

## Cross-resistance between cisplatin, antimony potassium tartrate, and arsenite in human tumor cells.

P Naredi, ... , R E Enns, S B Howell

*J Clin Invest.* 1995;**95**(3):1193-1198. <https://doi.org/10.1172/JCI117768>.

### Research Article

Cross-resistance between cisplatin (DDP) and metalloid salts in human cells was sought on the basis that mechanisms that mediate metalloid salt cross-resistance in prokaryotes are evolutionarily conserved. Two ovarian and two head and neck carcinoma cell lines selected for DDP resistance were found to be cross-resistant to antimony potassium tartrate, which contains trivalent antimony. The DDP-resistant variant 2008/A was also cross-resistant to arsenite but not to stibogluconate, which contains pentavalent antimony. A variant selected for resistance to antimony potassium tartrate was cross-resistant to DDP and arsenite. Resistance to antimony potassium tartrate and arsenite was of a similar magnitude (3-7-fold), whereas the level of resistance to DDP was greater (17-fold), irrespective of whether the cells were selected by exposure to DDP or to antimony potassium tartrate. In the resistant sublines, uptake of [3H]-dichloro(ethylenediamine) platinum(II) was reduced to 41-52% of control, and a similar deficit was observed in the accumulation of arsenite. We conclude that DDP, antimony potassium tartrate, and arsenite all share a common mechanism of resistance in human cells and that this is due in part to an accumulation defect.

**Find the latest version:**

<https://jci.me/117768/pdf>



# Cross-Resistance between Cisplatin, Antimony Potassium Tartrate, and Arsenite in Human Tumor Cells

Peter Naredi,\* Dennis D. Heath,\* Robert E. Enns,\* and Stephen B. Howell\*

\*Department of Surgery, Sahlgrenska University Hospital, S-41345 Göteborg, Sweden; and †Department of Medicine and the Cancer Center, University of California San Diego, La Jolla, California 92093

## Abstract

Cross-resistance between cisplatin (DDP) and metalloid salts in human cells was sought on the basis that mechanisms that mediate metalloid salt cross-resistance in prokaryotes are evolutionarily conserved. Two ovarian and two head and neck carcinoma cell lines selected for DDP resistance were found to be cross-resistant to antimony potassium tartrate, which contains trivalent antimony. The DDP-resistant variant 2008/A was also cross-resistant to arsenite but not to stibogluconate, which contains pentavalent antimony. A variant selected for resistance to antimony potassium tartrate was cross-resistant to DDP and arsenite. Resistance to antimony potassium tartrate and arsenite was of a similar magnitude (3–7-fold), whereas the level of resistance to DDP was greater (17-fold), irrespective of whether the cells were selected by exposure to DDP or to antimony potassium tartrate. In the resistant sublines, uptake of [<sup>3</sup>H]-dichloro(ethylenediamine) platinum(II) was reduced to 41–52% of control, and a similar deficit was observed in the accumulation of arsenite. We conclude that DDP, antimony potassium tartrate, and arsenite all share a common mechanism of resistance in human cells and that this is due in part to an accumulation defect. (*J. Clin. Invest.* 1995; 95:1193–1198.) Key words: cisplatin • antimonite • arsenite • resistance • cancer

## Introduction

Among several proposed mechanisms for natural and acquired resistance to cisplatin (DDP),<sup>1</sup> decreased drug accumulation is the most consistent finding both in vivo (1) and in vitro (2) (for reviews see references 3–7). The cause of this impairment in DDP accumulation is unknown, and in fact, the mechanism by which DDP enters or exits from cells remains poorly defined. DDP enters cells relatively slowly compared with the anticancer agents that participate in the multidrug-resistant phenotype, and at least one component of DDP uptake is likely to be mediated

by a transport mechanism or channel (6–10). DDP efflux is even slower, and it has been difficult to distinguish any major differences in efflux pattern between sensitive and resistant cell lines (11–13).

Several transport–protein complexes that mediate the detoxification of heavy metal salts have been identified in bacteria and yeast and appear to be structurally and functionally well conserved throughout evolution (14–20). During studies directed at identifying metal salts that demonstrated a pattern of cross-resistance similar to that of DDP, we found that a DDP-selected subline of the human ovarian carcinoma 2008 line was cross-resistant to antimony potassium tartrate, and an antimony potassium tartrate-selected variant was cross-resistant to DDP (Naredi, P., and S. B. Howell, unpublished data). In both resistant variants, there was an impairment of the uptake of the DDP analog [<sup>3</sup>H]-*cis*-dichloro(ethylenediamine) platinum(II) ([<sup>3</sup>H]DEP). Antimony potassium tartrate contains antimony in a trivalent form, and one mechanism of resistance to trivalent antimonials in bacteria is mediated by genes that make up the *ars* operon (21). In *Escherichia coli* and *Staphylococcus aureus*, this operon codes for a specific efflux–transport system that can export oxyanions of antimony but is best known for its ability to confer resistance to arsenic oxyanions (21). In the protozoa *Leishmania*, the *lmpgp* gene has also been reported to confer resistance to both antimony potassium tartrate and arsenite (19).

The aim of this study was to determine the extent to which human ovarian carcinoma cells selected for resistance to DDP are cross-resistant to oxyanions that share a common mechanism of resistance in bacteria. The results indicated that cells selected for resistance to DDP were cross-resistant not only to antimony potassium tartrate, but also to arsenite (see Fig. 1 for structures) and that cells selected for resistance to antimony potassium tartrate showed the same cross-resistant phenotype. In addition, both DDP- and antimony potassium tartrate-selected cells demonstrated impaired accumulation of radiolabeled arsenite that was of the same magnitude as the impairment in the uptake of the DDP analog [<sup>3</sup>H]DEP. The results provide evidence for a common mechanism of resistance to DDP and oxyanions of antimony and arsenic based on a defect in a shared accumulation mechanism system.

