0026-895X/01/6002-302–309
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 60:302–309, 2001

Vol. 60, No. 2
842/921115
Printed in U.S.A.

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Overexpression of Glutathione *S*-Transferase II and Multidrug Resistance Transport Proteins Is Associated with Acquired Tolerance to Inorganic Arsenic

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Received January 26, 2001; accepted May 7, 2001

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

Recent work shows that long-term exposure to low levels of arsenite induces malignant transformation in a rat liver epithelial cell line. Importantly, these chronic arsenic-exposed (CAsE) cells also develop self-tolerance to acute arsenic exposure. Tolerance is accompanied by reduced cellular arsenic accumulation, suggesting a mechanistic basis for reduced arsenic sensitivity. The present study examined the role of xenobiotic export pumps in acquired arsenic tolerance. Microarray analysis of CAsE cells showed increased expression of the genes encoding for glutathione *S*-transferase Π (GST-Π), multidrug resistance-associated protein genes (*MRP1/MRP2*, which encode for the efflux transporter Mrp1/Mrp2) and the multidrug resistance gene (*MDR1*, which encodes for the efflux transporter P-glycoprotein). These findings were confirmed at the transcription level by reverse transcription-polymerase chain reaction and at the translation level by Western-blot analysis. Acquired arsenic tolerance was abolished when cells were exposed to ethacrynic acid (an inhibitor of GST-Π), buthionine sulfoximine (a glutathione synthesis inhibitor), MK571 (a specific inhibitor for Mrps), and PSC833 (a specific inhibitor for P-glycoprotein) in dose-dependent fashions. MK571, PSC833, and buthionine sulfoximine markedly increased cellular arsenic accumulation. Consistent with a role for multidrug resistance efflux pumps in arsenic resistance, CAsE cells were found to be cross-resistant to cytotoxicity of several anticancer drugs, such as vinblastine, doxorubicin, actinomycin-D, and cisplatin, that are also substrates for Mrps and P-glycoprotein. Thus, acquired tolerance to arsenic is associated with increased expression GST-Π, Mrp1/Mrp2 and P-glycoprotein, which function together to reduce cellular arsenic accumulation.

Arsenic is a metalloid that naturally occurs in soil, water, and air. Arsenicals are also byproducts of production of copper, lead, and other metals and of coal consumption (Agency for Toxic Substances and Disease Registry, 1999; National Research Council, 1999). Arsenicals have been used since ancient times as therapeutic agents, as well as intentional poisons. Cells can develop tolerance to inorganic arsenicals, and several other inorganic compounds, at least in rodent cell lines (Lee et al., 1989; Wang et al., 1996; Romach et al., 2000). In current therapeutics, the medicinal use of arsenicals is largely confined to psoriasis, spirochetal, and protozoal diseases (Klaassen, 1996). However, arsenic trioxide (As_2O_3) has recently been used with remarkable success in the treatment of acute promyelocytic leukemia (Chen et al., 1997; Soignet et al., 1998). On the other hand, arsenic is also a human carcinogen, causing cancers of skin, lung, bladder, liver, and kidney after long-term exposure to arsenic-contaminated water or air (Abernathy et al., 1999; Agency for Toxic Substances and Disease Registry, 1999; Goering et al., 1999; National Research Council, 1999). Like other paradoxical chemotherapeutics/carcinogens, arsenic must be used with the realization of its potential adverse effects, including the possible iatrogenic induction of tumors (see Huff et al., 2000). Indeed, carcinogenic effects of arsenic in humans were first discovered in the late 19th century after its medicinal use for skin disorders (Huff et al., 2000). Therefore, although studies aimed at understanding the mech-

[1] This work was supported in part by National Cancer Institute contract No. N01-CO-56000 (J.E.S.) and National Institutes of Health Grant 07079 (D.R.J.), and ES09716 (C.D.K.).

ABBREVIATIONS: CAsE, chronic arsenic-exposed cells; MDR, multidrug resistance; MRP, multidrug resistance-associated protein; GST, glutathione *S*-transferase; RT-PCR, reverse transcriptase-polymerase chain reaction; TBST, Tris-buffered saline-Tween 20; MTS, methanethiosulfonate; P-gp, P-glycoprotein; GSH, glutathione; BSO, buthionine sulfoximine; EA, ethacrynic acid.

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anisms of arsenic carcinogenesis and arsenic tolerance address basic scientific issues, they also have clinical relevance.

We have recently shown that long-term exposure (18 or more weeks) of a rat liver epithelial cell line (TRL1215) to 125 to 500 nM sodium arsenite, a concentration range relevant to environmental arsenic contamination levels, resulted in malignant transformation (Zhao et al., 1997). In fact, these arsenic-transformed cells produce aggressive tumors capable of metastasis after inoculation into Nude mice (Zhao et al., 1997). Global DNA hypomethylation and aberrant gene expression, such as overexpression of metallothionein, oncogene c-*myc*, c-*met*, ErbB2, and c-K-*ras* are associated with arsenic-induced malignant transformation (Zhao et al., 1997; Chen et al., 2001). These chronic arsenic-exposed (CAsE) cells also show markedly enhanced cell proliferation, with up-regulation of cell cycle-regulated genes such as cyclin D1 and proliferating cell nuclear antigen (Chen et al., 2001).

