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Cross-Resistance to Methotrexate and Metals in Human Cisplatin-resistant Cell Lines Results from a Pleiotropic Defect in Accumulation of These Compounds Associated with Reduced Plasma Membrane Binding Proteins

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ABSTRACT

Cross-resistance to a wide array of toxic chemicals is a common phenomenon in cisplatin-resistant cell lines. In this study, two independently isolated cisplatin-resistant cell lines derived from a human hepatoma and a cervical adenocarcinoma were shown to be cross-resistant to methotrexate (MTX) and several metal salts, such as sodium arsenite, sodium arsenate, antimony potassium tartrate, and cadmium chloride. A pleiotropic defect resulting in reduced accumulation of cisplatin, ^{3}[H]MTX, $^{73}As^{3+}$, and $^{73}As^{5+}$ was found in both cisplatin-resistant cell lines. Analysis by immunoblot, indirect immunofluorescence, and Northern hybridization showed dramatically reduced expression of the folate binding protein that mediates MTX uptake in both human cisplatin-resistant cell lines. By photoaffinity labeling with UV irradiation, specific binding proteins of M_r 230,000 and M_r 48,000 for $^{73}As^{3+}$ and M_r 190,000 for $^{73}As^{5+}$ were found in enriched plasma membrane of both human cisplatin-sensitive parental cell lines. Expression of these specific binding proteins was decreased in cells selected for cisplatin resistance. A protein band at M_r 36,000 that binds to $^{73}As^{3+}$ was overexpressed in both human cisplatin-resistant cell lines. The finding of loss of distinct binding proteins for MTX, arsenate, and arsenite in association with decreased accumulation of these agents in cisplatin-resistant cells suggests a pleiotropic, possibly regulatory, alteration in these cells.

INTRODUCTION

cis-Diamminedichloroplatinum II (cisplatin) has become a major chemotherapeutic agent for treatment of many malignancies, particularly for head and neck, testicular, ovarian, bladder, esophageal, and small cell lung cancers. Intrinsic and acquired resistance to cisplatin occurs commonly both in patients and in cell culture. The mechanisms for resistance to cisplatin have been postulated to be associated with several different cellular changes, including reduced accumulation of the drug (1–3), increased levels of glutathione or enzymes involved in glutathione metabolism (4–8), as well as elevated expression of mRNA for γ-glutamylcysteine synthetase and γ-glutamyltransferpeptidase (9), increases in thymidylate synthase (10), and the presence of DNA-binding proteins recognizing damaged DNA (11–13). It was reported recently that increased expression of heat shock protein HSP60 and HSP70 was found in cisplatin-resistant human cell lines (14, 15). Alterations in apoptosis-related genes have also been described in association with cisplatin resistance (16–20).

A striking feature of many cisplatin-resistant cell lines is their cross-resistance to many different agents, including melphalan, amsacrine, 5-fluorouracil, 6-mercaptopurine, bleomycin, and UV irradiation (21–22). This spectrum of cross-resistance is markedly different from that due to expression of the multidrug transporter encoded by the *MDR*1 gene, which protects cells from cytotoxic natural products. There is, however, a recent report of cross-resistance to Adriamycin, Taxol, and mitoxantrone in cisplatin-resistant cells (23).

We and others have also described high level cross-resistance to MTX,[2] a folate antagonist, in cisplatin-resistant cells (15, 24, 25). However, no cross-resistance to cisplatin has thus far been found in cells selected for resistance to MTX and its analogues (26). The most common cellular alteration resulting in MTX resistance is elevated expression of the enzyme dihydrofolate reductase, which is not usually seen in acquired resistance to cisplatin (25).[3] This result suggests that there must be some other mechanism(s) responsible for acquisition of MTX resistance in cisplatin-resistant cells.

Recently, cross-resistance to metal salts in cisplatin-resistant cell lines has been noted. Elevated expression of MT II has been reported in cisplatin-resistant cells (27, 28). Nevertheless, results obtained from the introduction of the *MT II* gene into cisplatin-sensitive cells are quite controversial. Kelley *et al.* (29) reported that overexpression of the *MT II* gene confers resistance to cisplatin and melphalan, but others (30) reported that MT II overexpression does not confer resistance to cisplatin. Naredi *et al.* (31, 32) demonstrated that cross-resistance among cisplatin, antimony potassium tartrate, and sodium arsenite, but not arsenate, occurred in human tumor cells. Resistance to arsenite has been associated with expression of *MRP*, which encodes a GS-X pump (33). However, overexpression of *MRP* is not commonly observed in cisplatin-resistant cell lines (34). Transfection of the *MRP* gene does not confer resistance to cisplatin (35), and expression of *MRP* does not directly correlate with cisplatin resistance in clinical samples (36, 37).

The goal of this work was to characterize the cross-resistance to MTX and metal salts in cisplatin-resistant cell lines to improve understanding of the major transport routes of these compounds and cisplatin. The data presented here provide evidence that impaired accumulation of MTX, arsenate, and arsenite occur in human cisplatin-resistant cell lines in which cisplatin accumulation is also reduced. This decreased accumulation of MTX, arsenate, and arsenite is associated with the reduction of different specific plasma membrane binding proteins detectable by immunoblotting and radiochemical photoaffinity labeling, suggesting that selection for cisplatin resistance results in a pleiotropic defect in uptake of several different unrelated compounds.

MATERIALS AND METHODS

Cell Lines and Cell Culture. Two panels of cisplatin-resistant cell lines and their parental cell lines were studied: the human liver carcinoma cell line BEL-7404 and its cisplatin-resistant derivative 7404-CP20, and the human epidermoid carcinoma cell line KB-3-1 and its cisplatin-resistant derivative KB-CP20. Both human cisplatin-resistant cell lines were selected and maintained in 20 μg of cisplatin/ml medium as described previously (15). A partially revertant cell line, 7404-CP20^{-df180}, was isolated from the 7404-CP20 cells in the absence of cisplatin for 180 days. All cell lines were grown as monolayer cultures at 37°C in 5% CO_2, using DMEM (Quality Biological, Gaithersburg, MD) with 4.5 g/l glucose (Life Technologies, Inc.), supple-

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[2] The abbreviations used are: MTX, methotrexate; MT II, metallothionein II; FBP, folate binding protein.

[3] Unpublished data.

mented with L-glutamine, penicillin, streptomycin, and 10% fetal bovine serum (Bio Whittaker, Walkersville, MD).

