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Cross-resistance to *cis*-diamminedichloroplatinum(II) of a multidrug-resistant lymphoma cell line associated with decreased drug accumulation and enhanced DNA repair

Chuck C.-K. Chao *

Tumor Biology Laboratory, Department of Biochemistry, Chang Gung Medical College, Taoyuan 33332, Taiwan, ROC

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Abstract

HOB1/VCR, a multidrug-resistant subline of the immunoblastic B lymphoma cell line, was established by sequential selection in increasing concentrations of vincristine. The expression of the human *mdr1* gene, as analyzed by reverse transcription and polymerase-chain reaction (RT-PCR), revealed a 10–15-fold overexpression in this resistant cell line. A complete inhibition of vincristine resistance by verapamil was observed in the vincristine-resistant HOB1/VCR cells, which suggests that acquired resistance may be mainly due to P-glycoprotein. HOB1/VCR cells also developed a 67-fold cross-resistance to the anticancer drug cis-diamminedichloroplatinum (cisplatin). DNA repair of the resistant and the parental cell lines was investigated by in situ detection with a cisplatin-DNA adduct-specific antibody and by measurement of repair-associated host cell reactivation of damaged plasmid DNA. HOB1/VCR cells exhibited a 2-fold decrease in the level of cisplatin-DNA adducts, compared to the parental cells. The DNA repair rate following peak accumulation of cisplatin-DNA adducts (which took ~4 h) was also enhanced in the resistant cells. This was supported by the measurement of the cisplatin level remaining in cells by atomic absorption spectrophotometry, which showed a 2.7-fold reduction in the resistant cells. In addition, the acquired resistance and enhanced DNA repair in HOB1/VCR cells were partially reversed by nontoxic aphidicolin, a DNA polymerase-α and DNA repair inhibitor. Inhibition of the intracellular level of glutathione by DL-buthionine-[*S,R*]-sulfoximine demonstrated that cell viability was inhibited 4-fold more in the resistant cells than in the parental cells. The results suggest that the reduced formation of cisplatin-DNA adducts and the increased glutathione content of the multidrug-resistant cells play a major role in phenotypic cross-resistance to cisplatin.

Keywords: Cisplatin; DNA repair; Drug resistance; Multidrug resistance

1. Introduction

Cis-diamminedichloroplatinum(II) (cisplatin) is one of the most useful chemotherapeutic agents used against various malignant tumors such as brain, head and neck, ovarian, and testicular carcinomas (Loehrer and Einhorn, 1984; Rosenberg, 1985). Occasionally, resistant cells arise in residual tumor, causing the eventual failure of cancer therapy. A number of studies have described the isolation of cisplatin-resistant cells and have postulated different mechanisms for cisplatin resistance. The interaction of cisplatin with DNA has been implicated as the major cytotoxic action of the drug (Roberts and Thomson, 1979). Cisplatin generates various forms of cisplatin-DNA adducts, including intra- and interstrand DNA crosslinks, DNA-protein crosslinks, cisplatin-DNA-glutathione crosslinks and monoadducts (Hospers et al., 1988). Most cisplatin-resistant cells are also resistant to alkylating agents or even cadmium, which is consistent with the notion that cisplatin resistance is closely associated with DNA repair or an alteration in the level of free radical scavengers like glutathione or metallothionein (Hospers et al., 1988; Andrews and Howell, 1990; Timmer-Bosscha et al., 1992). Using a cell-free repair system, independent investigations demonstrated that cell extracts could carry out repair synthesis in DNA damaged by UV, psoralens and platinating

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agents (Wood, 1989; Hansson and Wood, 1989; Sibghat-Ullah et al., 1989) at sites of DNA damage (Hansson et al., 1989; Svoboda et al., 1989), whereas, extracts from some xeroderma pigmentosum cells failed to repair damaged plasmid DNA in vitro (Wood et al., 1988). We and others had previously demonstrated a cisplatin-resistant HeLa cell line (Chao et al., 1991a), which was cross-resistant to UV irradiation and which overproduced damaged-DNA recognition proteins (Chao et al., 1991b,c). Improved recognition and incision of cisplatin- or UV-DNA adducts were demonstrated in these resistant cells (Chao and Huang, 1993; Chao, 1994a,b), suggesting a close association between cisplatin resistance and efficient DNA repair.

In contrast to cisplatin resistance, multidrug resistance (MDR), which is mediated by a totally different resistance mechanism, is found in a wide spectrum of cell cultures and in clinical studies following exposure to MDR drugs. Numerous resistant cell lines selected in vitro by a single anticancer drug, such as an anthracycline, a Vinca alkaloid, or a taxane often show coordinate resistance to other structurally and functionally unrelated hydrophobic drugs. In many instances, MDR cells overexpress a 150–180 kDa integral membrane protein encoded by the *mdr1* gene, termed P-glycoprotein, which is believed to confer resistance by acting as a drug efflux pump. Resistant sublines show decreased intracellular drug accumulation due to ATP-dependent efflux of cytotoxic agents (reviewed by Endicott and Ling, 1989; Gottesman and Pastan, 1993). A non-P-glycoprotein-mediated MDR phenotype has also been demonstrated. A number of cell lines have been described, such as the human small cell lung cancer cell line H69AR (Mirski et al., 1987; Cole et al., 1991) and the human non-small cell lung cancer cell line PC-9 (Chiang et al., 1994), which both display MDR but do not overexpress P-glycoprotein. Multidrug resistance in the clinical setting appears to be multifactorial and may involve mechanisms besides P-glycoprotein overexpression (Murren and Hait, 1992).

An immunoblastic B-lymphoma cell line (termed HOB1), overexpressing c-*H-ras* and c-*myc* oncogenes, was established from a patient who did not respond to chemotherapy for an extranodal immunoblastic lymphoma (Ho et al., 1990). The patient failed to respond to a combination of CHOP (cyclophosphamide, adriamycin, vincristine and prednisone) and MOPP (nitrogen mustard, vincristine, procarbazine and prednisone) chemotherapy, and died 3 months following treatment. In this study, a vincristine-resistant HOB1 cell line (HOB1/VCR) was established and was found to have an unusual resistance to a spectrum of structurally and functionally unrelated drugs, including classical MDR drugs (e.g., Vinca alkaloids and anthracycline) and DNA-damaging agents (e.g., cisplatin). Collectively, the results suggest that reduced adduct formation, enhanced DNA repair, and increased glutathione content play critical roles in cisplatin resistance of the MDR cells.

