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Characterization of a Cisplatin-resistant Subline of Murine RIF-1 Cells and Reversal of Drug Resistance by Hyperthermia

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ABSTRACT

The development of tumor cell drug resistance is a major obstacle which often leads to failure of cancer chemotherapy. Therefore, reversing the cell drug resistance would have important implications in cancer treatment. We have developed a cisplatin-resistant mouse tumor cell line from the radiation induced fibrosarcoma (RIF-1) parental line; this line is named RIF/ptr1 *versus* the parental line RIF/pts1. It is shown that the formation of cisplatin-DNA interstrand cross-links is the same for both cell lines although the intracellular cisplatin concentrations of resistant line is significantly lower. The cytosolic activities of glutathione reductase, glutathione peroxidase, and DT-diaphorase were the same in two cell lines. However, the concentration of glutathione was significantly higher in the resistant line. The resistant line was shown to be more sensitive to the cytotoxicity of heat (43°C) but the combination of heat and drug had the same tumoricidal effect for both cell lines. The addition of verapamil also had a similar effect on both cell lines. We conclude that the major difference between these two lines was the glutathione-related detoxification of platinum. Regardless of drug resistance, the combination of drug and heat can effectively kill both cell lines. Elevated glutathione in RIF/ptr1 cells may be associated both with enhanced heat sensitivity and drug resistance such that combined treatments with drug and heat were equally effective in killing cells of either line.

INTRODUCTION

Cisplatin [*cis*-diamminedichloroplatinum(II)] is an effective drug in the treatment of several human cancers such as lung, bladder, head and neck, and germ cell tumors. It is one of the most widely used chemotherapeutic agents either singly or in combination. However, its use often leads to tumor drug resistance (1–3).

Although the exact mechanism of CDDP[2] action is not well understood, it is assumed, from the available experimental data, that the site of its action is the cellular DNA. Current understanding of the mechanism of development of tumor drug resistance and its reversal, however, is limited. We have developed a subline of CDDP-resistant RIF-1 tumor cells which has been designated RIF/Ptr1 (RIF/platinum-resistant subline 1); the CDDP-sensitive parental line is denoted by RIF/Pts1 (RIF platinum-sensitive line 1).

The present study was directed toward (*a*) the elucidation of specific characteristics of the RIF/ptr1 cell line that may underlie its resistance to CDDP, and (*b*) the reversal of CDDP resistance in RIF/ptr1 cells.

MATERIALS AND METHODS

Cisplatin was a gift from Bristol Laboratories, Inc., Syracuse, NY. 1-Chloro-2,4-dinitrobenzene, menadione, cumene hydroperoxide, ethacrynic acid, dicoumarol, NADPH, yeast glyoxalase I, methylglyoxal, GSH, GSSG, yeast GSSG reductase, and bovine serum albumin (crystalline) were purchased from Sigma Chemical Co., St. Louis, MO. Sucrose (enzyme grade) was obtained from Schwarz Mann Biotech, Cambridge, MA. The protein microassay reagent was from Bio-Rad Laboratories, Richmond, CA.

Methods

RIF-1 cells were originally obtained from the Laboratory of Dr. Dietmar Siemann (University of Rochester, Rochester, NY). The cells were isolated and maintained from mouse leg tumors by *in vitro* trypsinization and monolayer tumor cell culture as has been described previously (4).

RIF-1 CDDP-resistant variant cells were developed in the presence of increasing concentrations of CDDP with repeated subcultures until the cells became resistant to CDDP and could grow exponentially in the presence of 0.5 to 1 μg/ml of the drug.

Cell Survival. Both parental and resistant cells were plated in T-25 plastic tissue culture flasks at appropriate cell concentrations. The experiments were started about 48 h after plating. Cell survival was measured after exposure of replicate T-25 flasks containing 3×10^2 to 3×10^5 cells to CDDP, heat, or both. Feeder cells were added for cell concentrations of 3×10^3/flask or below. The flasks were incubated for 6–8 days at 37°C until macroscopic colonies were visible. Colonies were stained and counted and fractional cell survival was computed. Each experiment contained six replicate flasks for each experimental point. Colony numbers per flask ranged from 10 to 300.