An important feature of these CAsE cells is the development of self-tolerance to arsenite (As^{3+}), arsenate (As^{5+}), and dimethylarsinic acid and cross-resistance to other metals, such as antimony, nickel, and cadmium (Romach et al., 2000). There are several possible mechanisms for arsenic-induced metal-tolerance in these CAsE cells. In this regard, there seems to be an increase in the relative amount of methylation of arsenic in CAsE cells (Romach et al., 2000), and methylation is often thought to be a detoxication pathway for inorganic arsenicals under some conditions (Aposhian, 1997). Beyond this, there is also a hyperinducibility of the metallothionein gene, probably as a result of DNA hypomethylation (Zhao et al., 1997; Romach et al., 2000) and metallothionein is known to be important in acquired tolerance to metals, such as cadmium (Klaassen et al., 1999) and possibly arsenic (Liu et al., 2000). However, the most dramatic change in these CAsE cells is the marked reduction in cellular arsenic accumulation, caused by an apparent increase in arsenic efflux (Romach et al., 2000).

The current study was undertaken to examine further the molecular mechanism(s) underlying acquired arsenic tolerance in CAsE cells, focusing, after initial gene array screening, on cellular glutathione *S*-transferase Π (GST-Π) and multidrug resistance transporters. Our results demonstrated that the acquired self-tolerance to arsenic in this case is associated with increased expression of GST-Π and multidrug resistance transporters (Mrp1, Mrp2 and P-glycoproteins), which together contribute to arsenic tolerance by reducing cellular arsenic content. These data have important implications in the toxicology and pharmacology of arsenic.

Materials and Methods

Chemicals. Sodium arsenite ($NaAsO_2$), PSC833 (a specific inhibitor for P-glycoprotein), buthionine sulfoximine, ethacrynic acid, *cis*-diamminedichloroplatinum(II) (cisplatin), doxorubicin (Adriamycin), vinblastine, and actinomycin D were obtained from Sigma Chemical Company (St. Louis, MO). The cDNA expression arrays were purchased from CLONTECH (Palo Alto, CA). Rabbit polyclonal antibodies against Mrp1 and Mrp2 were a gift from author C. K. The mouse monoclonal antibody (C219) and rabbit polyclonal antibody (Ab-1) against MDR-encoded P-glycoproteins were purchased from Signet Laboratories (Dedham, MA) and Oncogene Research (Cambridge, MA), respectively. MK571, a specific inhibitor for Mrp1/Mrp2, was purchased from BIOMOL (Plymouth Meeting, PA). The mouse monoclonal antibody (G59720) against glutathione *S*-transferase-Π was obtained from BD PharMingen Signal Transduction Laboratories (San Diego, CA). Horse radish peroxidase-conjugated secondary antibodies against rabbit, mouse, and goat were purchased from Sigma, and the enhanced chemiluminescence (ECL) kits and [α-^{32}P]dATP were obtained from Amersham Pharmacia Biotech (Piscataway, NJ).

Cell Culture and Treatments. The rat liver epithelial cell line, TRL1215, was originally derived from the liver of 10-day-old Fischer F344 rats. The cells are diploid and normally nontumorigenic. Malignant transformation was accomplished by continuously culturing TRL1215 cells in arsenite-containing media (0, 125, 250, and 500 nM) for 18 or more weeks as described previously (Zhao et al., 1997). This treatment also induces tolerance to inorganic arsenicals in these CAsE cells (Romach et al., 2000). In the present study, CAsE cells were grown in the continuous presence of 125, 250, or 500 nM arsenite for up to 24 weeks and were compared with untreated, passage-matched control cells. Assays were performed with 70 to 80% confluent cell cultures.

Microarray Analysis. Total RNA in cultured cells was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA), according to manufacturer's instructions. The microarray analysis was according to manufacturer's instructions. Briefly, 1 μg of poly(A^+) RNA was converted to ^{32}P-labeled cDNA probes using Moloney murine leukemia virus reverse transcriptase and [α-^{32}P]dATP with the CLONTECH Atlas rat CDS primer mix. The ^{32}P-labeled cDNA probe was purified using CHROMA SPIN-200 (CLONTECH) columns, denatured in 0.1 M NaOH, 10 mM EDTA at 68°C for 20 min, followed by neutralization with an equal volume of 1 M NaH_2PO_4 for another 10 min. The microarray membranes were prehybridized with ExpressHyb (CLONTECH) containing sheared salmon testes DNA (100 μg/ml) for 30 to 60 min at 68°C, followed by hybridization overnight at 68°C with the cDNA probes. The array membranes were washed four times in 2× SSC/1% SDS, 30 min each, and two times in 0.1× SSC/0.5% SDS for 30 min. The array membranes were then sealed in plastic bags, and exposed to a PhosphorImage screen (Molecular Dynamics, Sunnyvale, CA) or X-ray film. The image was analyzed densitometrically using AtlasImage software (ver.1.5; CLONTECH). Four relatively consistent housekeeping genes (i.e., 40S ribosomal protein, β-actin, myosin heavy chain, and phospholipase A2 precursor) were used to normalize the hybrid intensity of each gene of interest.

RT-PCR Analysis. RT-PCR was performed using Advantage one-step RT-PCR kit from CLONTECH. The primer sequences for the GST-Π gene (GST7–7) (5′-GATGGGGTGGAGGACCTTCGATGC-3′; 5′-CTGAGGCGAGCCACATAGGCAGAG-3′), multidrug resistance gene (MDR1) (5′CTCACCAAGCGACTCCGATACATG-3′; 5′-GATAATTCCTGTGCCAAGGTTTGCTAC-3′), and multidrug resistance protein gene (MRP) (5′-GGAAGACAA-AGATTCTAGTGTTGGACG-3′; 5′-AGATATGCCAGAGATCAGTTC-ACACC-3′) were also obtained from CLONTECH, and synthesized by Operon (Alameda, CA). RT-PCR products were visualized by ultraviolet illumination after electrophoresis through 2% agarose gel, with 0.5 μg/ml ethidium bromide at 50 V at 2 h, and scanned using Kodak gel analysis software.