2. Materials and methods

2.1. Chemicals, media and antibodies

Adriamycin, aphidicolin, BSO (DL-buthionine-[*S,R*]-sulfoximine), colchicine, mitomycin C, puromycin, vincristine, and verapamil were purchased from Sigma, St. Louis, MO, cisplatin from Farmitalia, and RPMI 1640 medium from Hyclone (Logan, UT). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, and penicillin and streptomycin were obtained from Gibco, Gaithersburg, MD. Polyclonal antibody to P-glycoprotein, mdr (Ab-1), was purchased from Oncogene Science, Uniodale, NY. Peroxidase-conjugated goat anti-mouse immunoglobulins were purchased from Dako, Copenhagen, Denmark. Monoclonal antibody MAb 62-5 (Chao et al., 1994) was raised against cisplatin-treated calf-thymus DNA by the method of Sundquist et al. (1987). Others were from Sigma or Merck.

2.2. Cells and drug resistance

A human HOB1 cell line was established from a gingival biopsy of a Taiwanese male patient with immunoblastic lymphoma (Ho et al., 1990) and was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. Vincristine-resistant (HOB1/VCR) cells were derived after an initial exposure of HOB1 cells to a low concentration of vincristine (0.001 μg/ml), followed by a 10-fold increment of the drug concentration up to 0.1 μg/ml. HOB1/VCR cells were cultured at this drug concentration to maintain the resistant phenotype, or grown in drug-free medium for three population doublings before experiments. Colon cancer cells (SW620, SW620/MDR) and cervical carcinoma cells (HeLa and HeLa/CPR) were cultured as previously described (Chao et al., 1991a,1992). Cell viability was determined by drug-induced growth inhibition using MTT dye (3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide) (Sigma) as described before (Mosmann, 1983). The drug resistance of cellular clonogenicity was defined as the ratio of the IC_{50}, the drug concentration inhibiting 50% cell growth, of tested cells to that of control cells. Cell clonogenicity was determined in soft agar 2 weeks following treatment. The resistance of cells was calculated by the ratio of D_0 of the resistant and the parental cells, the drug concentration inhibiting 63% of cell proliferation. In some cases, a sublethal dose (10 μg/ml) of aphidicolin (a DNA α-polymerase inhibitor), or 50 μM of BSO (a glutathione synthesis inhibitor) was added to the culture medium during cisplatin treatment.

2.3. Reverse transcription and polymerase-chain reaction (RT-PCR)

Preparation of DNA and RNA, and the related enzymatic reactions for RT were prepared and processed by

standard methods (Sambrook et al., 1989). PCR was performed with cDNA derived from 50 ng of cellular RNA or otherwise indicated, 1 U of Taq DNA polymerase and reaction buffer (Stratagene) in a microprocessor-driven thermal cycler (Perkin-Elmer/Cetus) in a final volume of 25 μl as described (Saiki et al., 1988). Each cycle of PCR included 30 s of denaturation at 95°C, 1 min of primer annealing at 55°C, and 2 min of extension/synthesis at 72°C. PCR primers (amplifiers) were synthesized by using an Applied Biosystems DNA synthesizer (model 380B). The primer yield and quality were tested by UV spectroscopy and gel electrophoresis. *mdr1*-specific sequences were amplified by using the sense-strand primer GAGGTGAAGAAGGGCCAGACG (nucleotides 3175–3195 relative to the first nucleotide of translation initiation codon) and the antisense-strand primer TTCTGGATGGTGGACAGGCGGTGA (nucleotides 3716–3693) (Chen et al., 1986); PCR using these primers yielded a 542 bp product. Amplifiers used for amplification of β_2-microglobulin (β_2m)-specific sequences were ACCCCCACTGAAAAAGATGA (nucleotides 1544–1563; sense strand) and ATCTTCAAACCTCCATGATG (nucleotide 2253–2262 and 3507–3516; antisense strand) (Gussow et al., 1987); PCR using these primers yielded a 114 bp product. Each primer was added at 50 pmol per reaction. For quantitation, 2 μCi of [α-^{32}P]dCTP was added to each reaction mixture. PCR products were separated on 12% polyacrylamide gels, dried and exposed at −70°C to X ray films (X-AR5, Kodak) in cassettes with an intensifying screen. Autoradiographs were multiply scanned by one-dimensional densitometry (Hoefer GS300). Films and intensifying screens were calibrated by different time period of exposure of the standard [α-^{32}P]dCTP.

2.4. Isolation of cellular DNA and atomic absorption spectrophotometry

Cellular DNA was isolated by a standard method (Sambrook et al., 1989) in the presence of potassium phosphate (Tilby et al., 1991). By this method, recovery of RNA from the hydroxyapatite column was undetectable. In brief, cells were lysed, sonicated using an ultrasonic processor (Sonics and Materials VC-600), fitted with a cup-horn at maximum power for 2 min, and incubated with 10 μg of RNase at 37°C for 15 min. The lysate was brought to 5 ml with lysis buffer, followed by extraction with an equal volume of phenol, then the aqueous phase was mixed with 25 ml of 6 M urea/80 mM potassium phosphate (pH 6.8) and 0.5 g hydroxyapatite (DNA grade Biogel-HPT from Bio-Rad) for 15 min at room temperature. The gel suspension was transferred into and eluted from a spun column device. Specific binding of platinum to DNA was determined by atomic absorption spectrophotometry as previously described (Bungo et al., 1990). The cells were washed twice with cold phosphate-buffered saline, and the cell pellets were immediately dissolved in nitric acid at 80°C for 5 h. The platinum was chelated with sodium diethyldithiocarbamate followed by extraction with chloroform, and analyzed on an atomic absorption spectrophotometer. The number of platinum atoms was normalized to cellular protein determined by the Bradford assay, using the Bio-Rad dye reagent (Bradford, 1976).