## Methods

**Materials.** DDP (Pt(Cl)<sub>2</sub>(NH<sub>3</sub>)<sub>2</sub>) was obtained from the Bristol-Myers Co. (Syracuse, NY). Sodium arsenite (Na<sub>3</sub>AsO<sub>3</sub>) was purchased from Sigma Chemical Co. (St. Louis, MO). Antimony potassium tartrate (bis[μ-[2,3-dihydroxybutanedioato(4-)-01, 02:03, 04]]-diantimonate dipotassium trihydrate) (C<sub>8</sub>H<sub>4</sub>K<sub>2</sub>O<sub>12</sub>Sb<sub>2</sub>·3H<sub>2</sub>O) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Stibogluconate (C<sub>12</sub>H<sub>17</sub>O<sub>17</sub>Sb<sub>2</sub>·Na<sub>3</sub>) was obtained from Burroughs Wellcome (UK). [<sup>3</sup>H]DEP (PtCl<sub>2</sub>N<sub>2</sub>C<sub>2</sub>H<sub>8</sub>) was synthesized as previously described (22). Radioac-

Address correspondence to Dr. Stephen B. Howell, Department of Medicine 0812, University of California, San Diego, La Jolla, CA 92093. Phone: 619-543-5530; FAX: 619-543-5258.

Received for publication 12 July 1994 and in revised form 3 November 1994.

1. Abbreviations used in this paper: DDP, cisplatin; DEP, *cis*-dichloro(ethylenediamine) platinum(II).

*J. Clin. Invest.*

© The American Society for Clinical Investigation, Inc.

0021-9738/95/03/1193/06 \$2.00

Volume 95, March 1995, 1193–1198

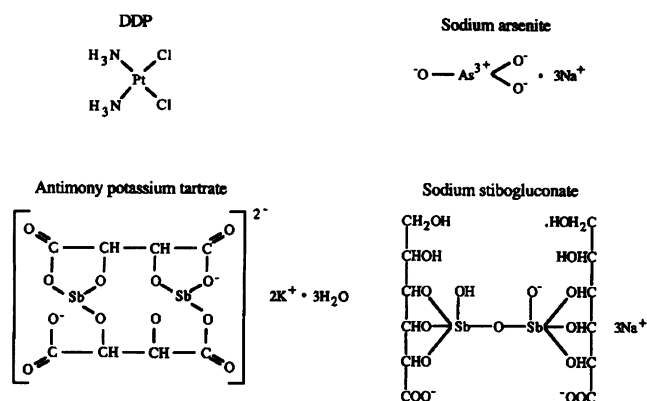


Figure 1. Structures of DDP, sodium arsenite, and antimony potassium tartrate, and stibogluconate.

tive arsenate ( $^{73}\text{AsO}_4$ ) was purchased from Los Alamos National Laboratory (Los Alamos, NM).

**Tumor cell lines.** The human cell line 2008 was established from a patient with a serous cystadenocarcinoma of the ovary (23). A resistant subline, designated 2008/A, was produced by four selections with 0.5  $\mu\text{M}$  DDP over a 1-mo period. 2008/H was obtained by chronic exposure of 2008 cells to antimony potassium tartrate, during which the drug concentration was increased stepwise to 140  $\mu\text{M}$  over 8 mo. A2780 is a human ovarian cancer cell line, and A2780/CP is a DDP-selected variant (24). UMSSC5 and UMSSC10b were derived from human squamous cell carcinomas in the head and neck region (25, 26). The DDP-resistant UMSSC5/DDP and UMSSC10b/DDP lines were selected by chronic exposure to DDP with incremental increases in DDP concentration to 0.6 and 0.8  $\mu\text{M}$ , respectively, over 5 mo (27). The present studies were performed after cells had been passaged in drug-free media a minimum of three times. The cells were grown on tissue culture dishes in a humidified incubator at 37°C in a 5% CO<sub>2</sub> atmosphere. They were maintained in complete medium consisting of RPMI 1640 (Irvine Scientific, Santa Ana, CA) supplemented with 5% (10% for UMSSC cells) heat-inactivated FCS and 2 mM glutamine. Cultures were routinely tested for mycoplasma using a kit from Gen Probe Inc. (San Diego, CA) and were found to be negative.

**Clonogenic assays.** Cells in exponential growth were trypsinized from monolayer culture and adjusted to a concentration of 60 cells per ml. A volume of 5 ml was plated onto 60-mm polystyrene tissue culture dishes (Corning Glass Works, Corning, NY). Stock solutions of drugs to be tested were added to triplicate plates at each drug concentration. Plates were incubated at 37°C in a 5% CO<sub>2</sub> humidified atmosphere, and after 10 d, the plates were fixed with methanol and stained with Giemsa. Colonies of over 50 cells were counted macroscopically. Each experiment was repeated a minimum of three times using triplicate cultures.

**[<sup>3</sup>H]DEP accumulation.** Uptake was measured with subconfluent cells by addition of 5  $\mu\text{M}$  [<sup>3</sup>H]DEP (5  $\mu\text{Ci}/\text{ml}$ ) to triplicate cultures for the indicated period of time. Immediately after completion of [<sup>3</sup>H]DEP exposure, the cells were washed six times with a total of 20 ml of 4°C PBS and lysed in 2 ml of 1 N NaOH overnight. An aliquot was removed for protein determination by the method of Bradford (28). Radioactivity was determined by liquid scintillation counting. Each experiment was performed a minimum of three times with triplicate cultures.

**Synthesis of <sup>73</sup>AsO<sub>3</sub>.** Radioactive arsenate was chemically reduced to arsenite as described previously (29). 40  $\mu\text{l}$  of <sup>73</sup>AsO<sub>4</sub> (0.5  $\mu\text{Ci}/\mu\text{l}$ ) was mixed with 40  $\mu\text{l}$  of a reducing solution consisting of 1 mM Na<sub>3</sub>AsO<sub>4</sub>, 66 mM Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub>, 27 mM Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, and 82 mM H<sub>2</sub>SO<sub>4</sub>. After 40 min at room temperature, > 99.5% of <sup>73</sup>AsO<sub>4</sub> was reduced to <sup>73</sup>AsO<sub>3</sub>, as verified by HPLC. Freshly reduced <sup>73</sup>AsO<sub>3</sub> was made each day.