Western-Blot Analysis. Cells were pelleted in lysis buffer (10 mM Tris-HCl, pH 7.4, 100 μM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin A, 2 μg/ml leupeptin, 2 μg/ml antipain, and 1 μg/ml aprotinin). Total protein (20–40 μg) was subjected to electrophoresis on Tris-glycine polyacrylamide minigels (4–20%; Invitrogen), followed by electrophoretic transfer to nitrocellulose membrane for 3 h. The membrane was blocked in 10% dried milk in TBST (15 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween 20) for 2 h at room temperature, followed by incubation with the appropriate primary antibody (1:500 to 1:2,000) in 3% milk in TBST overnight at 4°C. After four washes with TBST, the membranes were incubated in secondary antibody (1:5,000 to 1:10,000) for 60 to 120 min followed by another four washes with TBST. Signals were detected using the enhanced chemiluminescence Western blot detection system.

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Cytotoxicity Assays. The Promega nonradioactive cell proliferation assay was used to determine acute toxicity as defined by metabolic integrity. The assay measures the amount of formazan produced by metabolic conversion of Owen's reagent [(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt, MTS)] by dehydrogenase enzymes in the mitochondria of metabolically active cells. The quantity of formazan product, as measured by absorbance at 490 nm, is directly proportional to the number of living cells. To determine cytotoxicity, 70% confluent CAsE cells or passage-matched control cells were treated with various concentrations of arsenite in the presence or absence of ethacrynic acid (an inhibitor of GST-II), buthionine sulfoximine (a glutathione synthesis inhibitor), MK571 (a specific inhibitor for Mrp), PSC833 (a specific inhibitor for P-gp), or with various concentrations of anticancer drugs (cisplatin, doxorubicin, vinblastine, and actinomycin D) for 36 h, and metabolic integrity was determined. Data are expressed as metabolic integrity using untreated control levels as 100%.

Cellular Arsenic Accumulation. Control and CAsE cells were grown to 70% confluence in arsenic-free medium, and then cells were incubated with fresh medium containing 12.5 μM arsenite in the presence or absence of MK571 (a specific inhibitor for Mrp), PSC833 (a specific inhibitor for P-gp), or buthionine sulfoximine (a glutathione synthesis inhibitor). Twenty-four hours later, cells were harvested. After sonication, cellular protein was determined by Bradford dye-binding assay (Bio-Rad, Hercules, CA), and cell suspension was digested completely in nitric acid and dissolved in distilled water. Total arsenic, including inorganic and organic forms, was determined using graphite furnace atomic absorption spectrometry and normalized with cellular protein content.

Statistics. The gene array data represent the mean ± S.E.M. of RNAs derived from four separate experiments using Atlas Rat cDNA Expression Array, and two separate experiments using Atlas Rat Toxicology Array (CLONTECH). The data for cell cytotoxicity and cellular arsenic content represent the mean ± S.E.M. of three separate determinations. Student's t test was used to analyze differences between CAsE cells and control cells. The level of significance was set at $p < 0.05$. The LC_{50} was determined from regression analysis of the linear portion of the triplicate metabolic integrity curves.

Results

Microarray Analysis of Arsenic-Tolerant Cells Revealed the Up-Regulation of GST-II and Multidrug Resistance Transport Proteins. Consistent with previous studies (Romach et al., 2000), cells grown for 24 weeks in the presence of 500 nM arsenite exhibited increased tolerance to micromolar concentrations of arsenite (Figs. 4 and 5). Microarray analysis revealed that among ~70 differentially expressed genes (Chen et al., 2001), the expression of genes encoding for GST-IIpi, microsomal GST1, MDR1, MDR2, and MRP was increased in cells that have undergone long-term exposure to arsenite (CAsE cells, Fig. 1A). Expression of other plasma membrane proteins, such as Na^+/K^+-ATPase, chloride channel, water channel aquaporin, and monocarboxylate transporter, was unaltered. The constitutive expression for other transporters was too low to make valid comparisons (data not shown). RT-PCR analysis confirmed increased expression of the genes for GST-II, MDR1 and MRP1 in CAsE cells (Fig. 1B). Expression of β-actin, which was used to standardize load, was similar in control and CAsE cells.

To determine whether long-term exposure to low levels of arsenite also increased the above gene products at the protein level, cells were exposed to 0, 125, 250, or 500 nM arsenite for 24 weeks, and cell extracts were analyzed for expression of GST, Mrp1, Mrp2, and P-glycoprotein (MDR gene product) by Western-blot analysis. Figure 2 shows that expression of each of these proteins increased with increasing arsenite concentration. For each blot, the greatest increases in band density were apparent with 250 to 500 nM exposure. By using a Kodak Digital Science ID software, the band intensity was semiquantified. Long-term exposure of cells to arsenite (125, 250, and 500 nM) produced increases in GST-II (98, 155, and 190%), Mrp1 (266, 602, and 1020%), Mrp2 (280, 425, and 540%), and Pgp (136, 307, and 280%), respectively. The specificity of the antibody used for GST-Pi detection was verified using Hela cell lysate (BD PharMingen, control for GST-II), whereas the specificity of the C219 antibody against P-glycoprotein (P-gp, 170 kD) was verified with CH^RC5 cell lysate (provided by Dr. Victor Ling, Ontario Cancer Institute, Canada) and it reacts with the MDR1, MDR2, and sister of P-glycoprotein gene products, so it was not clear from the blot