2.5. Enzyme-linked immunosorbent assay (ELISA)

A monoclonal antibody, MAb 62-5, raised against cisplatin-DNA adducts (D/N = 0.2 cisplatin/phosphate) (Chao et al., 1994) was used to detect the level of DNA damage as previously described (Chao, 1994a). The procedure entailed the transfer of aliquots of antibody solution to DNA-coated polystyrene flat-bottomed 96-well microtiter plates (diameter 3.4 mm, Corning). $5–8 \times 10^4$ cells were evaluated per well. The nonspecific binding was blocked by adding 1% normal goat serum in phosphate-buffered saline at 37°C for 60 min. After removal of NGS with phosphate-buffered saline containing 0.05% Tween, the plate was incubated in 100 ml diluted MAb 62-5 (1/30) for 30 min at 37°C. 50 μl of secondary antibody, peroxidase-conjugated goat anti-mouse immunoglobulins, was added and incubated at 37°C for 30 min. Freshly prepared 1 mM ABTS (2,2′-azino-bis(3-ethy(benzthiazoline-6-sulfonic acid) in ABTS buffer (0.1 M citrate, 0.2 M disodium phosphate buffer, pH 4.2–4.8, 0.01% H_2O_2) was then added for 30 min and the absorbance at wavelength 405 nm was read with a Biotek microtiter plate reader as previously described (Chao et al., 1994).

2.6. DNA transfection and chloramphenicol acetyltransferase (CAT) assays

20 μg each of plasmid DNA, cisplatin-treated pRSVcat and untreated pSVβ (Clontech Laboratories) were co-transfected into cells using the electroporation technique in Hepes buffer as previously described (Chao et al., 1991a). Electroporation by GenePulser (Bio-Rad) with 1000 μF capacity and 200 voltage was conducted according to the supplier's instructions. Following 40 h of transient expression, cells were harvested for CAT and β-galactosidase activity assays (Sambrook et al., 1989) using 200 μg and 400 μg of cell extracts, respectively. The CAT reaction was at 37°C for 1 h, followed by development on a silica thin-layer chromatography (TLC) plate (Macherey-Nagel, Germany). After autoradiography, density on the X ray film was quantitated with a scanning densitometer (Hoefer GS300). The average of ten scans of each chloramphenicol spot was taken. CAT activity was calculated as percentage of chloramphenicol converted into acetylated derivatives. After being normalized to β-galactosidase activity, relative

Table 1
Susceptibility to anticancer agents of HOB1 and HOB1/VCR cells

Agents	IC_{50} (μM) [a]		
	HOB1	HOB1/VCR	Resistance [b]
Vincristine	0.072 ± 0.01	15 ± 2	208 ± 11
Puromycin	0.147 ± 0.03	31.5 ± 5	214 ± 15
Colchicine	0.048 ± 0.004	6 ± 0.2	125 ± 12
Adriamycin	0.11 ± 0.003	8.19 ± 0.5	74.5 ± 4
Cisplatin	0.15 ± 0.02	10 ± 2	67 ± 4
Mitomycin C	0.15 ± 0.04	5.92 ± 0.8	39.5 ± 2

[a] IC_{50} is the the drug concentration effective in inhibiting 50% of the cell growth measured by MTT assay after 4 days of continuous exposure to the drug. Mean ± S.D. ($n = 5$). [b] Resistance was determined by the ratio of IC_{50} of HOB1/VCR to the IC_{50} of HOB1 cells.

CAT activity was determined by setting untreated samples as 100%.

3. Results

3.1. Resistance of HOB1 / VCR cells to multiple drugs

Resistance of HOB1/VCR cells to anticancer drugs was determined by MTT assay. Calculated IC_{50} values are listed in Table 1. The resistant cells displayed a 208-fold resistance to the selecting agent, vincristin. HOB1/VCR cells also showed high resistance to MDR-type drugs: puromycin (214-fold), colchicine (125-fold), adriamycin (74.5-fold); and to DNA-damaging agents: cisplatin (67-fold), mitomycin C (39-fold). It appears that HOB1/VCR cells are resistant to a wide spectrum of anticancer drugs and cytotoxic agents. The effect of verapamil on drug sensitivity was also investigated. A sublethal dose of verapamil (1 μg/ml) was included in the culture in viability assays. The IC_{50} values of three pairs of parental and resistant cell lines were estimated (Table 2). Numbers in parentheses indicate the increased resistance of resistant cells relative to their parental cells. The verapamil effect was represented by the reduction in cytotoxicity, the IC_{50} without verapamil being divided by the IC_{50} with verapamil. As indicated, the sensitivity of HOB1 cells to vincristine was not affected by verapamil. In contrast, there was a 230-fold reduction in that of HOB1/VCR cells, indicating that the acquired resistance to the drug was completely abolished. Similarly, most of the vincristine resistance was inhibited by verapamil in SW620/MDR cells, a P-glycoprotein-mediated MDR control line (Chao et al., 1992). The negative control HeLa/CPR cells, which are MDR-independent resistant to cisplatin and other DNA-damaging agents (Chao et al., 1991a), showed little or no modification of vincristine sensitivity by verapamil. These results strongly suggest that MDR phenotype acquired by HOB1/VCR is mediated by P-glycoprotein.

HOB1 and HOB1/VCR cells were also analyzed for cytotoxicity induced by cisplatin (Fig. 1). Cells were treated with various concentrations of cisplatin for 5 h and were assayed for viability after 5 days (upper Panel) or for clonogenicity after 14 days (lower panel) incubation in growth medium. Relative survival, by setting untreated cells as 100% vs. cisplatin concentration, is indicated. There is a 'shoulder' in the survival curve of HOB1/VCR cells compared to the parental HOB1 cells. IC_{50}, the cisplatin concentration inhibiting 50% of cellular viability, was ~0.15 μM and 10 μM in HOB1 and HOB1/VCR

Table 2
Effects of verapamil on the susceptibility of parental and resistant cells to vincristine

Cell lines	IC_{50} (μM) [a]			
	− verapamil		+ verapamil	Reduction [b]
HOB1	0.072 ± 0.01		0.07 ± 0.004	1.03 ± 0.05
HOB1/VCR	15 ± 2	(208) [c]	0.065 ± 0.01	230 ± 15
SW620	0.18 ± 0.04		0.17 ± 0.02	1.06 ± 0.3
SW620/MDR	3.8 ± 0.25	(21)	0.22 ± 0.03	17.3 ± 0.1
HeLa	0.008 ± 0.001		0.007 ± 0.001	1.14 ± 0.07
HeLa/CPR	0.01 ± 0.002	(1.2)	0.11 ± 0.01	0.91 ± 0.05

[a] IC_{50} is the drug concentration effective in inhibiting 50% of the cell growth measured by the MTT assay after 4 days of continuous exposure to the drug; results are means ± S.D. ($n = 5$). [b] Reduction was determined from the ratio of IC_{50} without verapamil to IC_{50} with verapamil (1 μg/ml). [c] Numbers in parentheses are the increased resistance relative to that of the parental cells.