Platinum Determination. Cells were cultured and exposed to CDDP as described, trypsinized, and pelleted. The medium was replaced by distilled water. Samples were assayed on a Perkin Elmer 5000 atomic absorption spectrometer with a Zeeman furnace accessory and an AS40 auto sampler. Platinum content of samples was determined by comparison with an external standard, also prepared in distilled water.

Determination of Enzyme Activities. Packed, saline-washed cells were suspended in 0.6 ml of cold deionized water, subjected to 3 cycles of freezing and thawing, vortexed extensively, and centrifuged at 27,000 × *g* for 15 min at 4°C. Cold 0.25 M sucrose (0.2 ml) was added to each of the supernatant fractions. GSH transferase activities were measured with 1-chloro-2,4-dinitrobenzene and ethacrynic acid as substrates, using published methods (5). GSH peroxidase activities toward cumene hydroperoxide and hydrogen peroxide were measured by a modification (6) of the method of Paglia and Valentine (7). GSSG reductase activity was assayed as described by Carlberg and Mannervik (8) at 25°C in the presence of bovine serum albumin (0.5 mg/ml) (9). DT-diaphorase activity was measured by a modification (10) of the method of Ernster (11) as the dicoumarol-sensitive enzymatic reduction of menadione by NADPH. All enzyme activities have been corrected for nonenzymatic rates. Specific activities are based on protein concentrations measured by the dye-binding microassay of Bradford (12) with bovine serum albumin as a standard.

Measurement of GSH Concentrations. Cells were trypsinized and centrifuged. The medium was poured off and the packed cells were frozen on dry ice. GSH was measured by the glyoxalase method, as described by Ackerboom and Sies (13). The frozen cells were promptly treated with cold $HClO_4$ in EDTA, and the supernatants obtained by centrifugation were neutralized at 0°C and assayed for their GSH concentrations. An equal volume of medium, concurrently subjected to the same procedure as a negative control, was used as a blank. The assay was validated by use of a freshly prepared solution of GSH.

Measurement of DNA Cross-Linking. Cells were plated in T-25 flasks for 2 days. They were labeled on the third day with [2^{14}C]thymidine, 0.4 μCi/ml, and [*methyl*-1′,2′-^{3}H]thymidine, 1 μCi/ml for 18–21 h.

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[2] The abbreviations used are: CDDP, cisplatin [*cis*-diamminedichloroplatinum(II)]; GSH, glutathione; GSSG, oxidized glutathione; MDR, multidrug resistant.

Cisplatin was added from a stock solution to yield a final concentration of 30 μg/ml. The cells were incubated at 37°C or 43°C for 1 h. The alkaline elution was then performed according to the method of Kohn and Grimek-Ewig (14), as modified by Henle *et al.* (15). The presence of CDDP-DNA cross-links retarded the elution rate of the DNA from the filters. The retardation of DNA, therefore, was used as an index of CDDP-DNA cross-links.

RESULTS

Cell Survival. Cell survival was determined for both cell lines after exposure to heat (43°C) alone and to drug and heat combination. After 1 h of incubation at 43°C in the absence of CDDP, the surviving fraction for sensitive cells was 16 *versus* 3.5% for the resistant cells (Fig. 1), indicating high sensitivity of RIF/Ptr1 to heat. In the presence of 3 μg of CDDP, incubation of sensitive and resistant cells for 1 h at 37°C produced surviving fractions of 1.5 *versus* 16%, for sensitive and resistant cells, respectively (Figs. 2 and 3). However, when both cell lines were treated at 43°C for 1 h in the presence of 3 μg/ml of CDDP, the surviving fraction was about 0.01% for both cell lines, indicating that this combination of drug and heat produced equivalent cell killing in both cell lines (Figs. 1–3). The use of hyperthermia together with verapamil or ethanol decreased the surviving fraction for both parental and resistant cells, but the difference between two cell lines was not significant. However, spermidine was significantly more tumoricidal for CDDP-resistant cells (Figs. 4 and 5).