Drugs and Radiochemicals. Cisplatin was a gift from Bristol-Myers Research Laboratory and Johnson Matthey. Methotrexate, sodium arsenate, sodium arsenite, $CdCl_2$, $NiCl_2$, $ZnCl_2$, and others were purchased from Sigma Chemical Co., unless otherwise mentioned. Antimony potassium tartrate was purchased from Aldrich. [^{3}H]MTX (specific activity, 38.3 Ci/mmol) was purchased from DuPont NEN. Arsenic-73 (specific activity, 242 Ci/mmol) was provided by Los Alamos National Laboratory.

Synthesis of $^{73}As^{3+}$. $^{73}As^{3+}$ was prepared by reduction of $^{73}As^{5+}$. Fifty μl of reduction solution consisting of 0.1 mM $NaAsO_2$, 66 mM $Na_2S_2O_5$, 27 mM $Na_2S_2O_3$, and 82 mM H_2SO_4 were added to 50 μl of 50 μCi of $^{73}As^{5+}$ ($H_3{}^{73}AsO_4$) and incubated for 40 min at room temperature according to Reay and Asher (38).

Drug Sensitivity Assay. Dose-response curves of the human tumor cell lines were determined as described previously (15). An IC_{50} was measured as the concentration of drugs reducing the number of cells to 50% of that in control (drug-free) medium. A relative resistance factor for each drug was determined by dividing the IC_{50} of the drug for the cisplatin-resistant cell lines by that for the appropriate parental cell lines, BEL-7404 or KB-3-1, respectively. The values are means of triplicate determinations.

Measurement of Uptake and Efflux of Radiochemical Compounds. Dishes containing a subconfluent layer of cells were used for the assay. For [^{3}H]MTX uptake analysis, cells were washed once with folic acid-deficient DMEM and then incubated at 37°C with the same medium containing 1 μCi/ml [^{3}H]MTX. For determination of $^{73}As^{5+}$ uptake, cells were washed with TS buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$]; then $^{73}As^{5+}$ was loaded in TS buffer + 4.5 mg/ml glucose or TS buffer + 5 mM 2-deoxyglucose and 1 mM sodium azide (which deplete the cells of energy by reducing ATP) for 20 min at 37°C. For measurement of $^{73}As^{3+}$ uptake, cells were washed with PBS, then $^{73}As^{3+}$ was loaded in PBS + 4.5 mg/ml glucose or PBS containing 2-deoxyglucose/NaN_3 for 20 min at 37°C as described above. Cells were then incubated with the same buffer (with glucose or 2-deoxyglucose/NaN_3) + 0.5 μCi/ml of $^{73}As^{5+}$ or $^{73}As^{3+}$ at room temperature for the desired period. To measure efflux of $^{73}As^{3+}$, cells were incubated with $^{73}As^{3+}$ for 2 h at 37°C, then washed twice with ice-cold PBS, and reincubated with regular medium without $^{73}As^{3+}$ for the desired period of time. The radioactivity of cells at 2 h incubation with $^{73}As^{3+}$ was calculated as 100% of $^{73}As^{3+}$ remaining at 0 point of efflux time period. At various times of incubation, uptake or efflux was stopped by washing the dishes three times with ice-cold PBS, then harvesting cells by trypsinization. The cell suspensions were transferred from the dishes into counting vials with mixture Formula 989 (DuPont NEN) and counted in a Beckman LS3801 liquid scintillation counter. Triplicate dishes of cells for each time point were analyzed.

Preparation of Enriched Plasma Membrane Proteins. Membrane proteins were purified according to the method of Cornwell *et al.* (39). Briefly, 1×10^9 cells from each cell lines were disrupted on ice by nitrogen cavitation with constant stirring for 40 min. Two cycles of sucrose gradient ultracentrifugation were followed. The purified membrane pellets were resuspended in 3 ml of TSNa buffer [10 mM Tris-HCl (pH 7.45), 250 mM sucrose, 50 mM NaCl, and 1% aprotinin] and stored at −80°C until use.

Radiochemical Photoaffinity Labeling. UV irradiation was applied as modified from Cornwell *et al.* (39). A 200-W mercury lamp was used as a light source at a distance of 6–8 cm from microtubes containing a mixture of 100 μg of enriched plasma membrane proteins in 15 mM HEPES, 150 mM mannitol, 1 mM $MnCl_2$, and 1 mM phenylmethylsulfonyl fluoride with 4 μCi of $^{73}As^{3+}$ or $^{73}As^{5+}$ on ice for 10–30 min. After UV cross-linking, the proteins were separated on SDS-PAGE and then stained with Coomassie Blue. SDS-PAGE and immunoblotting were carried out as described previously (15). Minigels were run as recommended by the manufacturer (Novax). After electrophoresis, the gels were transblotted onto nitrocellulose membranes (S & S) at 4°C. Immunoreaction was performed with MOv19, a monoclonal antibody directed to human FBP as described in Mantovani *et al.* (40), a generous gift from Dr. Colnaghi. Pierce ECL reagents were used for developing signals, as described by the manufacturer.

Indirect Immunofluorescence. Cells were cultured in 35-mm dishes and fixed with 4% formaldehyde for 5 min and acetone:methanol (1:3) for another 5 min. The fixed cells were reacted with the primary mAb MOv19 followed by rhodamine-labeled anti-mouse IgG second antibody (Jackson Immuno-Research Laboratory). Immunofluorescence of cells was examined with a Zeiss Axiothot microscope.

Northern Blot Analysis. Poly(A)$^+$-enriched RNA was isolated from each cell line using the FastTrack mRNA isolation kit according to the manufacturer's instructions (Invitrogen, San Diego, CA). Northern blot hybridization was performed as described previously (41). A cDNA probe for the human *FBP* gene (42) was generated by using an RT-PCR kit according to the manufacturer's instructions (Perkin-Elmer, Foster City, CA). The cDNA fragment was labeled with ^{32}P by random primer extension (Lofstrand, Gaithersburg, MD).

RESULTS

Cross-Resistance of Cisplatin-resistant Cells to Methotrexate. The killing curves shown in Fig. 1 indicate the resistance of human cisplatin-resistant cell lines and their parental cell lines to MTX. The resistance to MTX for the human liver carcinoma 7404-CP20 cells was 42-fold higher than its parental cell line BEL-7404, whereas the human KB-CP20 cell line is 20-fold more resistant than its parental cell line KB-3-1 (Table 1). It is noted that there is a difference between the two parental cell lines in their intrinsic sensitivity to MTX; the liver carcinoma BEL-7404 cell line is more resistant to MTX than the cervical adenocarcinoma KB-3-1 cells (15).