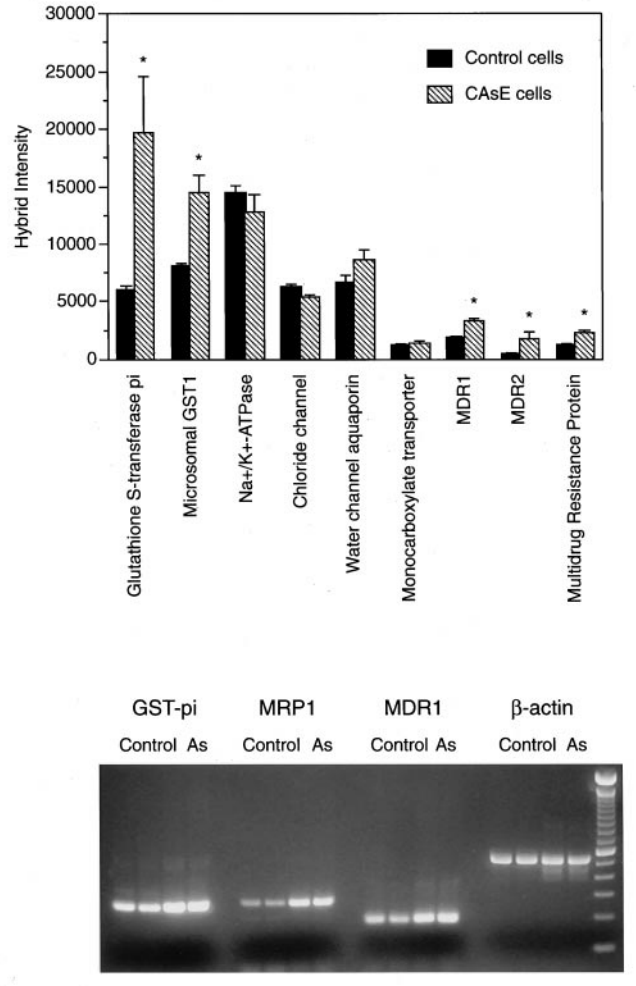

Fig. 1. The Atlas microarray analysis of the expression of glutathione *S*-transferase-II and transporter-related genes in CAsE cells after long-term exposure to arsenite (500 nM, 24 weeks) and passage-matched control cells (top). Data are mean ± S.E.M. of four separate experiments. *$p < 0.05$, significantly different from control cells. The details of cDNA microarray and data analysis were described under *Materials and Methods* (bottom). RT-PCR analysis of the *GST*-II gene (272 bp), multidrug-resistance protein gene (*MRP1*, 278 bp), and multidrug-resistance gene (*MDR1*, 194 bp) in CAsE cells and passage-matched control cells: 25 cycles for β-actin, 30 cycles for GST, 35 cycles for MDR1, and 37 cycles for MRP1.

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to what extent expression of the individual proteins was increased. The antibodies used to detect Mrp1 and Mrp2 were verified with their corresponding positive control at the University of Kansas Medical Center (data not shown).

Inhibition of Mrps and P-Glycoprotein and Depletion of GSH Increases Cellular Arsenic Accumulation. Previous studies showed that one notable feature of CAsE cells was reduced arsenic accumulation after acute exposure to arsenite (Romach et al., 2000). To assess whether inhibition of Mrps and P-glycoprotein transporters affects arsenic toxicity, we measured arsenic accumulation and cytotoxicity after incubating cells with MK571 (a specific inhibitor of Mrp1/Mrp2) and PSC833 (a specific inhibitor of P-gp), as well as with BSO, an inhibitor for GSH synthesis. For arsenic accumulation studies, we chose acute exposure conditions that produced only limited cytotoxicity in initial experiments with control and CAsE cells (i.e., 24-h exposure to 12.5 μM arsenite). As shown in Fig. 3, control cells accumulated about 10 ng of As/mg of protein, whereas CAsE cells accumulated about 4 ng of As/mg of protein (the assay measures total arsenic, but not individual arsenic species). Thus, arsenic accumulation in CAsE cells was only 40% of that in control cells, a finding consistent with previous experiments (Romach et al., 2000). With 50 μM MK571, cellular arsenic content was markedly increased from 9.7 to 90 ng/mg protein in control cells, and from 3.8 to 80 ng/mg protein in CAsE cells (Fig. 3A). PSC833 also produced a concentration-dependent increase in cellular arsenic content; at a concentration of 10 μM, PSC833 increased cellular arsenic from 9.7 to 75 ng/mg protein in control cells and from 3.8 to 39 ng/mg in CAsE cells (Fig. 3B). Inhibition of GSH synthesis with 3 μM BSO increased cellular arsenic content from 9.7 to 76 ng/mg protein in control cells, and from 3.8 ng to 30 ng/mg protein in CAsE cells (Fig. 3B).

Inhibition of GST-II and Depletion of GSH Eliminate Arsenic Resistance. Consistent with previous observations (Wang and Lee, 1993; Wang et al., 1996; Chen et al., 1998; Shimizu et al., 1998), ethacrynic acid (EA, an inhibitor for GST; Tew et al., 1997) and BSO greatly increased arsenic toxicity. EA at concentrations of 25 and 50 μM, did not produce cytotoxicity in either control or CAsE cells, but it reversed arsenic tolerance in both cell lines (Fig. 4). At higher concentration (75 μM), EA alone produced cytotoxicity in control cells (data not shown). BSO, at the concentrations used (0.3–3 μM), was cytotoxic to neither control nor CAsE