Enzyme Measurement and GSH. Markedly higher GSH levels were observed in the resistant cells than in the sensitive cells

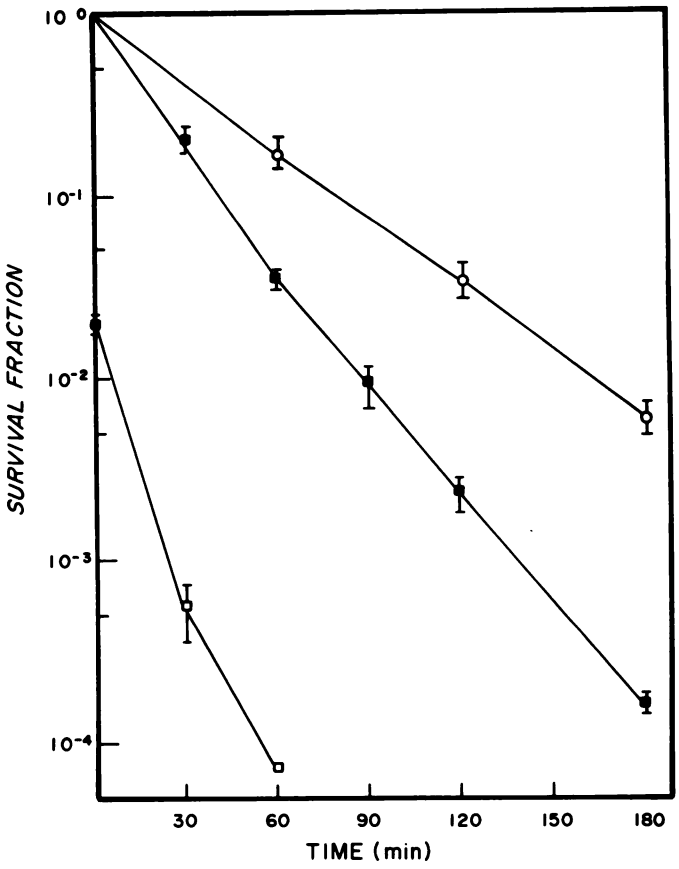

Fig. 1. Upper curve (○), CDDP-sensitive cells (RIF/pts1) were incubated at 43°C and survival fraction was measured at various time intervals. Middle curve (■) represents survival of CDDP-resistant (RIF/ptr1) at 43°C. Lower curve (□) represents survival of resistant cells at 43°C, following preincubation with 3 μg/ml of CDDP for 120 min at 37°C.

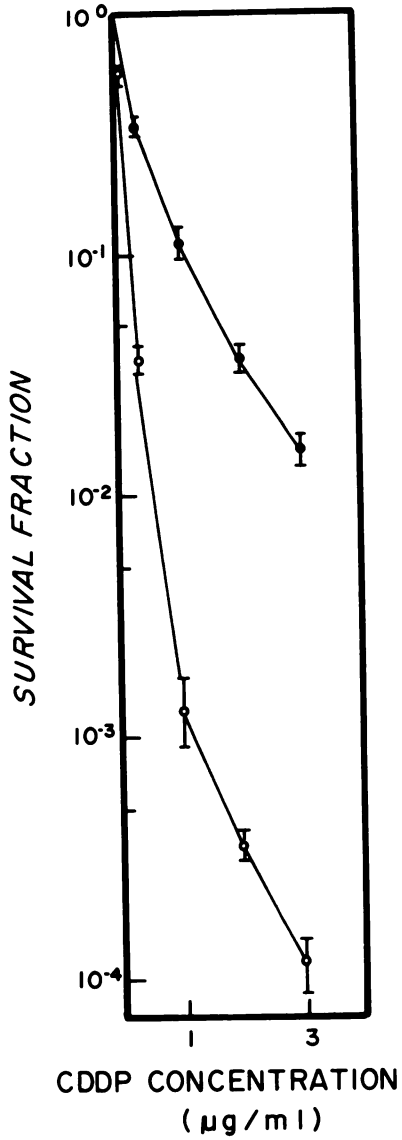

Fig. 2. Survival pattern of CDDP-sensitive cells (parent cell line) incubated for 1 h in the presence of various concentrations of CDDP at 37°C (●) and at 43°C (○).