Reduced Accumulation of [^{3}H]MTX in Cisplatin-resistant Cells. Time courses were performed for measurement of accumulation of [^{3}H]MTX in both cisplatin-resistant cell lines. Fig. 2 shows a considerable reduction of [^{3}H]MTX accumulation of about 7–12.3-fold in KB-CP20 and 7404-CP20 cell lines compared with their parental cell lines. Both the initial rate of accumulation and the extent of accumulation were reduced in the cisplatin-resistant cells, suggesting that uptake of MTX was decreased. No changes in [^{3}H]MTX uptake were observed in either the 7404-CP20 cells, which were maintained in cisplatin-free medium for 1–7 days, or the cisplatin-sensitive BEL-7404 cells, which were incubated with cisplatin for up to 4 h (data not shown), indicating that reduced accumulation of [^{3}H]MTX in cisplatin-resistant cells does not result from an acute effect of cisplatin exposure.

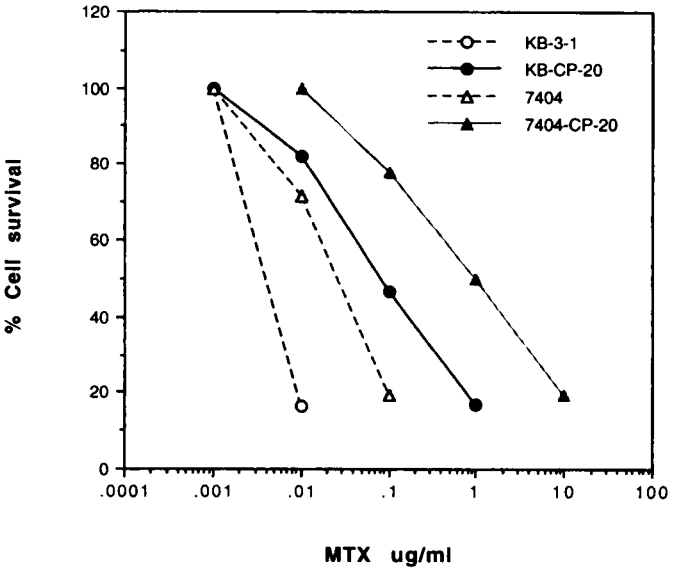

Fig. 1. Methotrexate killing curves of human cisplatin-resistant cell lines compared with their drug-sensitive parental cell lines were measured as described in "Materials and Methods." *KB-3-1*, human epidermoid carcinoma cells; *KB-CP20*, cisplatin-resistant cells maintained in cisplatin 20 μg/ml isolated from the KB-3-1 cells; *7404* (BEL-7404), human liver carcinoma cells; *7404-CP20*, cisplatin-resistant cells maintained in cisplatin 20 μg/ml isolated from the BEL-7404 cells.

Table 1 *Cross-resistance to MTX and metals*

	IC_{50} (μM)					
	BEL-7404	7404-CP20	RR[a]	KB-3-1	KB-CP20	RR[a]
Cisplatin	0.44	49	111	0.033	38	1152
MTX	0.024	1.0	42	0.004	0.08	20
Sodium arsenite	0.32	8.5	27	0.23	5.3	23
Sodium arsenate	71	957	14	46	1050	22
Antimony potassium tartrate	65	>100	>1.5	5	42	8.4
$CdCl_2$	7	83	12	23	81	3.5
$NiCl_2$	68	96	1.4	32	130	4.1
$ZnCl_2$	40	60	1.5	18	88	4.9

[a] RR (relative resistance) was determined by dividing the IC_{50} of the drug for cisplatin-resistant 7404-CP20 or KB-CP20 cells by that for the parental cell line, BEL-7404 or KB-3-1 cells, respectively. Both 7404-CP20 and KB-CP20 cells were maintained in medium containing 20 μg of cisplatin/ml.

Cisplatin Does Not Inhibit Accumulation of [^{3}H]MTX. To determine whether [^{3}H]MTX influx in both cisplatin-sensitive and -resistant cell lines was inhibited by cisplatin, cells were exposed to [^{3}H]MTX in the presence of cold nonradiolabeled cisplatin or MTX. No competition was found with cisplatin at concentrations of 0.01–10 μg/ml for all four cell lines tested here. Fig 3B shows a representative histogram of KB cell lines. However, as expected for a saturable uptake system, cold MTX significantly inhibited the uptake of [^{3}H]MTX at concentrations of 0.1, 1, and 10 μg/ml in KB-3-1 cells by 4.7-, 9-, and 18-fold, respectively (Fig. 3*A*). A similar result was also observed in KB-CP20 cells (Fig. 3*A*).

Expression of Folate Binding Protein. Three different means for detection of expression of FBP were used in this work. Fig. 4 shows an immunoblot analysis on expression levels of FBP using a monoclonal antibody MOv19 (a generous gift from Dr. M. I. Colnaghi) directed to the human FBP with visualization by ECL. Enriched membrane proteins were isolated from each cell line as described in "Materials and Methods." It is obvious that the cisplatin-sensitive parental cell lines BEL-7404 and KB-3-1 have abundant FBP, whereas the expression of the *FBP* gene in both the cisplatin-resistant cell lines 7404-CP20 and KB-CP20 was dramatically reduced below the limit of detection by this assay.

Observation by indirect immunofluorescence reaction with MOv19 demonstrates that the FBP was strongly expressed at the plasma membrane in the cisplatin-sensitive KB-3-1 cells (Fig. 5*A*). There is no detectable signal above background in the cisplatin-resistant KB-CP20 cells (Fig. 5*B*). Background fluorescence was determined by applying the second antibody only, a rhodamine-conjugated Affinipure goat anti-mouse IgG, showing some background fluorescence similar to that seen in Fig. 5*B* (data not shown).

These results were further confirmed by Northern hybridization as shown in Fig. 6. The expression of the FBP mRNA was also reduced in both human cisplatin-resistant cell lines 7404-CP20 and KB-CP20.

Interestingly, expression of the *FBP* gene appeared to be reactivated in a partially revertant cell line 7404-CP20^{-df180}, which was maintained in the absence of cisplatin for 180 days. The resistance level of this partially revertant cell line to cisplatin dropped from its original 112-fold to 30-fold, and the resistance to MTX was also reduced from 42-fold to 10-fold, suggesting that the expression of the *FBP* gene in cisplatin-resistant cells was coordinately regulated with resistance to cisplatin and MTX. When this revertant cell line was reexposed to cisplatin for 24 h, there was no acute effect on FBP

