Utilization of arsenic trioxide as a treatment of cisplatin-resistant non-small cell lung cancer PC-9/CDDP and PC-14/CDDP cells

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Abstract. Cisplatin is a commonly used drug in combination chemotherapy. However, various malignant tumors frequently acquire resistance to cisplatin. Arsenic trioxide (ATO) has been approved as a chemotherapeutic drug for the treatment of acute promyelocytic leukemia, and the combination of ATO and cisplatin has been revealed to demonstrate synergistic effects in ovarian and small cell lung cancer cells. Thus, it was hypothesized that ATO may also be active against cisplatin-resistant non-small cell lung cancer (NSCLC) PC-9/CDDP and PC-14/CDDP cells. The present study also evaluated the effects of ATO on the cisplatin-sensitive NSCLC PC-9 and PC-14 cell lines. Notably, ATO demonstrated a markedly decreased IC₅₀ in the cisplatin-resistant PC-9/CDDP and PC-14/CDDP cells compared with the IC₅₀ in the cisplatin-sensitive parental PC-9 and PC-14 cells. Additionally, it was found that arsenite accumulation in the PC-9 cell line was affected through the downregulation of GS-X pump systems. Although it is likely that cisplatin resistance in PC-9 cells does not depend on the GS-X pump systems, ATO was effective against cisplatin-resistant NSCLC PC-9/CDDP and PC-14/CDDP cells in combination chemotherapy.

Introduction

Since the late 20th century, a large number of molecular targeted therapeutic drugs, such as epidermal growth factor receptor tyrosine kinase inhibitors, have been developed for cancer chemotherapy. However, platinum-based combination chemotherapy continues to be important for the treatment of various cancers, including lung cancer. Cisplatin is a commonly used drug for combination chemotherapy (1). Although cisplatin is a potent anticancer drug, various malignant tumors frequently acquire resistance to cisplatin, limiting the clinical application of this agent (2,3).

Arsenite is a toxic metalloid that is widely distributed in the environment (4). Despite the toxicity, arsenic-containing compounds have been used in traditional Chinese medicine for >2,000 years (5). In addition, arsenic trioxide (ATO) has been approved in numerous countries for the treatment of acute promyelocytic leukemia (APL) (6). In vitro studies have revealed that a combination of ATO and cisplatin exerts a synergistic inhibitory effect on the growth of ovarian cancer and small cell lung cancer cells (7-9).

It is well known that adenosine triphosphate-binding cassette (ABC) transporters are important molecules for the resistance of cancer cells to chemotherapy agents and the detoxification of toxic metals in xenobiotic metabolism (10). Leslie et al previously reported that ABC subfamily C, member 1 (ABCC1) and ABC, subfamily C, member 2 (ABCC2) each play an important role in multidrug resistance and arsenic detoxification in cells, through the cellular efflux of the glutathione (GSH) conjugate (11).

The present study aimed to examine the sensitivity of the non-small cell lung cancer (NSCLC) PC-9 cell line and cisplatin-resistant PC-9/CDDP subline to toxic metals. The cisplatin-resistant PC-9/CDDP cell line demonstrated hypersensitivity to arsenite and arsenate. Furthermore, the accumulation of arsenite was decreased in PC-9/CDDP cells relative to the parental cells. Notably, the sensitivity to arsenite and accumulation of arsenite were markedly increased compared with the cisplatin-resistant cell line. Therefore, it was hypothesized that the GS-X pump systems were downregulated in the cisplatin resistant NSCLC cell line. In addition, the arsenic-containing chemotherapeutic agent ATO was applied to the cisplatin-sensitive and -resistant NSCLC cells, and it was found that the cisplatin-resistant NSCLC cell lines PC-9/CDDP and PC-14/CDDP were hypersensitive to ATO.