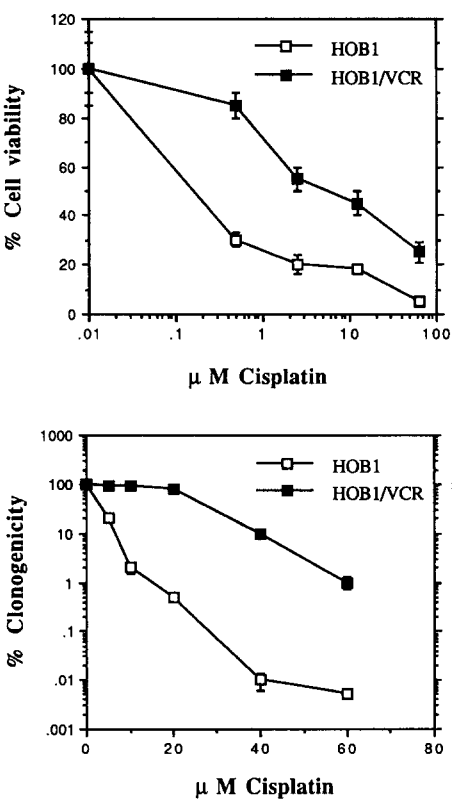

Fig. 1. Drug sensitivity and cell growth pattern in HOB1 and HOB1/VCR cells. Upper panel: %cell viability relative to that of mock-treated cells was determined by MTT assay. Points, means; bars, ±S.D.; $n = 5$. Lower panel: %cell clonogenicity relative to that of mock-treated cells by colony forming assay was estimated for 14 days (means ± S.D.; $n = 3$).

cells, respectively. Do, the cisplatin concentration inhibiting 63% of cellular proliferation, was ~2 μM and 10 μM in HOB1 and HOB1/VCR cells, respectively. There was 67- and 5-fold resistance as measured by viability ($n = 5$) and clonogenicity ($n = 3$), respectively.

3.2. Overexpression of mdr1 gene in resistant cells

The steady-state *mdr1* mRNA in HOB1 and HOB1/VCR cells was measured by RT-PCR (Fig. 2). RT-PCR assays for *mdr1* of HOB1/VCR (Fig. 2A, lanes 1, 3, 5, 7) or HOB1 (lanes 2, 4, 6, 8) cells yielded a single PCR product of the expected size of 543 bp (indicated with an arrowhead) over the range of 1-, 5-, 10-, or 50-fold dilution of RNA (30 PCR cycles) (Fig. 2A). The specificity of the amplified cDNA for *mdr1* was confirmed by partial sequence (data not shown) and by the same sized PCR product amplified from 50 ng of pGEM3Zf(−)-*mdr1*, a plasmid DNA containing full-length *mdr1* cDNA (a kind gift from Professor Piet Borst, The Netherlands Cancer Institute) (lane 9). The control reaction with buffer is also shown (lane 10). The PCR product of the expected size of 114 bp for β_2-microglobulin is also shown as an internal control (indicated with a star). The *mdr1* amplification (HOB1/VCR vs. HOB1) from three independent experiments was calculated (Fig. 2A, lower panel). The data were normalized to β_2-microglobulin. There was a 9–20-fold amplification of the *mdr1* mRNA in the resistant cells. PCR assays were also conducted in 20, 30, or 40 cycles using *mdr1* mRNA without dilution (Fig. 2B). Undiluted RNA transcribed from HOB1/VCR cells (lanes 1, 3, 5) or HOB1 cells (lanes 2, 4, 6) was amplified by PCR in 20 (lanes 1, 2), 30 (lanes 3, 4) or 40 (lanes 5, 6) cycles. PCR controls with *mdr1* cDNA template or buffer are also shown (lanes 7 and 8, respectively). The analysis revealed a by-product of β_2-microglobulin with a smaller size in HOB1 cells after 40-cycle PCR reactions for an unknown reason. The data indicated 5–9-fold amplification of *mdr1* when normalized to β_2-microglobulin (Fig. 2B, lower panel).

We had previously shown that a P-glycoprotein-mediated MDR human colon cancer cell line (SW620/MDR) acquires the ability to decrease intracellular drug accumulation by pumping the drug out of cells (Chao et al., 1992). This characteristic holds true for a variety of lipophilic compounds, which is typical of an MDR phenotype. It is reasonable to speculate that the HOB1/VCR