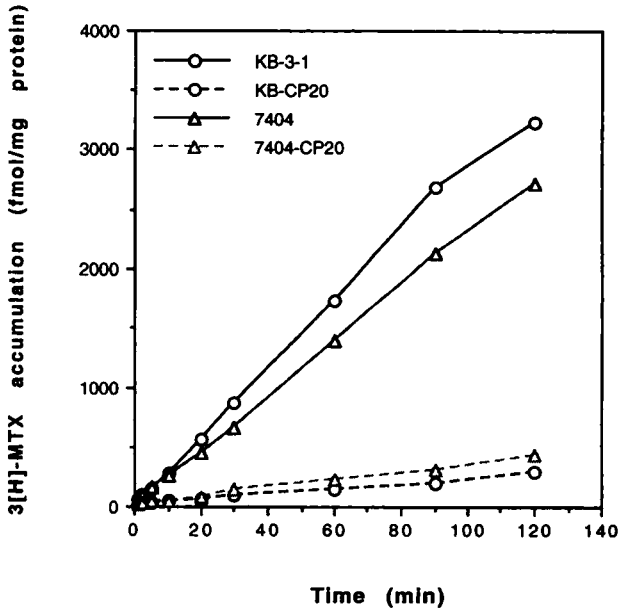

Fig. 2. Time course of [^{3}H]MTX uptake into human cisplatin-resistant cells compared to their parental cisplatin-sensitive cells. [^{3}H]MTX radioactivity was measured as described in "Materials and Methods." *KB-3-1,* human epidermoid carcinoma cells; *KB-CP20,* cisplatin-resistant cells maintained in cisplatin 20 μg/ml isolated from the KB-3-1 cells; *7404* (BEL-7404), human liver carcinoma cells; *7404-CP20,* cisplatin-resistant cells maintained in cisplatin 20 μg/ml isolated from the BEL-7404 cells.

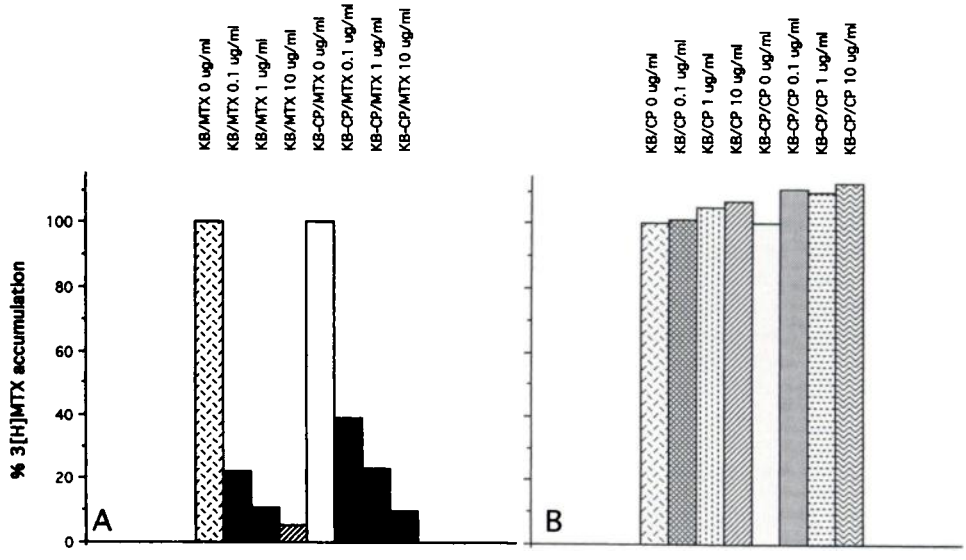

Fig. 3. Competition assay indicates an inhibition of [^{3}H]MTX influx by adding cold MTX at 0.1, 1, and 10 μg/ml (*A*) and no competition with cisplatin (*B*). *KB-3-1,* human epidermoid carcinoma cells; *KB-CP20,* cisplatin-resistant cells maintained in cisplatin 20 μg/ml derived from the KB-3–1 cells.

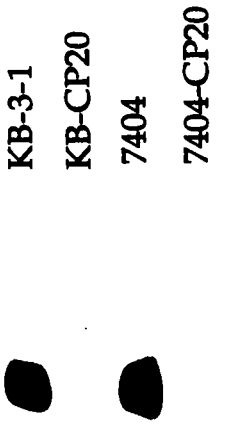

Fig. 4. Immunoblot analysis of FBP with MOv19 visualized by ECL. Enriched plasma membranes were transblotted on a nitrocellulose membrane after 10% SDS-PAGE as described in "Materials and Methods." *KB-3-1*, human epidermoid carcinoma cells; *KB-CP20*, cisplatin-resistant cells maintained in cisplatin 20 μg/ml isolated from the KB-3-1 cells; *7404* (BEL-7404), human liver carcinoma cells; *7404-CP20*, cisplatin-resistant cells maintained in cisplatin 20 μg/ml isolated from the BEL-7404 cells.

expression levels (data not shown), indicating that the reactivated expression of FBP in this revertant cell line is quite stable.

Cross Resistance of Cisplatin-resistant Cells to Metal Salts. To determine the patterns of cross-resistance of cisplatin-resistant hepatoma and KB cell lines to metal salts, several agents were examined. The relative resistance levels to sodium arsenate, sodium arsenite, antimony potassium tartrate, cadmium chloride, nickel chloride, and zinc chloride in parental and cisplatin-resistant cell lines are listed in Table 1. As can be seen, the pattern of cross-resistance to arsenic and antimony salts is similar in the two cisplatin-resistant cell lines. Both lines are also cross-resistant to cadmium, although KB-CP20 is less so than 7404-P20. KB-CP20 is somewhat resistant to nickel and zinc chloride, whereas 7404-CP20 is not.

Reduced Uptake of $^{73}As^{3+}$ or $^{73}As^{5+}$ in Cisplatin-resistant Cells. Using the human liver carcinoma cell line BEL-7404 and its cisplatin resistant variant 7404-CP20, the uptake of $^{73}As^{3+}$ or $^{73}As^{5+}$ was determined as described in "Materials and Methods." The results are shown in Fig. 7, *A* and *B*, respectively. The uptake of $^{73}As^{3+}$ or $^{73}As^{5+}$ was time dependent. During a 60-min incubation, the parental BEL-7404 cells took up 720 pmol/mg protein of $^{73}As^{3+}$, whereas only 220 pmol/mg protein of $^{73}As^{3+}$ was taken up in the cisplatin-resistant 7404-CP20 cells, a 3-fold reduction. Reduction of $^{73}As^{5+}$ uptake was also found in the cisplatin-resistant 7404-CP20 cells (Fig. 7*B*). The uptake of $^{73}As^{3+}$ and $^{73}As^{5+}$ was also energy dependent. The uptake was partially inhibited in both BEL-7404 and 7404-CP20 cells in the presence of sodium azide and 2-deoxy-glucose, which depletes cells of energy by reducing ATP levels. Measurement of ATP in BEL-7404 cells under these conditions showed a reduction to 20% of levels in cells incubated in the presence of glucose.