The results of the present study suggest that arsenite accumulation depends on the GS-X pump and that treatment with ATO may overcome certain cases of cisplatin resistance in NSCLC.
Materials and methods

Cell lines and materials. The NSCLC PC-9 and PC-14 cell lines, which were derived from untreated patients with pulmonary adenocarcinoma, were provided by Professor Y. Hayata (Tokyo Medical College, Tokyo, Japan). The cisplatin-resistant PC-9/DDP and PC-14/DDP sublines were established at the National Cancer Center Research Institute (Tokyo, Japan) (12).

The mouse anti-human ABCC1 monoclonal antibody (clone number, QCRLI; ALX-801-010) and mouse anti-human ABCC2 monoclonal antibody (clone number, M21-4; ALX-801-015) were obtained from Enzo Life Sciences, Inc. (Farmington, NY, USA). The mouse anti-human GST-π monoclonal antibody (clone number, GST-π3; 610718) was obtained from BD Biosciences (Franklin Lakes, NJ, USA). The Vectastain ABC kit and horseradish peroxidase-conjugated anti-mouse immunoglobulin G secondary antibody were obtained from Vector Laboratories (Burlingame, CA, USA). Enhanced chemiluminescence (ECL) reagent and Hyperfilm were obtained from GE Healthcare Biosciences (Pittsburgh, PA, USA). Sodium selenate, sodium selenite, sodium arsenite and sodium arsenate were obtained from Sigma-Aldrich (St. Louis, MO, USA). Antimycin and cadmium chloride were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Arsenic trioxide (Triseno) was obtained from Nippon Shinyaku Co. Ltd. (Kyoto, Japan). All other chemicals were obtained from Nacalai Tesque, Inc. or Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Cellular cytotoxicity assay to determine sensitivity to metals. Cell counting kit-8 was obtained from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan) and, with MTT, was used for colorimetric assays to determine the cell viability, as previously described (13). The cells were plated on 96-well microplates at a density of 2x10^3 cells per well, incubated overnight, and then treated with various concentrations of arsenate, arsenite, selenate, selenite, mercury, cadmium or antimony for 72 h.

Determination of arsenite accumulation. The cells were plated on 6-cm dishes at a density of 1x10^6 cells per dish, incubated overnight, and then treated with 100 µM sodium arsenite for 4 h. Subsequent to washing with ice-cold PBS (-), the cells were immediately harvested using a cell scraper. The cells were pelleted by centrifugation, and the cell pellets were extracted with 20 mM Tris-HCl (pH 7.4) containing 1% Triton-X 100 and 0.1% SDS. The arsenite concentrations in the cells were determined by atomic absorption spectrometry (AAS; Z-9000; Hitachi, Ltd., Tokyo, Japan).

Analysis of the GSH concentration and activity of GSH-associated enzymes. The intracellular GSH concentration was measured using 5,5-dithiobis(2-nitrobenzoic acid) (DTNB). Cell lysates were diluted in 100 mM sodium phosphate buffer (pH 7.4) containing 2.5 mM EDTA, 0.25 mM NADPH and 1 unit/ml glutathione reductase (Oriental Yeast, Co., Ltd., Tokyo, Japan). The reaction was then initiated by adding 200 µM DTNB, and the absorbance at 415 nm was subsequently measured. The amount of total GSH was determined by comparing the absorbance of the cell lysate with a GSH standard curve.

The activity of γ-glutamyl cysteine synthetase (γ-GCS) was measured using a previously reported high-performance liquid chromatography (HPLC) method (14), with slight modification. A 10-µl aliquot of cell lysate was diluted in 100 µl of assay mixture containing 75 mM KCl, 10 mM MgCl₂, 0.2 mM EDTA, 10 mM adenine triphosphate, 10 mM cysteine, 5 mM glutamate and 100 mM Tris-HCl buffer (pH 7.4). Following 20 min of incubation at 37°C, the reaction was terminated by the addition of 20 µl of 15% trichloroacetic acid, and the assay mixture was then chilled on ice. Subsequent to centrifugation, quantitation of GlnCys was conducted by post-labeled fluorescence detection HPLC using the ortho-ththaldehyde reagent (Nacalai Tesque, Inc., Kyoto,Japan).