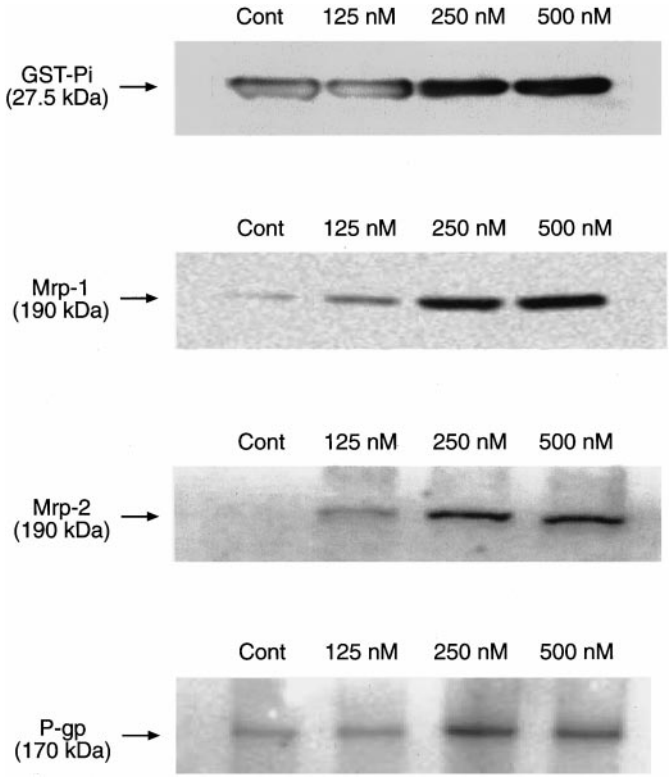

Fig. 2. Western-blot analysis of GST-II, multidrug resistance proteins (Mrp1, Mrp2), and multidrug resistance gene-encoded P-gps in CAsE cells after long-term exposure to arsenite (125, 250, and 500 nM for 24 weeks) and passage-matched control cells.

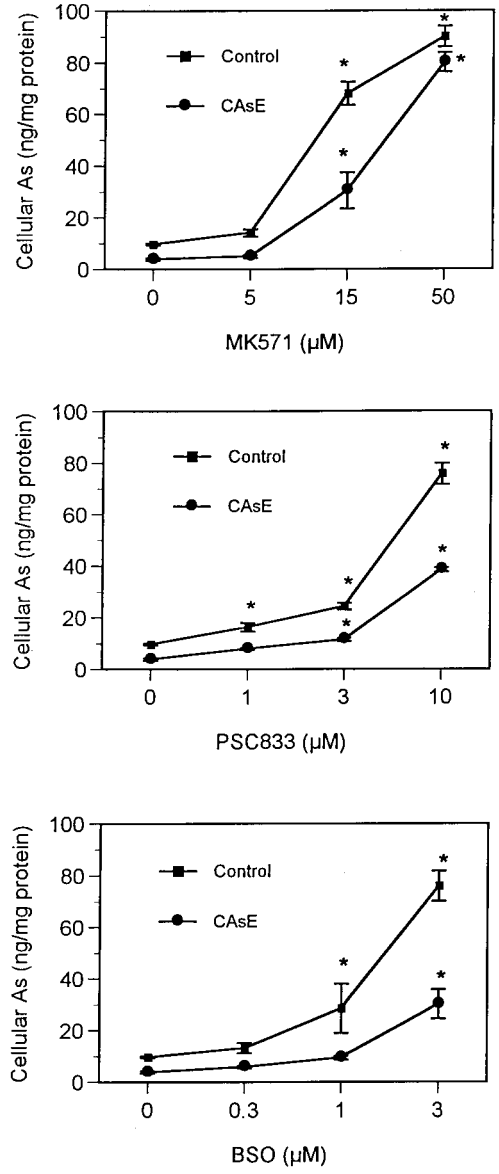

Fig. 3. The effects of MK571 (an inhibitor of Mrp1 and Mrp2), PSC833 (an inhibitor of P-glycoprotein), and BSO (an inhibitor of GSH synthesis) on cellular arsenic accumulation in control and CAsE cells. Cells were exposed to 12.5 μM arsenite, in the presence or absence of MK571 (5–50 μM), PSC833 (1–10 μM), or BSO (0.3–3 μM), and cellular arsenic was determined 24 h later by graphite furnace atomic absorption spectrometry. Data are mean ± S.E.M. of three determinations. *$p < 0.05$, significantly different from arsenic-only controls.

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cells, but greatly enhanced arsenic cytotoxicity in a dose-dependent manner in both control and CAsE cells (Fig. 4, C and D). Intracellular GSH content was depleted by BSO in a concentration-dependent manner. The basal GSH concentration in TRL1215 control cells was 9.82 ± 0.27 nmol/mg protein, whereas in CAsE cells, it was 14.1 ± 0.94 nmol/mg protein. BSO treatment at concentrations of 0.3, 1, 3, and 10 μM for 24 h resulted in 25 ± 2, 40 ± 5, 50 ± 6, and 75 ± 5 depletion of GSH levels in TRL1215 control cells, respectively; and 10 ± 2, 25 ± 4, 45 ± 7, and 65 ± 8% depletion of GSH levels in CAsE cells, respectively.

Inhibition of Mrps and P-Glycoprotein Eliminates Arsenic Resistance. Inhibition of Mrp1/Mrp2 function with 5 to 50 μM MK571 had no consistent effects on arsenic sensitivity in control cells (Fig. 5A). However, MK571 caused a concentration-dependent loss of arsenic tolerance in CAsE cells (Fig. 5B). Inhibition of P-glycoprotein function by 1 to 10 μM PSC833 increased arsenite sensitivity in both control and CAsE cells, but the effects of this drug were clearly greatest in the arsenic-tolerant CAsE cells (Fig. 5, C and D). In the arsenic-tolerant cells, all concentrations of PSC833 increased arsenic toxicity (Fig. 5D), whereas in control cells, increased toxicity was only seen with the highest PSC833 concentration used (Fig. 5C). Thus, inhibiting transport function of Mrps and P-glycoprotein reverses the acquired arsenic tolerance in CAsE cells.