One observation, suggesting that the mechanisms of uptake of $^{73}As^{5+}$ and $^{73}As^{3+}$ are different, was that the uptake of $^{73}As^{5+}$ was remarkably reduced in both BEL-7404 and 7404-CP20 cells when cells were incubated in PBS (Fig. 8*A*) containing sodium phosphate, whereas uptake of $^{73}As^{3+}$ was unchanged. Using 10 mM sodium phosphate or 150 mM *N*-methyl-D-glucamine-chloride (Sigma Chemical Co.), a large organic cation which is a substitute for sodium, the uptake of $^{73}As^{5}$ in BEL-7404 cells was reduced to 12% of control (in TS buffer containing no sodium phosphate; Fig. 8*B*), indicating that the main pathway for influx of $^{73}As^{5}$ in these cells is probably through the phosphate carrier or channel (43). However, there was no significant difference for the uptake of $^{73}As^{3+}$ in these cells in the presence or absence of sodium phosphate, suggesting that the uptake of $^{73}As^{3+}$ is not via the phosphate carrier.

Furthermore, it is interesting to note that there are both high- and low-affinity $^{73}As^{3+}$ uptake pathways in the cisplatin-resistant cell line. Fig. 9 shows somewhat reduced uptake of $^{73}As^{3+}$ in the 7404-CP20 cells at low unlabeled sodium arsenite levels but increased uptake of $^{73}As^{3+}$ when these cells were incubated in the presence of high concentrations of unlabeled sodium arsenite. Furthermore, the efflux of $^{73}As^{3+}$ in the cisplatin-resistant 7404-CP20 cells was somewhat slower than the sensitive parental BEL-7404 cells during a 2-h efflux period (Fig. 10). These results suggest that the decreased accumulation of arsenic in both cisplatin-sensitive and -resistant cell lines is due to reduced energy-dependent uptake via a high-affinity uptake system (Fig. 7), rather than increased efflux.

$^{73}As^{3+}$ or $^{73}As^{5+}$ Plasma Membrane Binding Proteins. Using UV cross-linking, it was possible to detect specific proteins binding to trivalent and pentavalent arsenic. Fig. 11*A* shows that two bands of M_r 230,000 and M_r 48,000, which bind $^{73}As^{3+}$ and are present in both cisplatin-sensitive parental cell lines BEL-7404 and KB-3-1, are

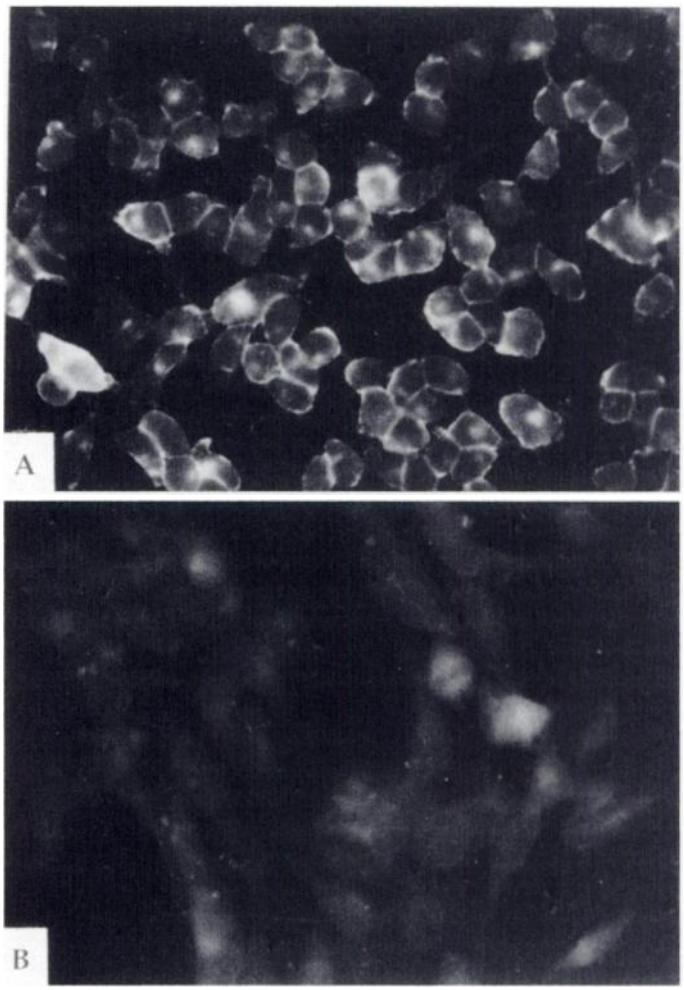

Fig. 5. Indirect immunofluorescence with the FBP-specific monoclonal antibody MOv19. *A*, KB-3-1: human epidermoid carcinoma cells show strong fluorescence on the plasma membrane of the cells; *B*, KB-CP20: cisplatin-resistant cells maintained in cisplatin 20 μg/ml isolated from the KB-3-1 cells show very weak or undetectable fluorescence on the cell surface. Exposure time for *A* and *B* was 1 s.

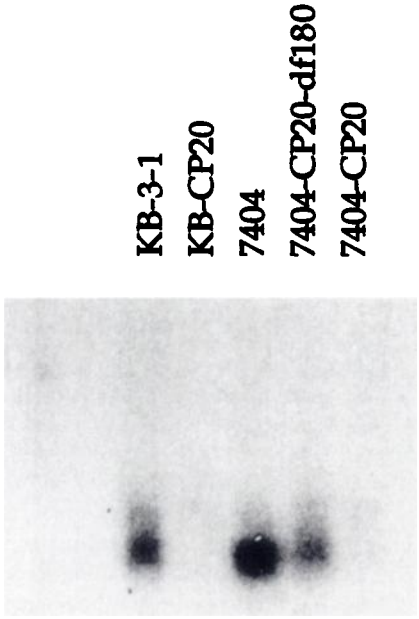

Fig. 6. Northern blot analysis of *FBP* with an *FBP*-specific cDNA probe. Total RNA from each cell line was transblotted onto nitrocellulose after electrophoresis. *KB-3-1*, human epidermoid carcinoma cells; *KB-CP20*, cisplatin-resistant cells maintained in cisplatin 20 μg/ml isolated from the KB-3-1 cells; *7404* (BEL-7404), human liver carcinoma cells; *7404-CP20*, cisplatin-resistant cells maintained in cisplatin 20 μg/ml isolated from the BEL-7404 cells; *7404-CP20-df180*, a partial revertant cell line of 7404-CP20 maintained in drug-free medium for 180 days.