(Table 1). In contrast to the 40% higher GSH levels, no difference in GSSG reductase activity was observed (Table 1).

The activities of several Phase II detoxication enzymes that are elevated in some "anticancer drug"-resistant phenotypes (16) were also examined. However, we found no significant increase in GSH peroxidase activity toward H_2O_2 or cumene hydroperoxide or in DT-diaphorase activity in cytosol fractions from resistant *versus* sensitive cells (Table 1). Since the expression of GSH transferase P_i may be a marker for inherent drug resistance (16), activity toward its characteristic substrate, ethacrynic acid, was also examined. Although this activity was too low relative to the rate of nonenzymatic reaction to permit very accurate measurement, no detectable difference in this activity was observed between the sensitive cells and the resistant cells (data not shown). GSH transferase activity toward 1-chloro-2,4-dinitrobenzene was significantly higher in the CDDP-sensitive cells as compared with the CDDP-resistant subline.

Platinum Concentration. Intracellular platinum concentration was shown to be approximately 60% higher in the sensitive

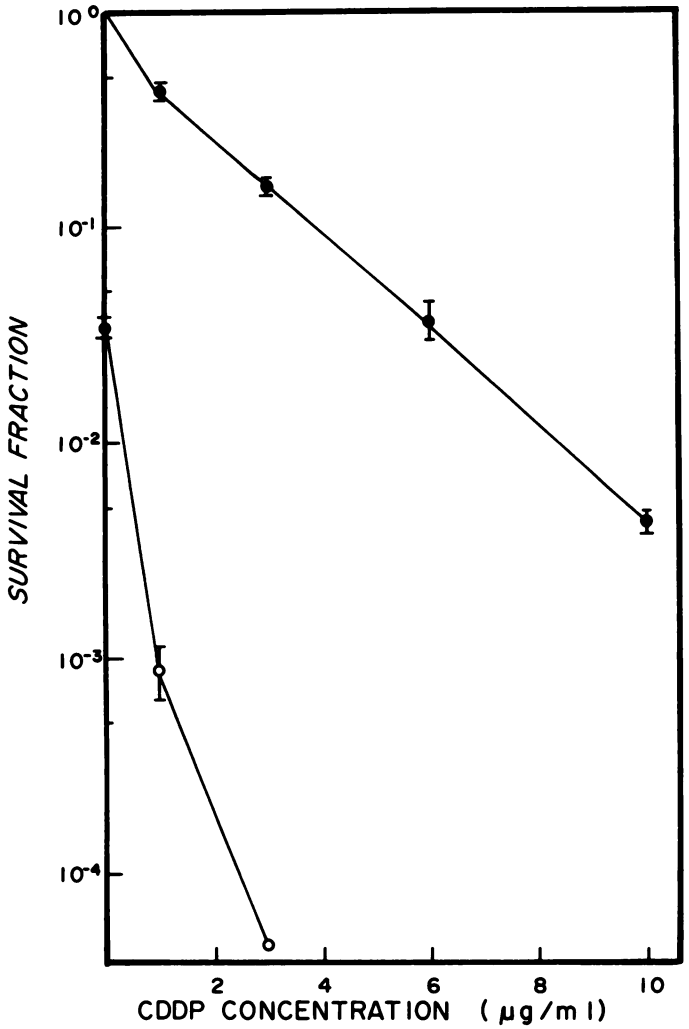

Fig. 3. Survival pattern of CDDP-resistant cells incubated for 1 h in the presence of various concentrations of CDDP at 37°C (●) and at 43°C (○).