CAsE Cells Are Cross-Resistant to Common Chemotherapeutic Drugs. Mrps and P-glycoprotein are membrane pumps that mediate the efflux of a wide variety of xenobiotics from cells (Keppler et al., 1998; Ambudkar et al., 1999; Kala et al., 2000). Increased expression of these transporters in CAsE cells implies that these cells also may be cross-resistant to a large number of compounds that are both cytotoxic and substrates for the transporters (Ambudkar et al., 1999). Table 1 shows that was indeed the case for selected anticancer drugs. The LC_{50} values for cisplatin, vinblastine, doxorubicin, and actinomycin D were three to five times greater in CAsE cells than in control cells, indicating that cells that have undergone long-term exposure to low levels of arsenic are multidrug-resistant.

Discussion

We have demonstrated previously that long-term exposure of cells to low levels of arsenic induces both malignant transformation (Zhao et al., 1997) and reduced sensitivity when cells are subsequently undergo acute exposure to higher levels of arsenic and other toxic metals (Romach et al., 2000). Arsenic tolerance seems to result in large part from increased arsenic efflux, resulting in a reduced cellular arsenic burden (Romach et al., 2000). In the present study, we demonstrated increased expression of genes encoding GST-Π and xenobiotic export transporters Mrp1, Mrp2, and P-glycoprotein in CAsE cells at both the transcriptional and translational levels. Most importantly, when CAsE cells were acutely exposed to arsenic, inhibiting function of any one of these proteins markedly increased arsenic accumulation and arsenic toxicity, indicating that their up-regulation is critical to acquired tolerance in CAsE cells.

It is not clear from the present data to what extent MK571 and PSC833 inhibited arsenic efflux through Mrp1/Mrp2 and arsenic efflux through P-glycoprotein, respectively. Nevertheless, each inhibitor was more effective in enhancing arsenite toxicity in CAsE cells than in control cells. For example, PSC833, at 1 to 3 μM, reversed tolerance in CAsE cells but did not affect the survival of control cells. In contrast, depletion of cellular GSH with BSO substantially reduced ability of control and CAsE cells to survive arsenic exposure, a finding consistent with the important role of GSH in acute arsenic toxicity (Shimizu et al., 1998). Beyond this, the clear up-regulation of GST-Π and transporter genes in CAsE cells implies mechanistic significance in acquired tolerance. Taken together, these observations indicate that acquired arsenic tolerance (and accompanying multidrug resistance) in CAsE cells is a consequence of increased expression of multiple genes. These include genes encoding for enzymes

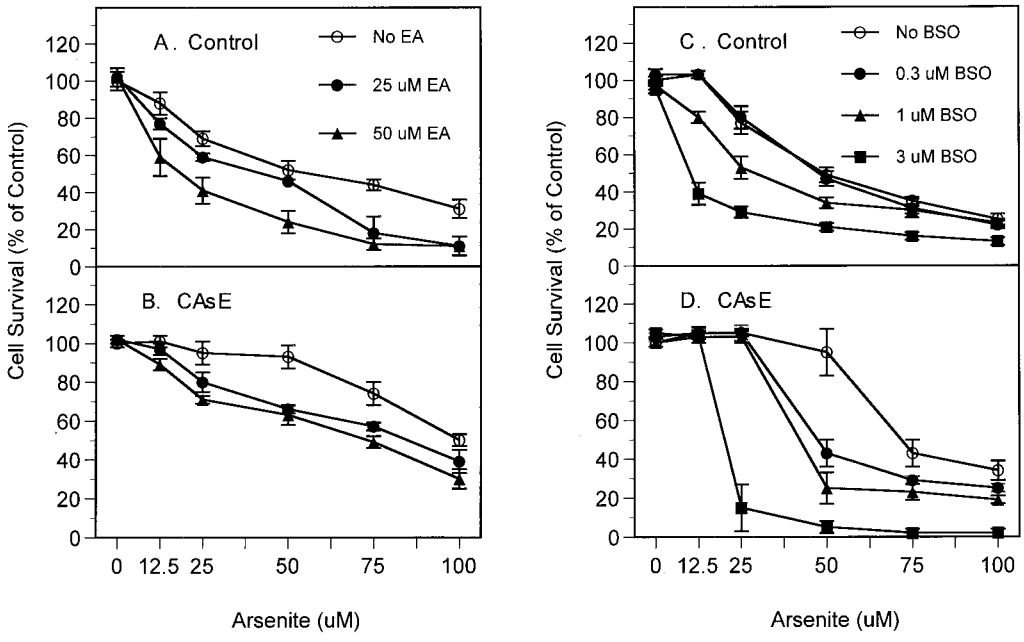

Fig. 4. The effects of EA (an inhibitor of GST) and BSO (an inhibitor of GSH synthesis) on arsenic cytotoxicity in control and CAsE cells. Cells were exposed to arsenic (0–100 μM), in the presence or absence of EA (25 or 50 μM) or BSO (0.3–3 μM), and cytotoxicity was determined 36 h later by the MTS assay. Data are mean ± S.E.M. of three determinations.

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that conjugate arsenic and genes encoding for efflux pumps capable of removing arsenic and its conjugates from the cells (Fig. 6). In this regard, we know that in intact animals, the transport of arsenic from the liver to bile is dependent on GSH (Gyurasics et al., 1991), and recent evidence indicates that the conjugation of arsenic with GSH is important for its biliary excretion via the MRP2/cMOAT transporter (Gregus and Gyurasics, 2000; Kala et al., 2000). The up-regulation of the genes encoding for these transport proteins could be envisioned as cellular adaptive mechanisms in response to continuous arsenic exposure, resulting in increased arsenic efflux from the cells and thus reducing cellular arsenic accumulation and, presumably, toxicity.