**<sup>73</sup>AsO<sub>3</sub> accumulation.** Uptake was measured using subconfluent cells growing in 60-mm dishes by addition of 3  $\mu\text{M}$  <sup>73</sup>AsO<sub>3</sub> (0.25  $\mu\text{Ci}/\text{ml}$ ) to duplicate cultures for the indicated period of time. Immediately after

Table I. Cytotoxicity of DDP and Antimony Potassium Tartrate against Human Ovarian and Squamous Head and Neck Carcinoma Cell Lines

Cell line	DDP		Antimony potassium tartrate	
	IC <sub>50</sub> ( $\mu\text{M}$ )	Fold resistant	IC <sub>50</sub> ( $\mu\text{M}$ )	Fold resistant
2008	0.13±0.01*		3.4±0.2	
2008/A	2.6±0.8	17±4 <sup>‡</sup>	17±1	4.9±0.4 <sup>‡</sup>
A2780	0.25±0.09		0.53±0.24	
A2780 <sup>CP</sup>	2.6±0.4	11±3 <sup>‡</sup>	4.8±1.3	7.2±0.3 <sup>‡</sup>
UMSCC 10b	0.27±0.01		4.0±1.1	
UMSCC10b/DDP	0.53±0.07	2.0±0.2 <sup>‡</sup>	8.4±1.4	2.1±0.2 <sup>‡</sup>
UMSCC5	0.27±0.02		4.0±1.1	
UMSCC5/DDP	0.62±0.06	2.3±0.3 <sup>‡</sup>	8.7±0.9	2.3±0.6 <sup>‡</sup>

\* Data are mean±SD of three determinations performed with triplicate cultures. <sup>‡</sup> Significantly different ( $P < 0.05$ ) compared with the parent cell line, as determined by paired Student's  $t$  test.

completion of <sup>73</sup>AsO<sub>3</sub> exposure, the cells were washed and lysed as for the [<sup>3</sup>H]DEP accumulation experiments. Protein determination and radioactivity measurements were also similar. Each experiment was repeated a minimum of three times.

**[<sup>3</sup>H]DEP and <sup>73</sup>AsO<sub>3</sub> efflux.** Subconfluent cells were prepared on dishes as for the accumulation experiments and loaded with drug by incubation with 5  $\mu\text{M}$  [<sup>3</sup>H]DEP (5  $\mu\text{Ci}/\text{ml}$ ) or 3  $\mu\text{M}$  <sup>73</sup>AsO<sub>3</sub> (0.25  $\mu\text{Ci}/\text{ml}$ ) for 120 min. At the end of the loading period, they were washed six times with a total of 20 ml of 37°C PBS. Drug-free medium was then added, and the cells were incubated at 37°C for the indicated efflux time. Efflux was terminated by washing six times with a total of 20 ml of PBS at 4°C. The monolayers were assayed for protein determination and radioactivity for the uptake experiments. Each experiment was repeated three times with duplicate cultures.

**Statistics.** IC<sub>50</sub> was defined as the concentration of drug that reduced the number of colonies to 50% of that in the untreated control cultures. Statistical significance of data was determined by paired Student's  $t$  test.  $P < 0.05$  was considered to be statistically significant. All values reported are mean±SD.

## Results

Two pairs of DDP-sensitive and -resistant human ovarian carcinoma cell lines and two pairs of human head and neck carcinoma cells lines were tested for cross-resistance to antimony potassium tartrate. The IC<sub>50</sub> values for the parental 2008 and A2780 human ovarian carcinoma cell lines were 0.13±0.01 and 0.25±0.09  $\mu\text{M}$ , respectively, for DDP and 3.4±0.2 and 0.53±0.24  $\mu\text{M}$ , respectively, for antimony potassium tartrate (Table I). The 17-fold DDP-resistant, DDP-selected subline 2008/A was 4.9-fold cross-resistant to antimony potassium tartrate ( $P < 0.05$ ), and the 11-fold DDP-resistant, DDP-selected subline A2780/CP was 7.2-fold cross-resistant to antimony potassium tartrate ( $P < 0.05$ ). Thus, both DDP-resistant variants of the ovarian carcinoma cell lines demonstrated substantial cross-resistance to antimonite. The same observation was made for the two head and neck carcinoma cell lines. The parental human squamous head and neck carcinoma cell lines UMSSC10b and UMSSC5 had similar IC<sub>50</sub> values for DDP (0.27±0.01 and 0.27±0.02  $\mu\text{M}$ , respectively) and for antimony potassium tartrate (4.0±1.1  $\mu\text{M}$  for both) (Table I). The DDP-

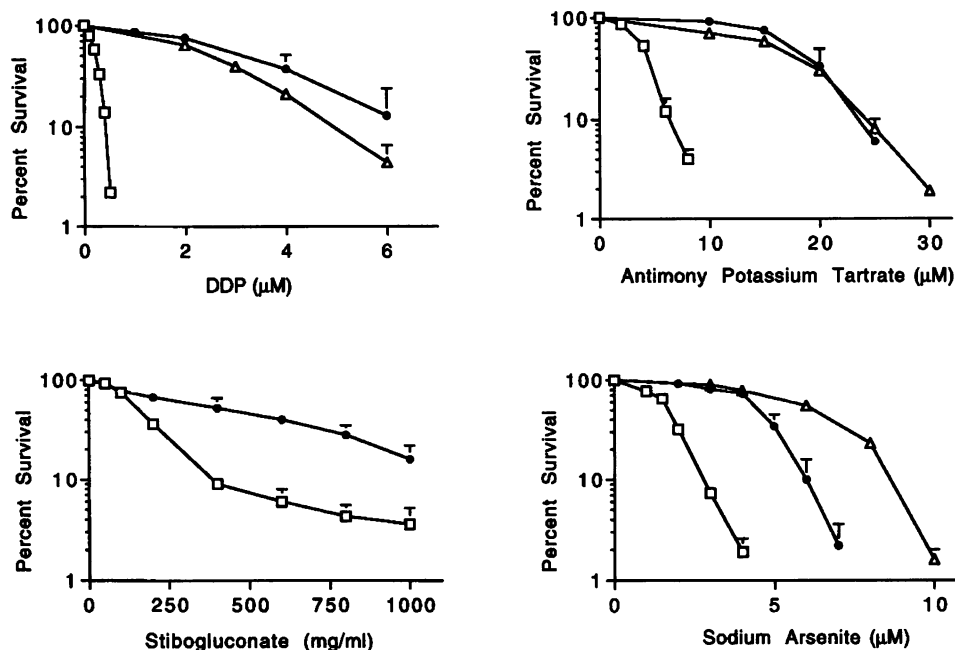


Figure 2. Dose-response curves for DDP (top left), antimony potassium tartrate (top right), stibogluconate (bottom left), and sodium arsenite (bottom right), as determined by clonogenic assay. (Open squares) 2008 cells; (closed circles) 2008/A cells; (open triangles) 2008/H cells. Data points represent the mean  $\pm$  SD of three experiments performed with triplicate cultures.