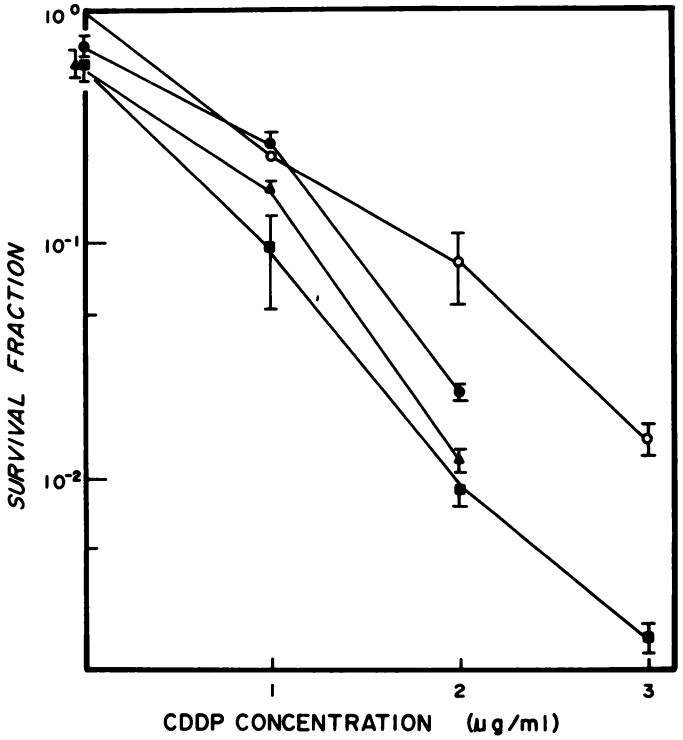

Fig. 4. Survival pattern of CDDP-sensitive cells incubated for 1 h at 37°C in the presence of various concentrations of CDDP alone (○), CDDP plus 5 μM verapamil (●), CDDP plus 1% ethanol (■), and CDDP plus 0.3 mM spermidine (▲).

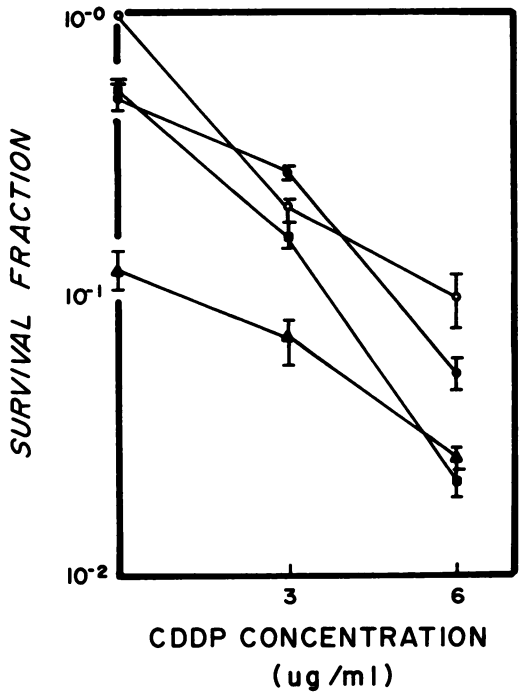

Fig. 5. Survival pattern of CDDP-resistant cells. Conditions and symbols are as described in the legend to Fig. 4.

cells compared to RIF/ptr1 cells after a 1-h exposure of cells to 50 μg/ml of CDDP, 40.22 *versus* 25.35 ng/10^6 cells. Exposure to 43°C for 1 h increased the platinum concentration in both cell lines to 53.94 *versus* 41.60 ng/10^6 cells, respectively, but reduced the difference in cellular CDDP content to 30% (Table 2).