markedly reduced in cisplatin-resistant cell lines 7404-CP20 and KB-CP20. A band of M_r 36,000 was significantly increased in both cisplatin-resistant cell lines. Competition assays with different metals, such as cisplatin, antimony, sodium arsenate, sodium arsenite, as well as MTX, indicated that these $^{73}As^{3+}$ plasma membrane binding proteins are very specific. The binding proteins seen here were reduced in a dose-dependent manner only in the presence of high concentrations of sodium arsenite (data not shown). Elimination of UV irradiation resulted in a loss or weakening of these signals associated with $^{73}As^{3+}$ binding. Fig. 11*B* shows specific sodium arsenate-binding proteins. A strong band at M_r 190,000 is seen in both cisplatin-sensitive parental cell lines KB-3-1 and BEL-7404. This band was decreased in both cisplatin-resistant cell lines KB-CP20 and 7404-CP20. Three protein bands smaller than M_r 190,000 could also be detected only in the KB-3-1 cells. Among the metal salts tested (*i.e.*, 0.3–30 μM cisplatin, 1–70 μM sodium arsenite, and 1–100 μM antimony) and 10 μM sodium phosphate, only sodium arsenate at 3 μM could inhibit binding by 80–90%; 30 μM could entirely abolish this labeling (data not shown).

DISCUSSION

Cross-resistance is a common phenotype in human cancers selected for resistance to a single cytotoxic agent. The best studied mechanisms of cross-resistance to natural product chemotherapeutic drugs result from expression of the *MDR*1 and *MRP*1 genes, which encode energy-dependent efflux pumps for many different drugs and/or drug conjugates (44, 45). Cross-resistance to a different variety of cytotoxic agents also occurs commonly in cisplatin-resistant cell lines. We show in this work that cross-resistance of cisplatin-resistant cells to MTX and metal salts results from a pleiotropic defect in accumulation of these compounds that is associated with multiple changes in the plasma membrane proteins that bind them.

Cross-Resistance to Methotrexate. Mechanisms of MTX resistance have been studied extensively because methotrexate is a clinically important antifolate for cancer chemotherapy and for treatment of rheumatoid arthritis, psoriasis, and autoimmune disease (46). Cross-resistance to MTX in cisplatin-resistant cell lines has been attributed to amplification of the *DHFR* gene (25) or overexpression of the *MDR*1 or *MRP*1 gene (47, 48). *MDR*1 overexpression does not usually result in resistance to MTX unless there is no carrier-mediated uptake of MTX (47). We found no detectable differences in mRNA levels of the *DHFR* gene between the human cisplatin-sensitive and -resistant cell lines (data not shown). Recently, three different human genes have been cloned and shown to be carriers mediating MTX transport, *i.e.*, the gene coding for FBP (49), the reduced folate carrier (RFC1; Ref. 50), and the MTX-carrier (51). Mutants lacking FBP are resistant to MTX but not cross-resistant to cisplatin.[3] The functional expression of different carriers mediating MTX transport is dependent on cell type. Transfection of any of the putative MTX carrier genes into mutant cells that are defective in MTX uptake results in cells being able to regain the ability to transport the drug resulting sensitivity to MTX (50, 51).

Reduced uptake of [^{3}H]MTX and decreased expression of the human *FBP* gene detected by indirect immunofluorescence, immunoblot, and Northern hybridization were found in both human cisplatin-resistant cell lines examined in this study. Southern blot hybridization

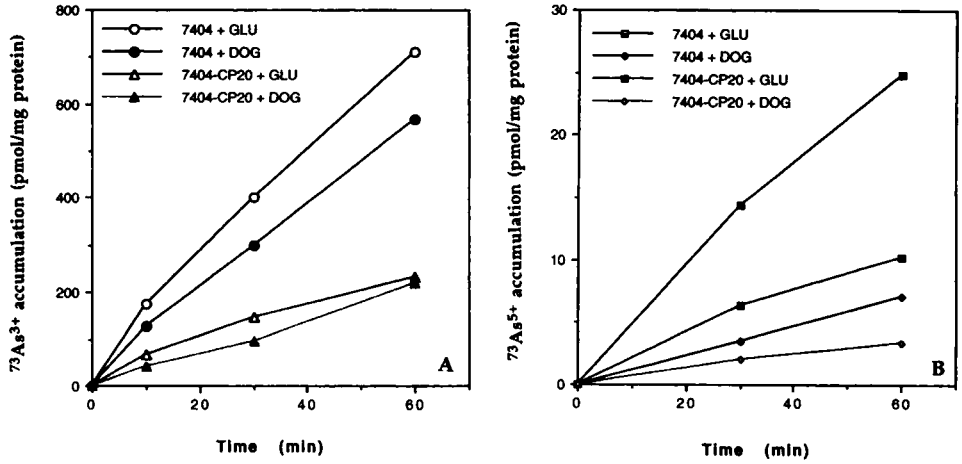

Fig. 7. Time course of $^{73}As^{3+}$ (*A*) and $^{73}As^{5+}$ (*B*) uptake. For determination of $^{73}As^{3+}$, which was synthesized by reduction of $^{73}As^{5+}$, cells were incubated in PBS buffer. For measurement of $^{73}As^{5+}$, cells were incubated in TS solution (details described in the text). Radioactivity was measured as described in "Materials and Methods." *7404* (BEL-7404), human liver carcinoma cells; *7404-CP20*, cisplatin-resistant cells maintained in cisplatin 20 μg/ml derived from the BEL-7404 cells. *GLU*, 4.5 mg glucose/ml; *DOG*, 5 mM 2-deoxyglucose and 1 mM sodium azide.

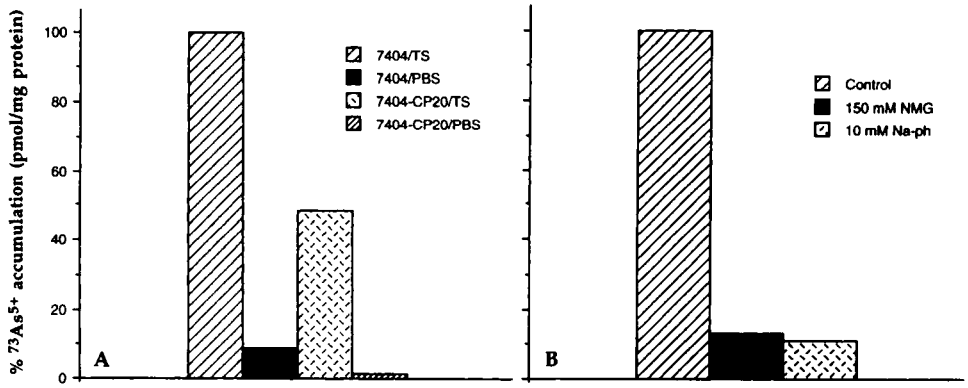

Fig. 8. Comparison of $^{73}As^{5+}$ uptake in TS or PBS buffer in BEL-7404 and 7404-CP20 cells (*A*). Inhibition of $^{73}As^{5+}$ uptake by 150 mM *NMG* (*N*-methyl-D-glucamine-HCl) or 10 mM sodium phosphate in BEL-7404 cells (*B*). The control cells were incubated in TS buffer only (see details in the text). *7404* (BEL-7404), human liver carcinoma cells; *7404-CP20*, cisplatin-resistant cells maintained in cisplatin 20 μg/ml derived from the BEL-7404 cells.

did not reveal any difference in DNA rearrangement or copy number (data not shown). Hsueh and Dolnick (52) reported that the levels of membrane-associated FBP protein and *FBP* mRNA were decreased 70–80% in MTX-resistant KB cells and noted that reduced FBP expression in MTX-resistant cells resulted from increased DNA methylation of the *FBP* gene.