selected variant UMSCC10b/DDP was 2.0-fold resistant to DDP and 2.1-fold cross-resistant to antimony potassium tartrate ( $P < 0.05$ ). The DDP-selected UMSCC5/DDP cells were 2.3-fold resistant to both DDP and antimony potassium tartrate ( $P < 0.05$ ). Thus, all four DDP-resistant variants demonstrated cross-resistance to antimony potassium tartrate, and there was a good correlation ( $r = 0.75$ ) between the magnitude of the DDP resistance and the magnitude of the cross-resistance to antimony potassium tartrate.

Antimony potassium tartrate contains antimony in its trivalent form. The 17-fold DDP-resistant 2008/A subline was tested for cross-resistance to stibogluconate, which contains antimony in its pentavalent form, and to arsenite, which contains arsenic in its trivalent form. Fig. 2 shows that 2008/A cells were cross-resistant to arsenite but not stibogluconate. The  $IC_{50}$  for stibogluconate tested against the parental 2008 cells was  $30 \pm 9 \mu\text{g/ml}$ , whereas for the 2008A cells, it was  $26 \pm 13 \mu\text{g/ml}$ . The  $IC_{50}$  for arsenite against the parental 2008 cells was  $1.5 \pm 0.1 \mu\text{M}$ , and for the 2008/A subline, it was  $4.5 \pm 0.1 \mu\text{M}$  (3.0-fold resistant) ( $P < 0.05$ ). Thus, the mechanism protecting 2008/A cells against DDP did not offer any protection against a pentavalent antimonial, but did offer a substantial degree of protection against a trivalent oxyanion of arsenic.

If DDP, antimony potassium tartrate, and arsenite share a common mechanism of resistance, then one might expect that cells selected for resistance to antimony potassium tartrate would also be resistant to DDP and arsenite, but not to stibogluconate. Fig. 2 shows the survival curves for the antimony potassium tartrate-selected 2008/H cells for DDP, antimony potassium tartrate, sodium arsenite, and stibogluconate. The  $IC_{50}$  for antimony potassium tartrate against 2008/H cells was  $14 \pm 1 \mu\text{M}$ , indicating that they were 6.6-fold resistant compared with the parental 2008 cells. They were also  $16 \pm 1$ -fold cross-resistant to DDP ( $P < 0.05$ ) and  $4.3 \pm 0.5$ -fold cross-resistant to sodium arsenite ( $P < 0.05$ ), but were not cross-resistant to stibogluconate. Thus, each of the predictions was substantiated, arguing strongly for a common underlying mechanism of resistance.

Previous studies with DDP-selected variants of 2008 cells had demonstrated impaired uptake of both DDP (2, 9, 11) and an analog of DDP, DEP, which can be labeled to high specific activity with  $^3\text{H}$  (30). Fig. 3 (top panel) shows the time course of [ $^3\text{H}$ ]DEP accumulation over 2 h in the parental 2008 cells and in DDP- and antimony potassium tartrate-selected variants. The rate of [ $^3\text{H}$ ]DEP accumulation in 2008 cells was  $0.56 \pm 0.21 \text{ pmol/mg protein per min}$ . In the 2008/A subline, the uptake rate was reduced to  $0.29 \pm 0.04 \text{ pmol/mg per min}$  (52% of control;  $P < 0.05$ ). Likewise, in 2008/H cells, the uptake rate was reduced to  $0.23 \pm 0.05 \text{ pmol/mg per min}$  (41% of control;  $P < 0.05$ ). Total accumulation of [ $^3\text{H}$ ]DEP at 2 h in 2008 cells was  $88 \pm 20 \text{ pmol/mg protein}$ . It was 67% of this control value in the 2008/A variant ( $59 \pm 4 \text{ pmol/mg protein}$ ) and 52% of control in the 2008/H subline ( $46 \pm 8 \text{ pmol/mg protein}$ ) ( $P < 0.05$  for both resistant cell types).

If impaired uptake is a central component of the mechanism underlying the shared DDP/antimonite/arsenite-resistant phenotype, then one might expect that both the DDP- and the antimony potassium tartrate-resistant sublines would also demonstrate an impairment in the uptake of antimony potassium tartrate and arsenite. A radiolabeled form of antimony potassium tartrate was not available for such studies, but radioactive  $^{73}\text{AsO}_3$  was successfully obtained. Fig. 3 (bottom panel) shows the time course for the uptake of  $^{73}\text{AsO}_3$  during a 2-h exposure. The rates of  $^{73}\text{AsO}_3$  accumulation were statistically significantly lower in the resistant sublines (2008 cells,  $2.7 \pm 0.4 \text{ pmol/mg per min}$ ; 2008/A cells,  $1.3 \pm 0.1 \text{ pmol/mg per min}$ ; and 2008/H cells,  $1.2 \pm 0.1 \text{ pmol/mg per min}$ ). The accumulation of  $^{73}\text{AsO}_3$  at 2 h was  $332 \pm 35 \text{ pmol/mg protein}$  in the parental 2008 cells. In the 2008/A cells, it was 52% lower ( $160 \pm 14 \text{ pmol/mg protein}$ ), and in the 2008/H cells, accumulation was 56% lower ( $146 \pm 10 \text{ pmol/mg protein}$ ) when measured at 2 h. To exclude the possibility that measurement of  $^{73}\text{AsO}_3$  uptake was confounded by the presence of contaminating  $^{73}\text{AsO}_4$ , the  $^{73}\text{AsO}_3$  was analyzed by HPLC and found to contain  $< 0.5\%$   $^{73}\text{AsO}_4$ . In addition, the uptake of  $^{73}\text{AsO}_4$  was found to be only 3.3–5.4% of that of  $^{73}\text{AsO}_3$ , making it very unlikely that the