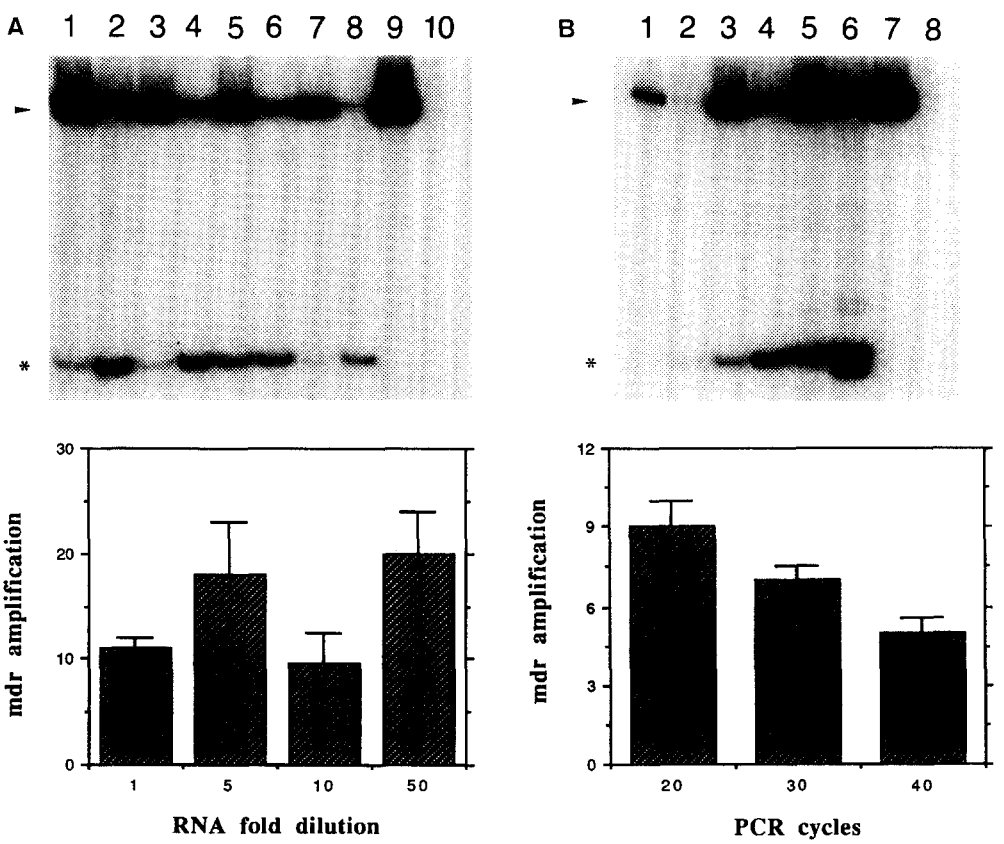

Fig. 2. Detection of *mdr1* expression. A: PCR in different mRNA concentration. mRNA dilution: 1-fold (lanes 1–2), 5-fold (lanes 3–4), 10-fold (lanes 5–6), or 50-fold (lanes 7–8). cDNA reverse transcribed from parental HOB1 (lanes 2, 4, 6, and 8) or HOB1/VCR (lanes 1, 3, 5, and 7) mRNA was amplified in PCR programmed in 30 reaction cycles. Lane 9, pGEM3(−)-*mdr1* DNA (50 ng) as PCR template; lane 10, without template added. PCR products for *mdr1* and β_2-microglobulin are indicated with arrowhead and star, respectively. The *mdr* amplification is shown in the lower panel. Points, means; bars, ±S.D.; $n = 3$. B: PCR in different reaction cycles. RT-PCR was conducted as for A except that the PCR reaction was carried out for 20, 30, or 40 cycles.

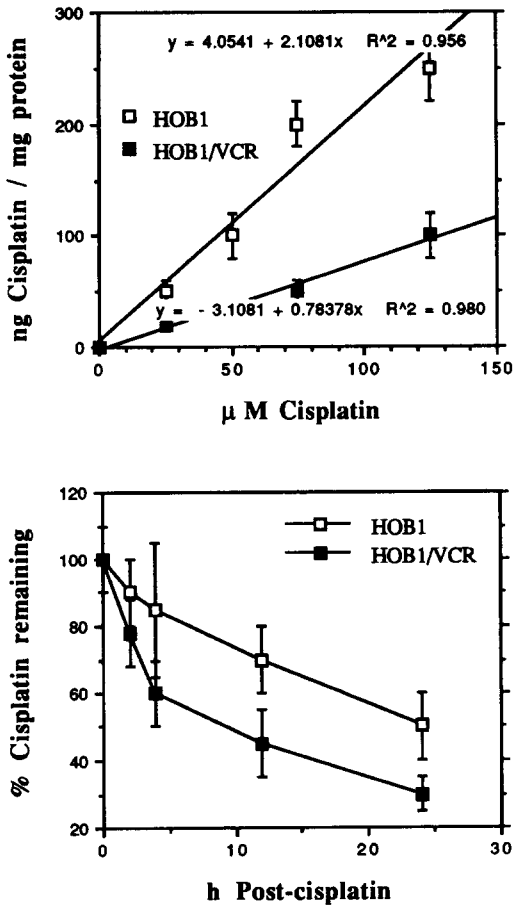

Fig. 3. Repair of cisplatin-DNA adducts in HOB1 and HOB1/VCR cells as measured by atomic absorption spectrophotometry. Upper panel: dose-response of the adduct frequency in cisplatin-treated cells. Cells were treated with various concentrations of cisplatin for 5 h, incubated for 24 h, and the relative adduct frequency was determined. The lines were calculated by linear regression. R is the correlation coefficient. Lower panel: repair kinetics post-cisplatin treatment. The % cisplatin remaining of average adduct frequency, with standard deviation ($n = 3$) (tested vs. untreated cells), normalized to the amount of protein, for each time point is shown. Cells were treated with 50 μM cisplatin for 5 h, and incubated in drug-free medium for various times. There was 200 and 145 ng cisplatin/mg protein in HOB1 and HOB1/VCR cells, respectively, immediately after drug treatment.

cells may overexpress P-glycoprotein because they develop an enhanced, verapamil-sensitive ability to pump vincristine out of cells (Chao, 1996). Immunostaining indicates that HOB1/VCR cells showed overexpression of protein bands with sizes comparable to that of P-glycoprotein (data not shown).

3.3. Reduced cisplatin-DNA adduct in resistant cells

The dose-response curve of cisplatin on intracellular platinum was determined by atomic absorption spectrophotometry (Fig. 3, upper panel). Cells were treated with 0, 25, 50, 75, or 150 μM of cisplatin for 5 h. Following 24 h of incubation, the level of cisplatin-DNA adducts in the cells was analyzed. The regression lines of the dose-response curves indicated a ~ 2.3-fold reduction of platinum in the resistant cells. The kinetics of removal of cisplatin-DNA adducts were also investigated (lower Panel). Cells were treated with 50 μM cisplatin for 5 h, and the amount of cisplatin-DNA adducts was measured at 0, 2, 4, 12, or 24 h after cisplatin treatment. It should be noted that cisplatin treatment (50 μM, 5 h) caused 250 and 150 ng cisplatin/mg protein in HOB1 and HOB1/VCR cells, respectively. The % platinum remaining was calculated. The repair rate was composed of two phases: an initial rapid phase within the first 4 h, followed by a terminal slow phase. The difference of repair rate in both cells was detected only in the initial phase. 15% and 40% adducts were removed within the first 4 h of incubation in HOB1 and HOB1/VCR cells, respectively. There was a ~ 2.6-fold reduction of adduct frequency in the resistant cells.