Overexpression of GST-II plays an important role in the acquisition of arsenic self-tolerance and antineoplastic drug resistance. In other arsenic-resistant cells, increases in GST-II gene expression have been reported (Lee et al., 1989; Wang and Lee, 1993; Wang et al., 1996). The increased GST-II expression may facilitate the formation of the putative arsenic-GSH conjugates, such as arsenic triglutathione and dimethylarsenic diglutathione (Dey et al., 1996; Kala et al., 2000), for more effective cellular efflux (Wang and Lee, 1993; Wang et al., 1996) or biliary excretion (Gregus and Gyurasics, 2000; Kala et al., 2000). Accordingly, inhibitors of the enzymatic activity of GST-II, such as ethacrynic acid and Cibacron blue, and inhibitors of glutathione synthesis, such as BSO, are capable of increasing arsenic toxicity and reducing arsenic efflux (Wang and Lee, 1993; Naredi et al., 1995; Wang et al., 1996; Chen et al., 1998; Shimizu et al., 1998). Consistent with the literature, the present study demonstrated that depletion of cellular GSH by BSO or inhibition of GST activity by ethacrynic acid (Tew et al., 1997) increased cellular arsenic accumulation and arsenic toxicity. It should also be pointed out that depletion of GSH by BSO could also affect Mrp activity (Vanhoefer et al., 1996). Taken together, the results of the present study and prior work reinforce the importance of up-regulation of the GST-II gene and GSH conjugation as integral parts of the acquired tolerance seen in the present study.

The majority of GS-X conjugates are pumped out of cells by ATP-dependent transporters, such as the MRP family. These transporters play a major role in the cellular efflux of GS-X conjugates (Keppler et al., 1998; Kala et al., 2000). In the present study, the up-regulation of MRP1 and MRP2 genes, encoding for Mrp1 and Mrp2 proteins, respectively, occurred in CAsE cells. Up-regulation of Mrps was confirmed at the transcriptional level by microarray analysis and RT-PCR and at the translation product level by Western blot analysis. Most importantly, MK571, the leukotriene LTD4 receptor antagonist for specific inhibition of the Mrp1/Mrp2 transporter (Gekeler et al., 1995), markedly reversed arsenic tolerance and increased cellular arsenic content. This strongly suggests that Mrp1 and Mrp2 transporters play a role in the reduction of cellular arsenic by increasing the efflux of a putative arsenic-GSH conjugate. In this regard, cisplatin-resistant human KB carcinoma cells (C-A120 cells), which overexpress MRP, were cross-resistant to both arsenite and arsenate (Chen et al., 1998). MRP1-overexpressing lung tumor cells also were markedly resistant (12- to 16-fold) to both arsenite and arsenate (Vernhet et al., 2000). Conversely, MRP (Mrp1) double-knockout ES cells showed an increased sensitivity to arsenite, arsenate, and antimony, a metalloid with biological properties similar to those of arsenic (Rappa et al., 1997). In intact animals, a dose of 15 mg arsenite/kg caused 50% lethality in MRP-null mice (Mrp1) but only 17% lethality in wild-type mice (Lorico et al., 1997). All these findings support the conclusion that the up-regulation of MRP proteins plays an important role in acquired arsenic-self tolerance in CAsE cells.

Expression of the multidrug-resistant gene, which encodes for P-glycoprotein (P-gp), was also increased in these arsenic-resistant CAsE cells. PSC833, an analog of cyclosporin A acting as a substrate and inhibitor of P-glycoproteins (Chen et al., 1999), greatly increased arsenic cytotoxicity and increased cellular arsenic accumulation in both control and CAsE cells in the present studies. Arsenite is known to induce human *MDR1* gene in vitro in a renal adenocarcinoma cell line (HTB-46; Chin et al., 1990), and in a cell line origi-

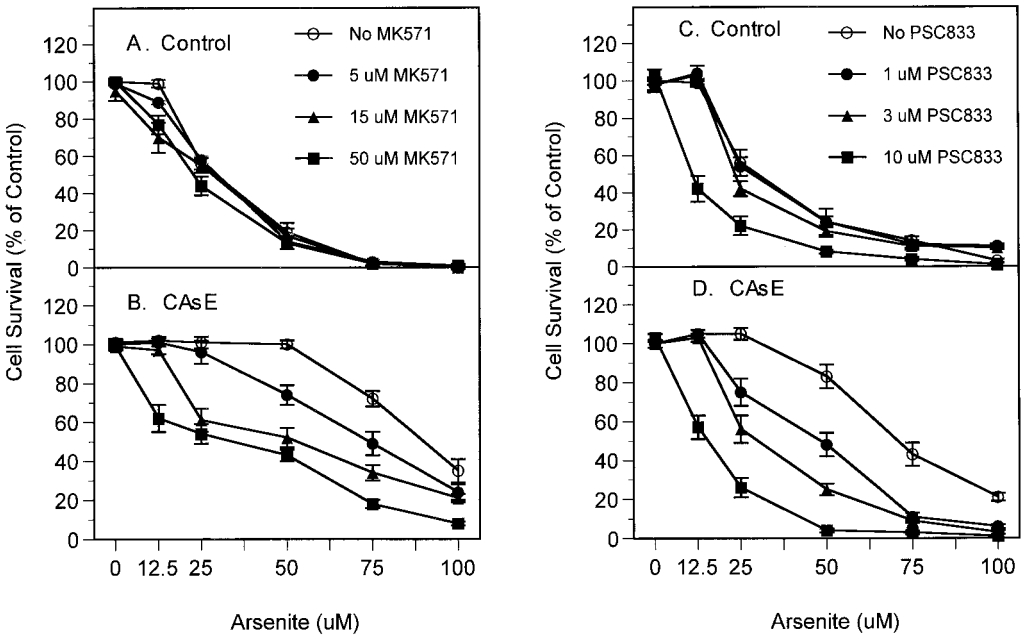

Fig. 5. The effects of MK571 (an inhibitor of Mrp1/Mrp2) and PSC833 (an inhibitor of P-glycoprotein) on arsenic cytotoxicity in control and CAsE cells. Cells were exposed to arsenic (0–100 μM), in the presence or absence of MK571 (5–50 μM) or PSC833 (1–10 μM), and cytotoxicity was determined 36 h later by the MTS assay.