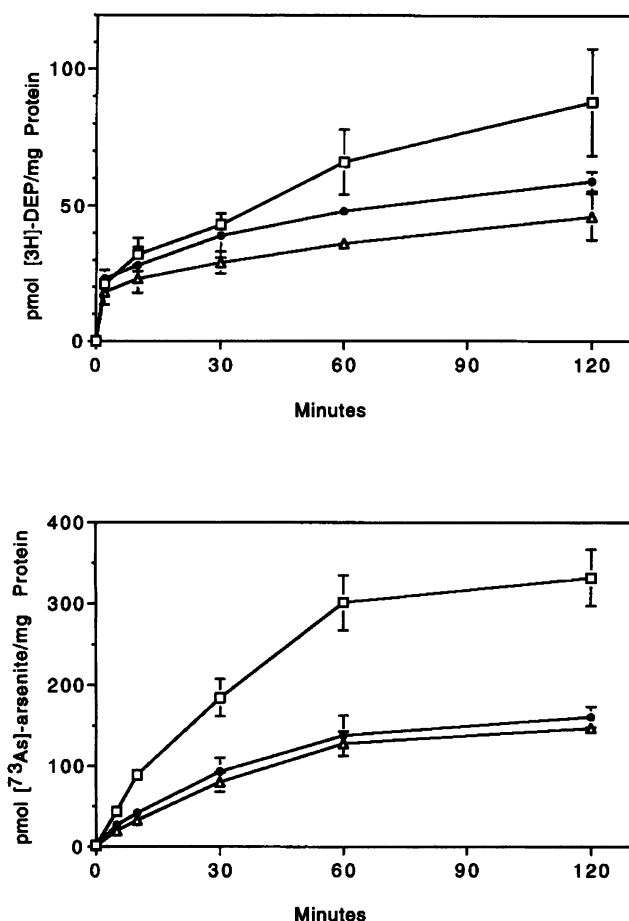


Figure 3. Time course of [ $^3\text{H}$ ]DEP (top panel) and  $^{73}\text{AsO}_3$  (bottom panel) uptake during exposure to  $5\text{ }\mu\text{M}$  [ $^3\text{H}$ ]DEP ( $5\text{ }\mu\text{Ci/ml}$ ) or  $3\text{ }\mu\text{M}$   $^{73}\text{AsO}_3$  ( $0.25\text{ }\mu\text{Ci/ml}$ ) for 2 h. (Open squares) 2008 cells; (closed circles) 2008/A cells; (open triangles) 2008/H cells. Data points represent the mean  $\pm$  SD of three experiments performed with triplicate ([ $^3\text{H}$ ]DEP) or duplicate ( $^{73}\text{AsO}_3$ ) cultures.

differences in uptake of  $^{73}\text{AsO}_3$  among the three cells could be attributed to differences in  $^{73}\text{AsO}_4$  uptake. We conclude that both the DDP- and the antimonite-selected sublines had a deficit in the uptake of both [ $^3\text{H}$ ]DEP and  $^{73}\text{AsO}_3$  and that the magnitude of this deficit was quite similar for both radiolabeled compounds.

To determine whether the impaired accumulation of [ $^3\text{H}$ ]DEP and  $^{73}\text{AsO}_3$  in the resistant sublines was due solely to a decrease in influx, the rate of efflux was compared in each of the lines. Fig. 4 (top panel) shows that the efflux of [ $^3\text{H}$ ]DEP from the parental 2008 cells was very slow, with 85% of the accumulated drug remaining in the cells after 2 h of efflux into drug-free medium. There was no difference in efflux in the 2008/A and 2008/H variants, which retained 83 and 79%, respectively, of the [ $^3\text{H}$ ]DEP present at the start of the efflux period. The efflux rate of  $^{73}\text{AsO}_3$  was 4-fold greater than that of [ $^3\text{H}$ ]DEP ( $0.72 \pm 0.05\%$ /min versus  $0.16 \pm 0.16\%$ /min;  $P < 0.05$ ), but there was no difference in the rate of efflux between the 2008, 2008/A, and 2008/H cells. Only 16% of the  $^{73}\text{AsO}_3$  remained in the 2008 cells after 2 h; in the case of the 2008/A and 2008/H cells, it was 14 and 13%, respectively. Thus, the mechanism underlying the DDP/antimony/arsenite-