We found in this work that reduction of *FBP* gene expression could be partially reversed after cells were allowed to revert to cisplatin sensitivity by growth in nonselective medium for 180 days (Fig. 6). These results suggest that MTX cross-resistance is due to reduced [^{3}H]MTX uptake tied to decreased expression of the *FBP* gene in cisplatin-resistant cells. Because selection of cells for reduced FBP expression does not result in cisplatin resistance, loss of FBP expression itself is not the cause of cisplatin resistance. The reduced expression of the *FBP* gene in cells selected for cisplatin resistance may be regulatory. The regulatory mechanism(s) responsible for reduction of FBP expression in cells are unknown, but these mechanisms, once deciphered, may help explain the basis for cisplatin resistance. Reduced expression and reexpression of the *FBP* gene in cisplatin-resistant cells and revertants could also be clinically significant.

Cross-Resistance to Metal Salts. Resistance to antimonials, arsenic, cadmium chloride, and others has been extensively studied in lower organisms, such as *Escherichia coli* (53), yeast (54), and *Leishmania* (55, 56), as well as in human cells (31, 32, 57), as models for understanding environmental toxins and carcinogenesis. Expression of the genes encoding MT, cadmium efflux ATPase, and *MRP* are associated with resistance to cadmium and other metals (58–60).

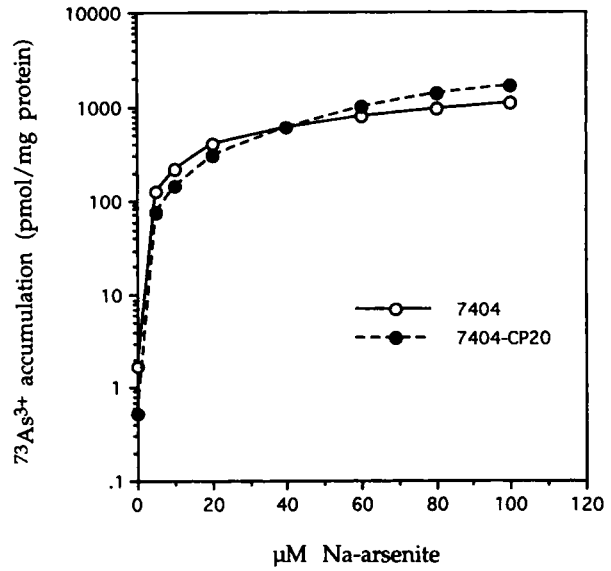

Fig. 9. Accumulation of $^{73}As^{3+}$ in the presence of high concentrations of sodium arsenite in the range of 0–100 μM. Cells were incubated at 37°C for 60 min. Radioactivity was measured as described in "Materials and Methods." *7404* (BEL-7404), human liver carcinoma cells; *7404-CP20*, cisplatin-resistant cells maintained in cisplatin 20 μg/ml derived from the BEL-7404 cells.

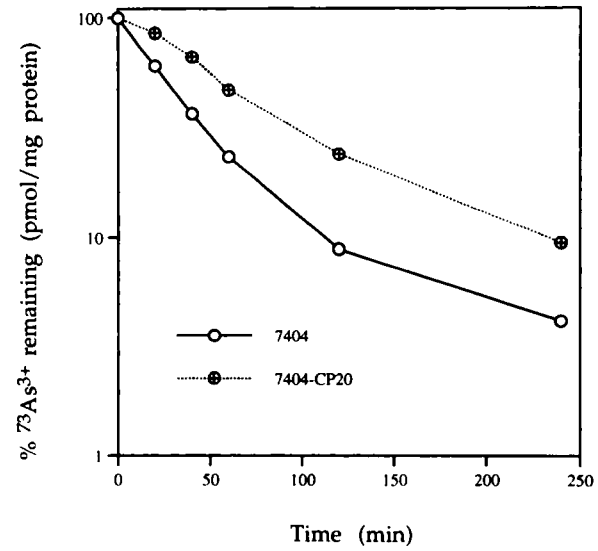

Fig. 10. Efflux of $^{73}As^{3+}$ in 7404 and 7404-CP20 cells. Cells were incubated with $^{73}As^{3+}$ at 37°C for 2 h. The radioactivity at this time point was calculated as 100% of $^{73}As^{3+}$ remaining for each of the cell lines. For 7404 cells, there were 274.8 pmol/mg proteins associated with the cells at the beginning of the efflux experiment and for 7404-CP20 cells; there were 233.6 pmol/mg proteins associated with the cells. After washing twice with ice-cold PBS, cells were reincubated with prewarmed regular medium for up to 2 h at 37°C for efflux measurement. The description of the cell lines is the same as in Fig. 9.

In this study, both human cisplatin-resistant cell lines are significantly cross-resistant to both arsenite and arsenate as well as antimony potassium tartrate. The human hepatoma 7404-CP20 cells were more resistant to cadmium (12-fold) than the KB-CP20 cells (3.5-fold), probably due to the origin of the cells from the liver, which functions as a detoxification organ in the body. However, lower level cross-resistance to $NiCl_2$ and $ZnCl_2$ were only detected in the KB-CP20 cells. Using radioactive As-73, it was found that uptake of both $^{73}As^{5+}$ and its reduced form $^{73}As^{3+}$ were decreased in the human hepatoma cisplatin-resistant 7404-CP20 cells in comparison to the parental cell line BEL-7404. *In situ* autoradiography further demonstrated that accumulation of $^{73}As^{3+}$ occurred only in the cisplatin-sensitive cells, particularly in the region of the nucleus (data not shown). This result is consistent with the observation that arsenic is genotoxic to mammalian cells by inducing chromosomal endoreduplication and formation of micronuclei (61, 62).