Glutathione-S-transferase (GST) activity was determined using standard spectrophotometric assays by monitoring the formation of the conjugate of 1-chloro-2,4-dinitrobenzene (CDNB) and reduced glutathione (GSH) (15).

GST-π protein expression was determined using immunoblotting. In total, 20 µg of protein was loaded onto 12.5% (w/v) SDS-polyacrylamide gels and subjected to electrophoresis. Subsequent to electrotransfer onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA, USA), the membranes were blocked with Block Ace (Yukijirushi-Nyugyo, Co., Sapporo, Japan) according to the manufacturer's instructions. Probing was performed overnight at 4°C with the monoclonal mouse anti-GST-π antibody diluted with PBS containing 0.1% Tween-20 (1:200; clone number, 3; BD Biosciences, Franklin Lakes, NJ, USA). Subsequent to washing, specific signals were detected using horseradish peroxidase-conjugated anti-mouse immunoglobulin G secondary antibody diluted with PBS containing 0.1% Tween-20 (1:10,000) and ECL reagent according to the manufacturer's instructions (GE Healthcare Biosciences, Pittsburgh, PA, USA).

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) for mRNA. Total RNA was isolated using an RNasy Mini kit (Qiagen, Hilden, Germany). Reverse transcription was performed on 1 µg of total RNA using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Foster City, CA, USA). RT-qPCR was performed using converted cDNA and the following reagents: TaqMan Gene Expression Assays for human ABCC1 (assay ID, Hs00219900_m1), ABCC2 (assay ID, Hs00166123_m1), ABCC3 (assay ID, Hs00978473_m1), ABCC4 (assay ID, Hs00988717_m1), ABCC5 (assay ID, Hs00981087_m1) and GAPDH (assay ID, Hs99999901_s1) genes, and the TaqMan Universal PCR Master Mix, no AmpErase UNG (Thermo Fisher Scientific Inc., Waltham, MA). An ABI PRISM 7300 Sequence Detector System (Thermo Fisher Scientific Inc.) was used to collect the signals according to the manufacturer's instructions. The relative gene expression was determined using the comparative cycle threshold (CT) method based on the normalization of the target gene against the GAPDH reference gene. ΔCT was calculated by subtracting the CT of GAPDH from the CT of the target genes.

Immunoblotting of ABCC1 and ABCC2. Plasma membrane fractions were prepared by ultracentrifugation, as previously described (16). Plasma membrane fractions (50 µg protein)
Statistical analysis. The results are expressed as the mean ± standard deviation. Statistical evaluations were performed using Student's t-tests with Microsoft Excel 2013 (Microsoft Corporation, Redmond, WA, USA) for the analysis.

Table I. Cross-sensitivity for various metals in the cisplatin-resistant PC-9/CDDP cell subline.

<table>
<thead>
<tr>
<th>Metal compounds</th>
<th>IC\textsubscript{50}, µM</th>
<th>Relative resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antimony (IV)</td>
<td>97.5±5.0</td>
<td>0.26±0.10</td>
</tr>
<tr>
<td>Arsenite (III)</td>
<td>22.5±5.0</td>
<td>0.14±0.03</td>
</tr>
<tr>
<td>Arsenate (V)</td>
<td>240.0±89.4</td>
<td>0.09±0.02</td>
</tr>
<tr>
<td>Selenite (IV)</td>
<td>9.8±0.4</td>
<td>1.00±0.00</td>
</tr>
<tr>
<td>Selenate (VI)</td>
<td>140.0±54.8</td>
<td>1.90±0.55</td>
</tr>
<tr>
<td>Mercury (II)</td>
<td>92.5±9.6</td>
<td>0.25±0.14</td>
</tr>
<tr>
<td>Cadmium (II)</td>
<td>62.5±22.2</td>
<td>1.15±0.24</td>
</tr>
<tr>
<td>Cisplatin (II)</td>
<td>4.1±0.3</td>
<td>5.76±1.20</td>
</tr>
</tbody>
</table>

IC\textsubscript{50}, half maximal inhibitory concentration. *P<0.05 vs. PC-9 cells.