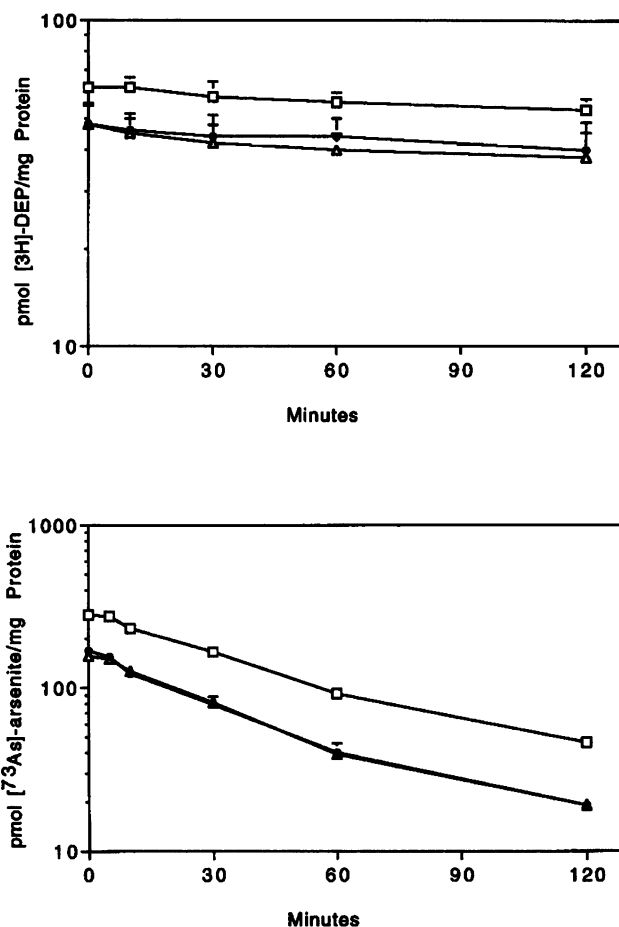


Figure 4. Time course of efflux of [ $^3\text{H}$ ]DEP (top panel) and  $^{73}\text{AsO}_3$  (bottom panel) after loading cells by exposure to  $5\text{ }\mu\text{M}$  [ $^3\text{H}$ ]DEP ( $5\text{ }\mu\text{Ci/ml}$ ) or  $3\text{ }\mu\text{M}$   $^{73}\text{AsO}_3$  ( $0.25\text{ }\mu\text{Ci/ml}$ ) for 2 h. (Open squares) 2008 cells; (closed circles) 2008/A cells; (open triangles) 2008/H cells. Data points represent the mean  $\pm$  SD of three experiments performed with triplicate ([ $^3\text{H}$ ]DEP) or duplicate ( $^{73}\text{AsO}_3$ ) cultures.

resistant phenotype impacted exclusively on accumulation and had no discernable effect on efflux.

## Discussion

The results reported here disclose a novel aspect of the DDP-resistant phenotype by demonstrating that the phenotype includes cross-resistance to antimony potassium tartrate and arsenite. The DDP-resistant phenotype has been characterized in several human carcinomas, both in vitro and in vivo, and in some circumstances, it has been shown to include cross-resistance to gamma radiation, UV light, other heavy metals and platinum-containing drugs, methotrexate, and alkylating agents (for reviews see references 3–5). However, DDP resistance is not accompanied by resistance to drugs that participate in the multidrug-resistant phenotype mediated by the *mdr1* gene product (31, 32); nor has it previously been reported to be accompanied by resistance to antimony- or arsenic-containing agents.

In this study, four different human DDP-resistant tumor cell lines representing two different histologic types of cancer were found to be cross-resistant to antimony potassium tartrate. There was a correlation between the level of DDP resistance and the

degree of cross-resistance to antimony potassium tartrate ( $r = 0.75$ ). In addition, primary resistance to DDP and cross-resistance to antimony potassium tartrate were accompanied by cross-resistance to arsenite, and the same pattern of cross-resistance to DDP and arsenite was observed in 2008 cells selected for primary resistance to antimony potassium tartrate. In 2008 cells demonstrating the DDP/antimonite/arsenite-resistant phenotype, the level of resistance to antimony potassium tartrate and arsenite was of a similar magnitude (3–7-fold), whereas the level of resistance to DDP was somewhat greater (17-fold), irrespective of whether the cells were selected by exposure to either DDP or antimony potassium tartrate. Interestingly, 2008 cells expressing the DDP/antimonite/arsenite-resistant phenotype, whether selected primarily with DDP or with antimony potassium tartrate, were not cross-resistant to antimony in its pentavalent state, as found in stibogluconate. We now refer to the phenotype of cells cross-resistant to DDP, arsenite, and antimony potassium tartrate as the RASP phenotype (Resistance to As/Sb/Pt)

Impairment in the uptake of DDP and [ $^3\text{H}$ ]DEP has been observed in many types of cells selected for resistance to DDP (9, 13, 33), as well as the 2008/A subline. The antimony potassium tartrate-selected 2008/H subline not only had a cross-resistance profile similar to that of the DDP-selected 2008/A subline, but also had the same degree of impairment in [ $^3\text{H}$ ]DEP uptake. In addition, both the DDP-resistant and antimony potassium tartrate-resistant variants demonstrated an impairment in the uptake of radiolabeled arsenite, and as for [ $^3\text{H}$ ]DEP, the impairment in arsenite accumulation was of the same magnitude in both resistant variants. In contrast, there was no evidence of an alteration in efflux. Whereas the efflux of arsenite was four times faster during the 2-h observation period than the efflux of [ $^3\text{H}$ ]DEP, no difference was observed for either agent in the efflux rate between the sensitive and resistant cells. It is clear that resistance to DDP is multifactorial (5) and not due solely to decreased accumulation. It appears that this is also the case for arsenite and that for this drug too there is not tight linkage between the magnitude of the decrease in uptake and the degree of resistance.

The mechanism by which DDP enters or exits from cells remains poorly defined (3). DDP uptake is not saturable within the limits of its solubility in tissue culture medium, which argues that either DDP enters cells by passive diffusion, or saturation occurs only at concentrations above its maximum solubility (11, 33). Thus far, no membrane changes of a magnitude sufficient to account for the observed decrease in uptake due to a change in passive diffusion of DDP have been found in DDP-resistant 2008 sublines (34). On the other hand, there is increasing evidence that DDP accumulation is mediated in part by an active transporter or channel that is dependent on a functional ( $\text{Na}^+/\text{K}^+$ )-ATPase, membrane potential, extracellular pH, and extracellular osmolality (for review see reference 3). In addition, DDP accumulation can be inhibited by ~50% by a variety of agents (8). Current evidence supports a model of DDP accumulation that involves contributions from both passive diffusion and a transporter or gated channel (3). Although antimony potassium tartrate and stibogluconate have structures quite different from those of cisplatin and sodium arsenite, the structure of the former two drugs in an aqueous environment is not known, and it is unclear what form of the drug is actually taken up into the cell. At physiologic pH, DDP probably crosses the membrane as a neutral drug and arsenite is present largely as

arsenous acid ( $\text{As}(\text{OH})_3$ ), which is also neutral. There is a strong suspicion that antimony potassium tartrate and stibogluconate come apart in an aqueous environment and that they cross the membrane as oxyanions, but no information is currently available as to the actual charge or molecular weight of the form that gets into the cell.