DNA Cross-Links. Sensitive and resistant cells were labeled with ^3H and ^{14}C and incubated with CDDP (final concentration of 30 μg/ml) at 37°C for 1 h; DNA cross-linking was measured by alkaline elution as described above. The amount of radioactivity that was retained on the filter (proportional to DNA cross-links) was not significantly different in parental *versus* resistant cells. When the two cell lines were exposed to CDDP at 43°C, both lines increased retention of DNA on filters (cross-links) by a similar factor (11.7 *versus* 13.7%).

DISCUSSION

There is now considerable evidence that DNA is a principal intracellular target of CDDP action, and that the formation of CDDP-DNA cross-links can inhibit DNA replication (17). However, the mechanism of the development of tumor cell CDDP resistance is poorly understood. The design of effective clinical strategies for overcoming tumor drug resistance would be greatly facilitated by a more complete understanding of the various mechanisms that may operate in different cell lines.

There appear to be multiple mechanisms of CDDP resistance. Some studies have shown a decrease in DNA interstrand cross-links proportional to the degree of tumor cell CDDP resistance, whereas others have shown that this is not always the case (18). In the present investigation we observed that CDDP resistance in a RIF-1 tumor cell subline was not accompanied by a decrease in DNA cross-links. Similarly, in studies on a CDDP-resistant

Table 1 *GSH levels and cytosolic enzyme activities in parental and CDDP-resistant cells*

GSH or enzyme	Substrate	Parental	CDDP resistant
GSH		5.40 ± 0.22[a]	7.54 ± 1.09
GSSG reductase	GSSG	90 ± 3[b]	99 ± 6
GSH peroxidase	Cumene hydroperoxide	14.9 ± 1.3	14.5 ± 2.0
GSH peroxidase	Hydrogen peroxide	11.50 ± 0.7	10.9 ± 1.5
GSH transferase	1-Chloro-2,4-dinitrobenzene	145 ± 8	86 ± 4[c]
DT-diaphorase[d]	Menadione	903 ± 59	1023 ± 120

[a] GSH concentrations are expressed as nmol per 10^6 cells ± SEM ($n = 4$).
[b] All enzyme activities are expressed as nmol/min/mg of cytosolic protein, and are the means ± SEM of the values obtained in 3 experiments.
[c] Significantly lower than the activity in the parental cells ($P < 0.01$).
[d] The enzymatic reduction of menadione by NADPH was measured in the presence of 10 μM dicoumarol and in its absence. The dicoumarol-sensitive portion of the activity is given.

Table 2 *Effect of temperature on CDDP concentration*

CDDP concentration ± SEM in ng/10^6 cells. The cells were suspended in culture medium containing 50 μg CDDP/ml for 1 h at 37 and 43°C.

	37°C	43°C
Parental cells	51.0	35.8
	38.3	66.0
	31.3	60.0
Mean ± SEM	40.2 ± 7.2	53.9 ± 12.1
CDDP-resistant cells	23.3	37.7
	25.6	46.2
	27.2	40.9
Mean ± SEM	25.4 ± 1.4	41.6 ± 3.1

subline of mouse L1210 leukemia cells, Micetich *et al.* (19) did not find a significantly reduced number of DNA cross-links in the resistant cells compared to the sensitive parent cells. Thus it appears that CDDP resistance in at least two cell lines may be due to mechanisms independent of the extent of cross-linkage of DNA. However, in our study one would expect a higher number of DNA cross-links in the sensitive cells which maintained a higher level of platinum. The only explanation for this discrepancy is that the experiments measuring the platinum concentration were carried out with a much higher CDDP concentration than those determining the DNA cross-links.