The dose-response curve was also determined by ELISA using cisplatin-DNA adduct specific MAb62-5 (Fig. 4,

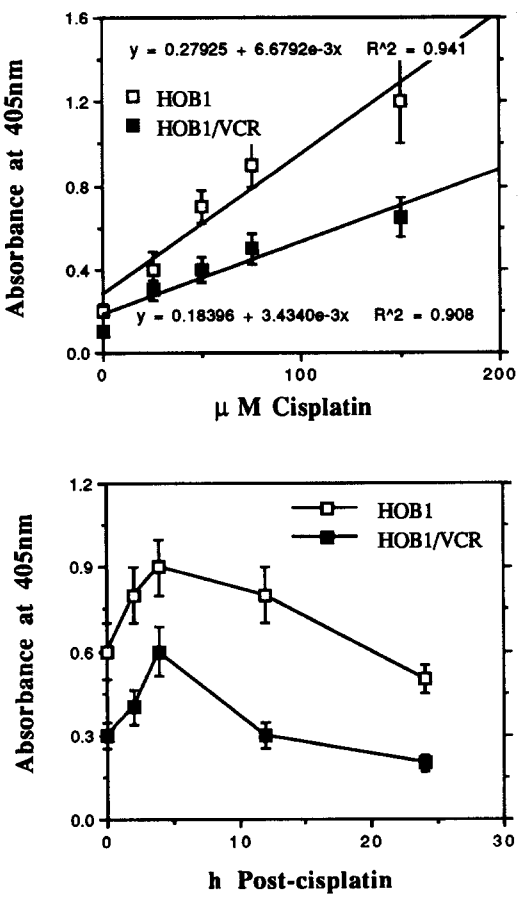

Fig. 4. Repair of cisplatin-DNA adducts in cells as measured by ELISA. Upper panel: dose-response curve of MAb binding to cisplatin-treated cells. Cells were treated with various concentrations of cisplatin for 5 h, incubated for 24 h, and the relative absorbance at 405 nm (mean ± S.D.) ($n = 3$) was determined. The lines were calculated by linear regression. R is the correlation coefficient. Lower panel: repair kinetics post-cisplatin treatment. The absorbance at 405 nm (mean ± S.D.) ($n = 3$) (tested vs. untreated cells) for each time point is shown. Cells were treated with 50 μM cisplatin for 5 h, and incubated in drug-free medium for 0, 2, 4, 12, or 24 h.

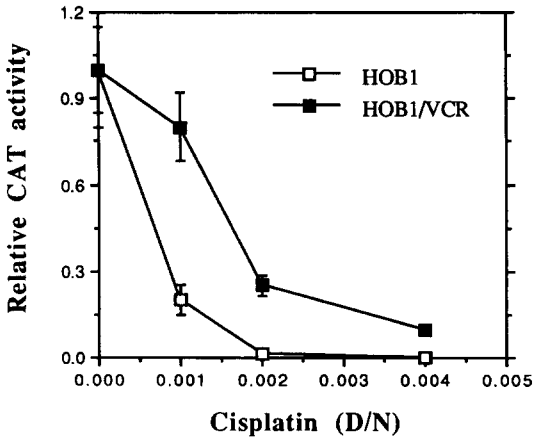

Fig. 5. Repair of cisplatin-DNA adducts in HOB1 and HOB1/VCR cells as measured by plasmid reactivation. Twenty μg of pRSVcat was platinated in vitro to generate different amounts of cisplatin-DNA adduct (D/N) before being introduced into cells for transient expression. Relative CAT activity, by setting untreated pRSVcat as 1, was calculated (mean $\pm$ S.D.; $n = 3$).

upper Panel). Cells were treated with 0, 25, 50, 75, or 150 μM of cisplatin for 5 h. Following 24 h of incubation in drug-free medium, the level of cisplatin-DNA adducts in cells was analyzed. The regression lines of the dose-response curves indicated a ~ 2-fold reduction of cisplatin-DNA adducts in HOB1/VCR cells (also see Table 3). The kinetics of cisplatin-DNA adducts remaining in cells were also compared in HOB1 and HOB1/VCR cells (Fig. 5, lower panel). To analyze repair kinetics, cells were treated with 50 μM cisplatin for 5 h, and the amount of cisplatin-DNA adducts was measured at 0, 2, 4, 12, or 24 h after cisplatin treatment. The patterns showed a slight increase with a peak accumulation at 4 h followed by a decrease of the relative absorbance at 405 nm. The peak accumulation of the cisplatin-DNA adducts (at ~ 4 h after treatment) for both cell lines was 0.9, and 0.6 A405 nm, respectively. The relative absorbance at 405 nm of HOB1/VCR cells was ~ 50% less than that of HOB1 cells. In any case, the initial slope of the curves following the peak of cisplatin-DNA accumulation was greater in the resistant cells than in the parental cells (0.038 vs. 0.013 A405 nm per h), suggesting an enhanced repair rate in resistant cells. Collectively, the results indicated that the amount of cisplatin-DNA adducts in chromosomes was reduced by 2–3-fold in HOB/VCR cells.

3.4. Enhanced host cell reactivation of damaged plasmid DNA in resistant cells

To further analyze whether DNA repair is associated with cisplatin resistance, plasmid reactivation was used to compare removal of cisplatin-DNA adducts generated in vitro. Plasmid DNA pRSVcat with 0, 0.001, 0.002, or 0.004 D/N was co-transfected with untreated pSVβ for transient expression. CAT and β-galactosidase activities were measured 40 h after transfection of the cells. After being normalized to the β-galactosidase activity, the relative CAT activity was calculated, by setting the untreated pRSVcat as 100%. Shown is a typical example of CAT activity vs. cisplatin-DNA adducts in pRSVcat from 0, 0.001, 0.002, or 0.004 cisplatin-DNA adducts. The IC_{50} value, the extent of cisplatin modification that inhibited CAT activity by 50%, for HOB1 and HOB1/VCR cells was 0.0006 and 0.0015 cisplatin-DNA adducts, respectively. There was a 2.5-fold enhancement of the plasmid reactivation in the resistant cells. The levels of cisplatin-DNA adducts in cells measured by different methods were compared (Table 3). The data indicates that the acquired drug resistance of HOB1/VCR cells ranged from 2- to 2.7-fold as measured by three different methods.