Impairment in arsenite accumulation, as well as that of antimony potassium tartrate, is also observed in drug-resistant strains of bacteria and the promastigote *Leishmania*; however, in these organisms, the impaired accumulation is due to enhanced efflux rather than decreased influx. In bacteria there is a well-defined plasmid-encoded efflux system belonging to the ATP-binding cassette family that exports arsenite and metabolites of antimony potassium tartrate. The efflux transporter consists of the membrane-spanning subunit, ArsB, a specific protein for the reduction of pentavalent anions to the trivalent state, ArsC, and in gram-negative bacteria, also a third protein, ArsA, with ATPase activity (21, 35). No example of resistance due to decreased influx of arsenite or antimony-containing anions has been reported in bacteria. In fact, whereas pentavalent arsenic (arsenate) is known to be taken up by the phosphate transport system in both bacteria and human cells (21, 36), nothing is known about the uptake of the trivalent forms of arsenic and antimony, such as arsenite and antimony potassium tartrate.

In the protozoa *Leishmania*, the *lmpgp* gene has been reported to confer resistance to the trivalent antimony compound antimony potassium tartrate, but not to stibogluconate, which contains antimony in the pentavalent state (19). In contrast, resistance of *Leishmania* to pentavalent antimonials has been associated with the expression of a *P*-glycoprotein-like molecule coded for by the *lpgpA* gene (37), and such resistance can be reversed by verapamil (38). Stibogluconate-resistant *Leishmania* were reported to accumulate two to five times less [ $^{125}\text{Sb}$ ]stibogluconate than sensitive clones, and the *P*-glycoprotein-specific antibody C219 detected a protein in *Leishmania* whose molecular weight was the same as that of *P*-glycoprotein in the multidrug-resistant human KB carcinoma sublines (39). Thus, the phenotype generated by amplification of the *lmpgp* gene appears to be closer to the RASP phenotype in human cells than that produced by the *lpgpA* gene.

The discovery that the DDP-resistant phenotype also includes resistance to antimony potassium tartrate and arsenite begs the question of whether this form of resistance extends to other metalloid salts, such as selenite or tellurite, in particular because biochemical mechanisms for the detoxification of several of these salts are well described and may be relevant to the detoxification of DDP in some tissues. The fact that the phenotype includes impaired uptake of arsenite is also of interest because it provides an additional putative substrate for the DDP uptake pathway, available in an easily usable radiolabeled form, with which to study the specificity of the accumulation mechanism.

## Acknowledgments

This work was supported by grants from the National Institutes of Health (CA-55725), the American Cancer Society (DHP26F), the Swedish Medical Research Council, the Assar Gabrielsson's Foundation for Cancer Research, the Swedish Institute, and the King Gustav V Jubilee Clinic Cancer Research Foundation in Göteborg. This work was conducted in part by the Clayton Foundation for Research—California Division. Dr. Howell is a Clayton Foundation Investigator.

