed with the C70 cells. The GSH levels of 2780/C70 ovarian cell lines are difficult to compare to the data reported by Godwin *et al.* [9] since these authors reported GSH concentration per cell. Unfortunately, the cell volume of the resistant (C70) cells is substantially greater than the parental (2780) cell line and as a result the data presented here are expressed relative to the total cellular protein. Expressing the data in this fashion still results in the resistant cells (C70) possessing substantially higher intracellular concentrations of GSH (fivefold) when compared to those in the sensitive cells. The differences observed in cisplatin sensitivity between the 2780 human ovarian carcinoma cells and its resistant counterpart thus appear to be due to differences in GSH metabolism [9,11] and it is interesting to note that the only collateral resistance observed with these lines was with ADR, a drug where GSH metabolism has been implicated in its cytotoxic effects [13, 31]. These data correlate well with those reported by Hamaguchi *et al.* [11]. The similar levels of GSH in 2008 and 2008/C13* cells correspond well with the data reported by Andrews *et al.* [1,2].

BSO, an inhibitor of GSH metabolism, produced a profound effect on the cytotoxicity of ADR. With all three cell lines, the effect was most striking with the 2780 and C70 cells. BSO had no effect on the cytotoxicity of COL, VCR, and TAX. These results are not unexpected since ADR cytotoxicity has been reported to be associated with GSH metabolism. However, GSH has not to date been reported to play a role in the cytotoxicity of mitotic spindle poisons or TAX. This is in keeping with the ineffectiveness of BSO to potentiate their cytotoxicity. Furthermore, the increase in ADR cytotoxicity in the presence of BSO was sequence-dependent; if tumor cells were treated simultaneously with BSO and ADR, no potentiation of ADR cytotoxicity was observed, a result which is to be expected due to the inhibition kinetics of BSO on GSH metabolism.

The expression of GST-π has been associated with resistance to ADR and cisplatin [12, 29, 31]. Analysis of the GST-π enzyme, an enzyme in the GSH salvage pathway most commonly implicated in drug cytotoxicity, showed that the enzyme was present in all the cell lines except for almost undetectable levels in the WS

cells. Clapper *et al.* [7] have previously reported increases in GST-π in the Walker cells pretreated with chlorambucil. In the present study, a marked increase in the basal, intracellular levels of this enzyme in the WR cells (fivefold) compared to the WS cells was observed. Furthermore, a minimal difference was observed between its levels in the C70 cells as compared to the parental (sensitive) cells, data in agreement with those reported by Hamaguchi *et al.* [11]. No difference was observed between the 2008 and 2008/C13* cells. This latter result is expected since the 2008/C13* cell line appears to be a cisplatin transport mutant [5, 15, 21].

Irrespective of the primary mechanism of cisplatin resistance, this study brings to light two salient points: (a) cisplatin-resistant cells may be more sensitive to mitotic spindle poisons; and (b) the effect of the vinca alkaloids, such as VCR, can be further potentiated if verapamil is included in the combination chemotherapy. The therapeutic importance of these findings has to await further clinical studies.

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