3.5. Inhibition of enhanced DNA repair and drug resistance by aphidicolin

The effect of a nontoxic concentration of aphidicolin (10 μg/ml) on cellular sensitivity to cisplatin was investigated by MTT assay. The average IC_{50} of cells from three independent experiments in response to cisplatin in the presence or absence of aphidicolin is shown (Table 4). The modification factor, estimated by the ratio of the IC_{50} without aphidicolin divided by that with aphidicolin, was 1.07 and 4.76 for HOB1 and HOB1/VCR cells, respectively. The results suggest that DNA metabolism was involved in the acquired cisplatin resistance in HOB1/VCR cells because it is known that aphidicolin is a potent inhibitor of DNA polymerase-α (Ikegami et al., 1978) and DNA repair (Berger et al., 1979; Yamada et al., 1985). Aphidicolin's effect on plasmid reactivation was assayed in parallel with that on cytotoxicity. The modification factor was 0.86 and 1.67 for HOB1 and HOB1/VCR cells, respectively. The data indicate that aphidicolin si-

Table 3
Comparison of cisplatin-DNA adducts measured by different methods in HOB1 and HOB1/VCR cells

	AA spectrophotometry [a] AFF	Immunoassay [a] AFF	Plasmid reactivation [b] IC_{50} (D/N)
HOB1	0.78 $\pm$ 0.1 (1)	0.0067 $\pm$ 0.0006 (1)	0.0006 $\pm$ 0.0001 (1)
HOB1-VCR	2.1 $\pm$0.3 (2.69 $\pm$ 0.2)	0.0034 $\pm$ 0.0003 (1.97 $\pm$ 0.1)	0.0015 $\pm$ 0.0003 (2.5 $\pm$ 0.1)

[a] The data were expressed as AFF $\pm$ S.D. ($n = 3$). AFF, average adduct formation frequency or ng adduct/mg protein/μM cisplatin as assayed by atomic absorption (AA) spectrophotometry, or absorbance at 405 nm/μM cisplatin as assayed by ELISA. Numbers in the parentheses are the increase or decrease by setting HOB1 cells as 1. [b] The data were expressed as IC_{50} $\pm$ S.D. ($n = 3$), estimated cisplatin-DNA adducts (D/N) required to inhibit 50% CAT activity, as assayed by plasmid reactivation in cells. Numbers in parentheses are the fold enhancement by setting HOB1 cells as 1.

Table 4
Effect of aphidicolin (aph) on the cytotoxicity and DNA repair induced by cisplatin in HOB1 and HOB1/VCR cells [a]

	Cytotoxicity IC_{50} (μM)	CAT activity IC_{50} (D/N)
HOB1	0.15±0.02 (1) [b]	0.0006±0.0001 (1) [b]
HOB1+aph	0.14±0.02 (0.93±0.05)	0.0007±0.0001 (1.17±0.1)
HOB1/VCR	10 ±2 (66.7±4.3)	0.0015±0.0002 (2.5±0.3)
HOB1/VCR+aph	2.1 ±0.4 (14±0.6)	0.0009±0.0001 (1.5±0.1)

[a] The data were expressed as average IC_{50} ±S.D. ($n = 3$). IC_{50}, cisplatin concentration causing 50% inhibition of cell viability (μM) or CAT activity (D/N). [b] Numbers in parentheses are the resistance (cytotoxicity) or enhancement (CAT activity) by setting HOB1 cells as 1.

Table 5
Effect of BSO on cisplatin-induced cytotoxicity and glutathione level

Cell lines	Cytotoxicity [a] IC_{50} (μM)	Glutathione content [b] nmol/mg protein
HOB1	0.15±0.02 (1) [c]	80±14 (1) [c]
HOB1+BSO	0.16±0.02 (1.07±0.05)	10± 2 (0.125±0.02)
HOB1/VCR	10 ±2 (67±4)	120±12 (1.5±0.12)
HOB1/VCR+BSO	2.5 ±0.3 (16.7±1.2)	16± 2 (0.2±0.01)

[a] The data were expressed as average IC_{50} ±S.D. ($n = 5$). IC_{50}, cisplatin concentration causing 50% inhibition of cell viability. [b] The data were expressed as means±S.D. ($n = 3$). Cells were mock-treated or treated with 25 μM of BSO for 24 h before being assayed for glutathione level. [c] Numbers in parentheses are the resistance or fold increase by setting mock-treated HOB1 cells as 1.

multaneously inhibits enhanced DNA repair and drug resistance, exerting a greater modification in the resistant cells. However, there was very little or no aphidicolin effect in HOB1 cells. These cells also repair cisplatin adducts and one would have expected, therefore, aphidicolin to have an effect. It is possible that this drug has unknown or confounding effects other than those that had previously been suggested for it.

3.6. Reduction of glutathione content and drug resistance by BSO

The level of glutathione was measured in cells in the presence or absence of DL-buthionine-[*S*,*R*]-sulfoximine (BSO). A sublethal concentration of BSO (50 μM) was used to test its effect on cytotoxicity. The intracellular content of glutathione decreased with time following BSO inclusion in the culture medium (Fig. 6). The kinetics of BSO inhibition of intracellular glutathione, in pattern and extent, were the same in parental and resistant cells. In both cell lines, about 50% of the glutathione content inhibited by BSO within 4 h; whereas less than 20% remained by 24 h. Glutathione content in HOB1 and HOB1/VCR cells was 80 and 120 nmol/mg protein, respectively (Table 5). The increase in glutathione levels in resistant cells was inhibited by BSO. The modification factor, glutathione content without BSO divided by glutathione content with BSO, was 8 and 7.5 for HOB1 and HOB1/VCR cells, respectively.