Reduced uptake of $^{73}As^{3+}$ and $^{73}As^{5+}$ could result either from defective uptake or active efflux, or both. To address this question,

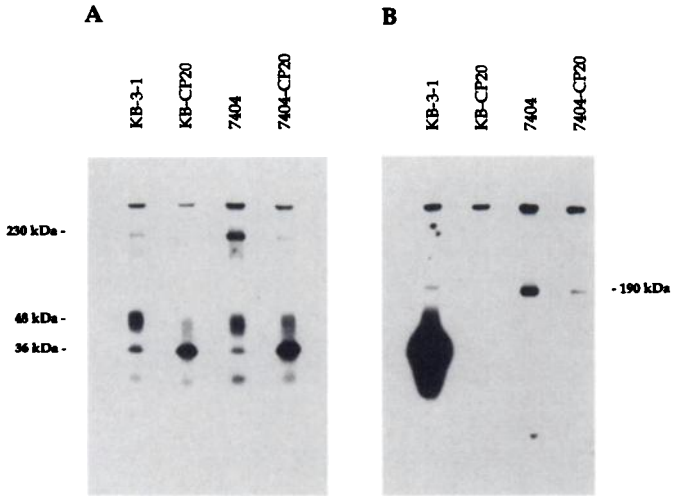

Fig. 11. Arsenic labeling of enriched plasma membrane proteins. *A*, $^{73}As^{3+}$; *B*, $^{73}As^{5+}$. Preparation of membrane proteins and UV cross-linking were performed as described in "Materials and Methods." *KB-3-1*, human epidermoid carcinoma cells; *KB-CP20*, cisplatin-resistant cells maintained in cisplatin 20 μg/ml isolated from the KB-3-1 cells; *7404* (BEL-7404), human liver carcinoma cells; *7404-CP20*, cisplatin-resistant cells maintained in cisplatin 20 μg/ml derived from the BEL-7404 cells.

two experiments were performed: (*a*) preincubation of cells with 2-deoxyglucose and sodium azide to deplete energy resulted in reduced uptake in the cisplatin-sensitive cells and also slightly in cisplatin-resistant cells, suggesting the presence of an energy-dependent uptake system in the sensitive cells that is missing in the resistant cells; and (*b*) an efflux assay using $^{73}As^{3+}$ showed reduced efflux of $^{73}As^{3+}$ in cisplatin-resistant 7404-CP20 cells compared with the cisplatin-sensitive BEL-7404 cells, presumably due to the lower initial accumulation of $^{73}As^{3+}$ in 7404-CP20 cells. These results argue that reduced accumulation of As-73 in our human cisplatin-resistant cells resulted from defective uptake, not from active efflux. This feature is consistent with observations on impaired uptake of [^{3}H]dichloro(ethylenediamine) platinum(II) and platinum measured by atomic absorption spectrometry in human cisplatin-resistant tumor cells (3, 63).

The next question we addressed was the entry route of the arsenic into cells. Because uptake of arsenic into cells is energy dependent, at least in part, as discussed above, it is reasonable to believe that there might be a carrier-mediated influx transporter that may exist in wild-type cisplatin-sensitive parental cells for arsenic, in analogy to FBP-mediated MTX uptake. If there is such a carrier for arsenic, then: is it the same carrier for both sodium arsenate and sodium arsenite, or are there are different carriers? Using arsenic photoaffinity labeling of purified plasma membrane proteins, the data presented here demonstrate that there are specific binding proteins for sodium arsenite and sodium arsenate appearing in both human hepatoma and KB-3-1 cisplatin-sensitive cell lines. Two major $^{73}As^{3+}$-binding proteins of M_r 230,000 and M_r 48,000 were detected in both human parental cisplatin-sensitive cell lines (BEL-7404 and KB-3-1), whereas the intensity of the two proteins was significantly reduced in both human cisplatin-resistant cell lines (7404-CP20 and KB-CP20). $^{73}As^{5+}$-labeling indicates that one major protein band at M_r 190,000 was detected in both the human hepatoma BEL-7404 cell line and the KB cell line. In addition, three more protein bands were seen only in the human KB cell line. It is not clear yet whether these additional proteins in the KB-3-1 cells were proteolyzed from the M_r 190,000 protein or whether they are cell type specific. Nevertheless, expression of all of these $^{73}As^{3+}$- or $^{73}As^{5+}$-binding proteins were dramatically reduced in cells selected for cisplatin resistance. Lack of competition with other metals salts indicates the specificity of labeling of these binding proteins with $^{73}As^{3+}$- and $^{73}As^{5+}$. It is tempting to speculate that these proteins are involved somehow in the high-affinity uptake of $^{73}As^{3+}$ and $^{73}As^{5+}$ into cells, and more experiments need to be done for clarification.

Implication of These Studies for Understanding Cisplatin Resistance. These findings suggest several speculations about the mechanism of cisplatin resistance in our cell lines. The finding of a pleiotropic defect in accumulation of MTX, arsenate, and arsenite in these two different cisplatin-resistant cell lines suggests a common mechanism that might also reduce cisplatin accumulation. The most obvious possibility is that there is a regulatory mutation(s) that turns off expression of a set of plasma membrane binding proteins needed for uptake of MTX, arsenate, arsenite, and cisplatin. However, it has been postulated that cisplatin enters cells by passive diffusion, based on observations that cisplatin accumulation is proportional to the drug concentrations and that accumulation is not saturated and not inhibited by structural analogues of cisplatin (64). There are two alternative possibilities consistent with these data for cisplatin. One is that at low concentrations, where most cell lines are sensitive to cisplatin toxicity and below levels that can be measured with existing techniques, cisplatin entry is energy dependent and requires a plasma membrane binding protein as for MTX, arsenate, and arsenite uptake. This pathway could be defective in our resistant cells. Another possibility is that cisplatin is being rapidly effluxed in resistant cells and that the putative regulatory system that turns on efflux also turns off uptake of other cytotoxic compounds. Another explanation for the pleiotropic defect in these cisplatin-resistant cell lines is that there is loss of a common uptake pathway (*e.g.*, a defect in receptor mediated uptake for a whole class of plasma membrane binding proteins), resulting in negative feedback on expression of these binding proteins. Any of these hypotheses are consistent with our observation that there are many differences in protein expression when cisplatin resistant and sensitive cells are compared using two-dimensional gel electrophoresis (15).

These studies suggest several important new approaches to understanding the mechanism by which cells regulate uptake and efflux of toxic compounds and normal nutrients. The possibility that there are high-affinity uptake systems for cisplatin, arsenite, and arsenate is worthy of further study. More comprehensive analyses of other cross-resistant human cisplatin-selected cell lines isolated from different sources and the association of these cell surface proteins with drug uptake would be needed to confirm and extend the results suggested in this work.

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