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nally derived from a hepatoma (HepG2; Kioka et al., 1992). The up-regulation of the *MDR1* gene seen in the present study may play, in turn, an important adaptive role by pumping arsenic out of cells. Indeed, targeted inactivation of a member of the P-glycoprotein family, *pgp-1*, in *Caenorhabditis elegans* resulted in increased sensitivity to arsenic and cadmium (Broeks et al., 1996). Similarly, mdr1a/1b double-knockout mice, which lack expression of MDR1-encoded P-glycoprotein, showed increased sensitivity to acute arsenic toxicity, with increased arsenic accumulation in tissues (J. Liu Y. Liu, C. D. Klaasen, M. P. Waalkes, manuscript in preparation). On the other hand, mouse cell lines lacking both P-glycoprotein and Mrp1 become hypersensitive to arsenic (Allen et al., 2000). Although the role of the *MDR* gene in arsenic efflux is as yet not definitively demonstrated, the up-regulation of the *MDR1* gene in CAsE cells coincides with acquired resistance to arsenic, strongly suggesting that this event is a major contributing factor.

Overexpression of the GST-Π gene was associated with malignant transformation in these arsenic-transformed cells. There is an increasing body of evidence suggesting that overexpression of GST-Π is associated with carcinogenesis (Henderson et al., 1998). Indeed, the organic arsenical, dimethylarsinic acid, increases GST-Π expression and promotes hepatocarcinogenesis in rats (Wanibuchi et al., 1997). Clearly, long-term arsenic-induced overexpression of GST-Π, together with the activation of oncogenes such as c-*myc*, c-*met*, c-K-*ras* as revealed by microarray analysis of CAsE cells (Chen et al., 2001), could play integral roles in arsenic-induced malignant transformation.

Overexpression of the *GST*-Π, *MRP*, and *MDR* genes has been implicated in acquired resistance to various cancer chemotherapeutics (Tew et al., 1997; Henderson et al., 1998; Ambudkar et al., 1999). In the present study, long-term arsenic exposure resulted in cross-resistance to several commonly used anticancer drugs, including cisplatin, doxorubicin, vinblastine, and actinomycin D. This is probably mediated through the same mechanism as that for acquired arsenic resistance; i.e., the increased expression of *GST*-Π, *MRP* and *MDR* genes in these CAsE cells makes them resistant to these various chemotherapeutics by enhancing efflux of the native drug or a conjugate metabolite. Because arsenic is now used in the treatment of promyelocytic leukemia (Soignet et al., 1998) and has been proposed for use against other tumors (Chen et al., 1997), care should be taken when using long-term or repeated arsenic therapy because loss of efficacy could occur. Furthermore, caution is needed when arsenic is given in combination with other chemotherapeutics, as there may be a potential for development of multidrug resistance. Additionally, this cross-resistance may well make arsenic-induced tumors occurring from occupational, environmental, or therapeutic exposures more difficult to treat and could reduce success rates of cancer chemotherapy in areas of high environmental arsenic exposure. It is alarming indeed to think that an environmental carcinogen could actually disrupt the pharmacological intervention directed toward the tumors it produces. Further research is required to define the effects of acquired arsenic tolerance or long-term arsenic exposure on the chemotherapy of cancer.

TABLE 1

Cytotoxicity (LC_{50} in μM) of arsenic and common chemotherapeutic agents in TRL1215 and CAsE cells

	Control Cells	CAsE cells	Ratio (CAsE/Control)
Arsenite	30.0 ± 1.61	85.5 ± 2.41	2.85
Cisplatin	46.0 ± 4.21	246 ± 12.6	5.34
Doxorubicin	5.21 ± 0.34	14.6 ± 1.16	2.80
Vinblastine	27.5 ± 1.72	104 ± 10.3	3.78
Actinomycin D	0.40 ± 0.02	1.19 ± 0.09	2.97

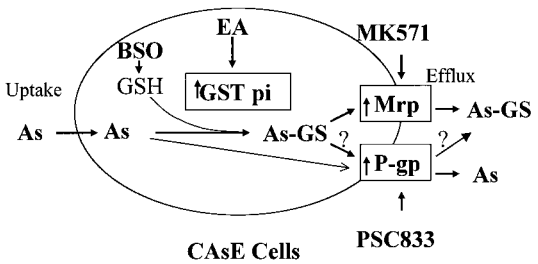

Fig. 6. The proposed mechanism for acquired arsenic tolerance in CAsE cells. Acquired arsenic tolerance involves the up-regulation of the *GST*-Π gene, which facilitates the formation of an As-GSH complex for efflux via up-regulated transporters (Mrp1/Mrp2 and/or P-gp). Abbreviations: As-GS, arsenic-glutathione conjugates; Mrp, multidrug resistance proteins; MK571, a specific inhibitor for Mrp; P-gp, multidrug resistance gene encoded P-glycoprotein; PSC833, an inhibitor for P-gp.

Acknowledgments

We thank Yanjie Qi for her summer internship work on this project and Drs. William Achanzar, Wei Qu, and Eduardo Brambila for their critical comments and assistance during the preparation of this manuscript.

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