Our data show consistently higher levels of cellular GSH in CDDP resistant-RIF-1 cells. The nature and the extent of biological significance of this finding are under investigation. There is mounting evidence for the involvement of cellular thiols in some forms of drug resistance. Although GSH plays an important role in the determination of cell sensitivity to alkylating agents (20), studies on the relationship of GSH levels to CDDP resistance have yielded both positive and negative results (19–23). Since the effect of CDDP on DNA is similar to that of some alkylating agents, Micetich *et al.* (19) have hypothesized that the increased thiol groups might be quenching DNA-CDDP monoadducts in CDDP-resistant cells. In studies on L1210 leukemia cells that exhibited CDDP resistance and a 74% elevation in GSH content, Hromas *et al.* (21) showed that treatment with D,L-buthionine-*S,R*-sulfoximine, a specific inhibitor of GSH synthesis, lowered the cellular GSH concentration to nearly that of the CDDP-sensitive L1210 cells, reversed CDDP resistance, and increased DNA cross-links resulting from exposure of the cells to CDDP. However, in another study (22), D,L-buthionine-*S,R*-sulfoximine pretreatment failed to reverse drug resistance in CDDP-resistant L1210 cells. In studies on a CDDP-resistant subline of human ovarian cancer cells, Behrons *et al.* (23) found higher GSH content as well as several other differences from the parental cell line. Human ovarian cancer cells have also been reported to contain increased levels of metallothionein, a metal-chelating sulfhydryl protein (24). The diversity of experimental findings in different cell lines suggests the existence of multiple mechanisms of CDDP resistance, such as the alteration of glutathione (or thiol-containing proteins) oxidation-reduction cycle, overexpression of membrane glycoprotein with a molecular weight of 170,000 (common MDR) or accelerated DNA repair. In the present studies on RIF-1 cells we found that, in contrast to the consistently higher GSH levels in the CDDP-resistant cells, no substantial difference existed in the activities of several enzymes related to GSH metabolism, except that GSH transferase activity was lower in the resistant cells. Although there appears to be no known basis for a causal relationship between decreased GSH transferase activity and CDDP resistance, further investigation will be required.

A consistent finding in tumor cells resistant to various drugs has been the lower concentration of the drug in resistant cells. We found the concentration of CDDP in resistant cells to be about 60% of that in the sensitive parent cell line. This observation is in agreement with the work of Hromas *et al.* (25) and Kraker and Moore (26), who demonstrated decreased CDDP uptake by a CDDP-resistant L1210 leukemia cell subline. Similar results were reported with anthracycline derivatives (27, 28). One of the major mechanisms of tumor drug resistance has been shown, in several cell lines, to be a decrease in drug accumulation which is associated with increased production of a membrane glycoprotein with a molecular weight of 170,000 (29). A major characteristic of this MDR phenotype is the reversal of drug resistance by calcium channel blockers such as verapamil.

Our resistant cell line showed lower concentrations of platinum but differed from the MDR phenotype in that the addition of verapamil did not change cellular resistance to CDDP, suggesting another mechanism for drug resistance. It has been suggested that altered glutathione oxidation-reduction cycle is an important pathway of drug resistance (30, 31). Our resistant cells exhibited two characteristics of MDR, *i.e.*, lower intracellular CDDP and high GSH. In addition they have two other interesting characteristics, namely increased sensitivity to heat and to spermidine.

Our data showed CDDP resistance in the RIF-1 cell subline to be reversible by hyperthermia. The studies of Wallner *et al.* (32, 33) using CDDP-sensitive and -resistant Chinese hamster ovary cell lines also showed reversal of resistance by application of heat. The data in Table 2 indicate that increasing the temperature to 43°C increased the amount of platinum measured in both RIF-1 cell lines. Thus increased drug concentration appeared to be a factor both in the increased cytotoxicity of CDDP in RIF-1 cells and in the reversal of CDDP resistance by hyperthermia. Heat alone had significant tumoricidal activity (Figs. 1–3), as has also been shown by Neilan *et al.* (34) in RIF-1 cells as well as in Chinese hamster fibroblasts. The present studies also showed that the tumoricidal activity of heat was higher for CDDP-resistant cells than for the parental cells. Based on our data we attribute the hyperthermic reversal of CDDP resistance to reduction of differences in intracellular CDDP levels between sensitive and resistant cells. The possibility that elevated heat sensitivity may be related to alterations in GSH metabolism is under investigation.

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