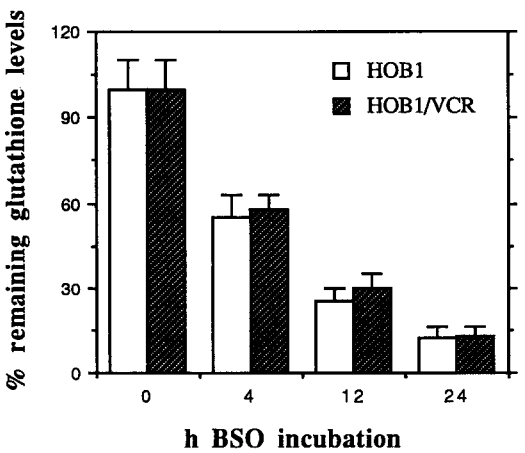

Fig. 6. Time dependent depletion of intracellular glutathione by BSO (25 μM) in HOB1 and HOB1/VCR cells. % remaining glutathione level is presented (mean±S.D.; $n = 3$) relative to the glutathione content (nmol/mg protein) of mock-treated cells.

The IC_{50} for cisplatin-induced cytotoxicity with or without BSO is shown in Table 5. The IC_{50} for HOB1 and HOB1/VCR cells was 0.15 and 10 μM, respectively. In the presence of BSO, the IC_{50} for the two cell lines was 0.16 and 2.5 μM, respectively. The modification factor, IC_{50} without BSO divided by IC_{50} with BSO, was 9.4 and 4 in parental and resistant cells, respectively. The results indicate that inhibition of glutathione synthesis by BSO renders both HOB1 and HOB1/VCR cells sensitive to the drug, affecting the resistant cells more than the parental cells. However, the same BSO effects were not seen with drugs such as vincristine which also produce MDR (data not shown), suggesting that the BSO effects are probably only seen with drugs that form free radicals. The data indicate that inhibition of intracellular glutathione content renders cells more sensitive to the drug, with being a greater effect in resistant cells.

4. Discussion

In this study, I present an MDR subline derived from an immunoblastic B lymphoma cell line by selection with vincristine. The MDR cell line displayed *mdr1* gene overexpression (Fig. 2) and P-glycoprotein-mediated MDR phenotype. This MDR cell line (HOB1/VCR) also showed cross-resistance to cisplatin. In an immunoassay with a cisplatin-DNA-specific monoclonal antibody (Chao et al., 1994), HOB1/VCR cells demonstrated a 2-fold reduction in the accumulation of cisplatin-DNA adducts. The initial level of cisplatin-DNA adducts was ~50% lower in the resistant cells than in the parental cells. A number of reasons (e.g., enhanced removal from DNA, increased drug efflux, altered uptake, or reduced conversion to an

active form) could have accounted for this effect. A 2.5-fold enhancement in DNA repair was detected, by the plasmid reactivation assay, in the resistant cells, based on the assumption that the CAT activity would not be efficiently expressed in transfected cells should cisplatin-DNA adducts remain on the gene. The results were supported by the direct measurement of the number of platinum atoms by atomic absorption spectrophotometry, which showed 2.7-fold less platinum in HOB1/VCR cells. In contrast to the immunoassays, kinetic studies using the latter method demonstrated two phases of adduct removal: a rapid phase within the initial 4 h, followed by a slow phase. There was a 2.7-fold reduction in the initial level of cisplatin-DNA adducts in HOB1/VCR cells. This is supported by the studies showing that inhibition of DNA repair by aphidicolin was associated with restoration of drug sensitivity (Table 4). Taken together, these results strongly suggest that the accumulation of cisplatin-DNA adducts plays a critical role in the overall response of HOB1/VCR cells to the drug. It has been demonstrated that xeroderma pigmentosum group A or E cells display defective excision repair (Friedberg et al., 1995); cell extracts from these mutant cells demonstrate a reduced recognition of UV-damaged DNA (Chu and Chang, 1988; Kataoka and Fujiwara, 1991; Chao, 1992). Thus, HOB1/VCR cells probably acquire an enhanced capacity for DNA repair that is deficient in xeroderma pigmentosum cells.

Despite the demonstration in this study that cisplatin resistance can be acquired through selection by MDR drugs in the immunoblastic lymphoma cells, the mechanism for the development of cisplatin resistance by this process is unclear. The decreased uptake or increased cisplatin conjugation with metallothioneins or glutathione, or the differences in the types of cisplatin-DNA lesions formed in cells may also contribute to the resistant phenotype. This study also demonstrated that a free radical scavenger is involved in cisplatin resistance in HOB1/VCR cells. A 1.5-fold increase in glutathione content suggests that the latter mechanism is of only marginal importance. However, inhibition of glutathione level by BSO in both cell lines (7.5–8-fold) resulted in little effect on cytotoxicity in the parental cells, whereas it caused a 4-fold reduction in cell viability in HOB1/VCR cells. The results demonstrate a causal relationship between glutathione content and cisplatin resistance of the lymphoma cells. Accumulated evidence suggests that cellular glutathione is a critical determinant in cell resistance to chemotherapeutic agents, such as nitrogen mustards, chloroethyl nitrosoureas, and cisplatin (Hamilton et al., 1985; Godwin et al., 1992). Evidence that glutathione-drug conjugates are potentially cytotoxic suggests that elimination of glutathione-drug conjugates is an important factor in the cytoxicity of anticancer drugs (Ishikawa and Ali-Osman, 1993; Ishikawa et al., 1994). A panel of cisplatin-resistant ovarian cancer cell lines have been demonstrated to have cross-resistance to natural product drugs, including adriamycin, mitoxantrone, and taxol (Hamaguchi et al., 1993). Different treatment strategies in chemotherapy may affect the type of resistant tumor cells. Perhaps, HOB1 cells acquired a potential for cisplatin resistance during chemotherapy by exposure of the patient to drugs such as nitrogen mustard. Some of these cells may have been promoted to become cisplatin resistant following exposure of cultured HOB1 cells to vincristine. It appears that HOB1/VCR cells acquired more than one cisplatin-resistance-associated mechanism such as enhanced DNA repair, reduced adduct formation, and increased glutathione content. Thus, cross-resistance to diverse drugs including cisplatin is associated with primary vincristine resistance in immunoblastic B lymphoma cells.

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