## References

- Loehrer, P. J., and L. H. Einhorn. 1984. Cisplatin. *Ann. Intern. Med.* 100:704–713.
- Andrews, P. A., J. A. Jones, N. M. Varki, and S. B. Howell. 1990. Rapid emergence of acquired *cis*-diamminedichloroplatinum(II) resistance in an *in vivo* model of human ovarian carcinoma. *Cancer Commun.* 2:93–100.
- Gately, D. P., and S. B. Howell. 1993. Cellular accumulation of the anticancer agent cisplatin: a review. *Br. J. Cancer.* 67:1171–1176.
- Timmer-Bosscha, H., N. H. Mulder, and E. G. E. DeVries. 1992. Modulation of *cis*-diamminedichloroplatinum(II) resistance: a review. *Br. J. Cancer.* 66:227–238.
- Andrews, P. A., and S. B. Howell. 1990. Cellular pharmacology of cisplatin: perspectives on mechanism of acquired resistance. *Cancer Cells.* 2:35–42.
- Dornish, J. M., E. O. Pettersen, and R. Oftebro. 1989. Modifying effect of cinnamaldehyde and cinnamaldehyde derivatives on cell inactivation and cellular uptake of *cis*-diamminedichloroplatinum(II) in human NHIK 3025 cells. *Cancer Res.* 49:3917–3921.
- Andrews, P. A., and K. D. Albright. 1991. Role of membrane ion transport in cisplatin accumulation. In *Platinum and Other Metal Coordination Compounds in Cancer Chemotherapy*. S. B. Howell, editor. Plenum Publishing Corp., New York. 151–159.
- Andrews, P. A., S. C. Mann, H. H. Huynh, and K. D. Albright. 1991. Role of  $\text{Na}^+$ ,  $\text{K}^+$ -adenosine triphosphatase in the accumulation of *cis*-diamminedichloroplatinum(II) in human ovarian carcinoma cells. *Cancer Res.* 51:3677–3681.
- Andrews, P. A., S. Velury, S. C. Mann, and S. B. Howell. 1988. *cis*-Diamminedichloroplatinum(II) accumulation in sensitive and resistant human ovarian carcinoma cells. *Cancer Res.* 48:68–73.
- Mann, S. C., P. A. Andrews, and S. B. Howell. 1991. Modulation of *cis*-diamminedichloroplatinum(II) accumulation and sensitivity by forskolin and 3-isobutyl-1-methylxanthine in sensitive and resistant human ovarian carcinoma cells. *Int. J. Cancer.* 48:866–872.
- Mann, S. C., P. A. Andrews, and S. B. Howell. 1990. Short-term *cis*-diamminedichloroplatinum(II) accumulation in sensitive and resistant human ovarian carcinoma cells. *Cancer Chemother. Pharmacol.* 25:236–240.
- Shionoya, S., Y. Lu, and K. J. Scanlon. 1986. Properties of amino acid transport systems in K562 cells sensitive and resistant to *cis*-diamminedichloroplatinum(II). *Cancer Res.* 46:3445–3448.
- Waud, W. R. 1987. Differential uptake of *cis*-diamminedichloroplatinum(II) by sensitive and resistant murine L1210 leukemia cells. *Cancer Res.* 47:6549–6555.
- Ortiz, D. F., L. Kreppel, D. M. Speiser, G. Scheel, G. McDonald, and D. W. Ow. 1992. Heavy metal tolerance in the fission yeast requires an ATP-binding cassette-type vacuolar membrane transporter. *EMBO (Eur. Mol. Biol. Org.) J.* 11:3491–3499.
- Conklin, D. S., J. A. McMaster, M. R. Culberston, and C. Kung. 1992. COT1, a gene involved in cobalt accumulation in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 12:3678–3688.
- Kamizono, A., M. Nishizawa, Y. Teranishi, K. Murata, and A. Kimura. 1989. Identification of a gene conferring resistance to zinc and cadmium ions in the yeast *Saccharomyces cerevisiae*. *Mol. Cell. Genet.* 219:161–167.
- Nies, D. H. 1992. CzcR and CzcD, gene products affecting regulation of resistance to cobalt, zinc, and cadmium (czc system) in *Alcaligenes eutrophus*. *J. Bacteriol.* 174:8102–8110.
- Nies, D. H. 1992. Resistance to cadmium, cobalt, zinc, and nickel in microbes. *Plasmid.* 27:17–28.
- Callahan, H. L., and S. M. Beverley. 1992. Heavy metal resistance: a new role for *P*-glycoproteins in Leishmania. *J. Biol. Chem.* 266:18427–18430.
- Wu, J., and B. P. Rosen. 1993. Metalloregulated expression of the ars operon. *J. Biol. Chem.* 268:52–58.
- Kaur, P., and B. P. Rosen. 1992. Plasmid-encoded resistance to arsenic and antimony. *Plasmid.* 27:29–40.
- Eastman, A. 1983. Characterization of the adducts produced in DNA by *cis*-diamminedichloroplatinum(II) and *cis*-dichloro-(ethylene-diamine)platinum(II). *Biochemistry.* 22:3927–3933.
- Disaia, P. J., J. G. Sinkovics, F. N. Rutledge, and J. P. Smith. 1972. Cell-mediated immunity to human malignant cells. *Am. J. Obstet. Gynecol.* 114:979–989.
- Behrens, B. C., T. C. Hamilton, H. Masuda, K. R. Grotzinger, J. Whang-Peng, K. G. Louie, T. Knutzen, W. M. McKoy, R. C. Young, and R. F. Ozols. 1987. Characterization of a *cis*-diamminedichloroplatinum(II)-resistant human ovarian cancer cell line and its use in evaluation of platinum analogues. *Cancer Res.* 47:414–418.
- Krause, C. J., T. E. Carey, R. W. Ott, C. Hurbis, K. D. McClatchey, and J. A. Regezi. 1981. Human squamous cell carcinoma: establishment and characterization of new permanent cells lines. *Arch. Otolaryngol.* 107:703–710.
- Grenman, R., D. Burk, E. Virolainen, R. N. Buick, J. Church, D. R. Schwartz, and T. E. Carey. 1989. Clonogenic cell assay for anchorage-dependent squamous carcinoma cell lines using limiting dilution. *Int. J. Cancer.* 44:131–136.
- Nakata, B., R. Barton, S. B. Howell, and G. Los. 1994. Association between over-expression of hsp60 and cisplatin resistance in head and neck cancer cells. *Proc. Am. Assoc. Cancer Res.* 35:466. (Abstr.)
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.
- Rosen, B. P., and M. G. Borbolla. 1984. A plasmid-encoded arsenite pump produces arsenite resistance in *Escherichia coli*. *Biochem. Biophys. Res. Commun.* 124:760–765.
- Christen, R. D., A. P. Jekunen, J. A. Jones, F. B. Thiebaut, D. R. Shalinsky, and S. B. Howell. 1993. In vitro modulation of cisplatin accumulation in human ovarian carcinoma cells by pharmacologic alteration of microtubules. *J. Clin. Invest.* 92:431–440.
- Licht, T., H.-H. Fiebig, K. J. Bross, F. Herrmann, D. P. Berger, R. Shoemaker, and R. Mertelsmann. 1991. Induction of multiple-drug resistance during anti-neoplastic chemotherapy in vitro. *Int. J. Cancer.* 49:630–637.
- Toffoli, G., A. Viel, L. Tumiotto, G. Biscontin, C. Rossi, and M. Boiocchi. 1991. Pleiotropic-resistant phenotype is a multifactorial phenomenon in human colon carcinoma cell lines. *Br. J. Cancer.* 63:51–56.
- Hromas, R. A., J. A. North, and C. P. Burns. 1987. Decreased cisplatin uptake and binding as a mechanism of resistance in L1210 leukemia cells. *Cancer Lett.* 36:197–201.
- Mann, S. C., P. A. Andrews, and S. B. Howell. 1988. Comparison of lipid content, surface membrane fluidity, and temperature dependence of *cis*-diamminedichloroplatinum(II) accumulation in sensitive and resistant human ovarian carcinoma cells. *Anticancer Res.* 8:1211–1216.
- Broer, S., G. Ji, A. Broer, and S. Silver. 1993. Arsenic efflux governed by the arsenic resistance determinant of *Staphylococcus aureus* plasmid p1258. *J. Bacteriol.* 175:3480–3485.
- Pison, R. L. 1991. Characterization of a phosphate transport system in human fibroblast lysosomes. *J. Biol. Chem.* 266:979–985.
- Papadopoulou, B., S. Dey, G. Roy, K. Grondin, D. Dou, B. P. Rosen, and M. Ouellette. 1993. Oxyanion resistance and *P*-glycoprotein gene amplification in Leishmania. *Gen. Motors Cancer Res. Found. Meeting*, Toronto. (Abstr.)
- Neal, R. A., J. van Bueren, N. G. McCoy, and M. Iwobi. 1989. Reversal of drug resistance in *Trypanosoma cruzi* and *Leishmania donovani* by verapamil. *Trans. R. Soc. Trop. Med. Hyg.* 83:197–198.
- Grogl, M., R. K. Martin, A. M. J. Oduola, W. K. Milhous, and D. E. Kyle. 1991. Characteristics of multidrug resistance in Plasmodium and Leishmania: detection of *P*-glycoprotein-like components. *Am. J. Trop. Med. Hyg.* 45:98–111.