Table II. Concentration of GSH and activity of associated enzymes in the cisplatin-resistant PC-9/CDDP cell subline.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>GSH, nmol/mg</th>
<th>GCS, unit/mg</th>
<th>GST, unit/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC-9</td>
<td>144.0</td>
<td>1.70</td>
<td>0.397</td>
</tr>
<tr>
<td>PC-9/CDDP</td>
<td>95.5</td>
<td>1.13</td>
<td>0.016</td>
</tr>
<tr>
<td>Ratio, % of PC-9</td>
<td>66.3</td>
<td>66.5</td>
<td>4.0</td>
</tr>
</tbody>
</table>

GSH, glutathione; GCS, glutamyl cysteine synthetase; GST, glutathione-S-transferase.

Table III. Sensitivity to arsenic trioxide in the parent and cisplatin-resistant non-small cell lung cancer PC-9 and PC-14 cell lines.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC\textsubscript{50}, µM</th>
<th>Relative resistance, fold of parent</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC-9</td>
<td>Parent</td>
<td>1.4±0.2</td>
</tr>
<tr>
<td>PC-9</td>
<td>Cisplatin resistant</td>
<td>1.1±0.7</td>
</tr>
<tr>
<td>PC-14</td>
<td>4.5±2.7</td>
<td></td>
</tr>
</tbody>
</table>

IC\textsubscript{50}, half maximal inhibitory concentration.

Results

Cross-resistance to metals. To elucidate the mechanism underlying cisplatin resistance, an analysis of the sensitivity of the PC-9 and PC-9/CDDP cell lines to various metals was performed. The IC\textsubscript{50} values of various metal compounds in the parental PC-9 and cisplatin-resistant PC-9/CDDP cell lines are reported in Table I. Only selenate (VI) demonstrated an increased cross-resistance in PC-9/CDDP cells compared with parental PC-9 cells, with a 1.90-fold increase. By contrast, antimony (IV), arsenate (V), arsenite (III) and mercury (II) were revealed to be more potent inhibitors of PC-9/CDDP cells compared with the effect on the parental PC-9 cell line. No difference in sensitivity was observed for the other metals between the PC-9/CDDP and parental PC-9 cells.

Arsenite accumulation. The PC-9/CDDP cells demonstrated hypersensitivity to arsenite and arsenate. To elucidate the mechanisms of arsenite sensitivity in the cisplatin-resistant PC-9/CDDP cell line, the accumulation of arsenite was measured. When the cells were incubated in medium containing 100 mM sodium arsenite for 4 h, the accumulation of arsenite in PC-9/CDDP cells was increased ~1.7-fold compared with the accumulation demonstrated by PC-9 cells (Fig. 1). When the cisplatin accumulation in the cells was determined under the same conditions, the cisplatin concentration in the PC-9/CDDP cells was found to be markedly decreased, demonstrating an accumulation of ~20% of the cisplatin found in the parental PC-9 cells (data not shown).

Analysis of the GSH concentration and activity of GSH-associated enzymes. GS-X pump systems are an important mechanism by which metals, including cisplatin and arsenite, are transported (17,18). To investigate the molecular basis for the decreased accumulation of arsenite, the GS-X pump systems were analyzed, including cellular GSH levels, activity of the GSH synthesis rate-limiting enzyme γ-GCS, GST activity and expression of the ABC transporters. Cellular GSH levels in PC-9/CDDP cells were 65% of those measured in the parental PC-9 cell line. γ-GCS activity was also downregulated in PC-9/CDDP cells, where it was 67% of the activity measured in PC-9 cells. GST activity was also extremely decreased to ~4.0% of the activity observed in the parental
cell line (Table II). The protein expression of GST-π was also downregulated in PC-9/CDDP cells compared with the parental cell line (Fig. 2).

Analysis of the expression of ABCC transporters. It has previously been indicated that arsenite is a substrate for the GS-X pump, as a GS-As conjugate (11). As shown in Fig. 3, the expression of ABCC1, 2 and 3 was downregulated in the PC-9/CDDP cells, with mRNA expression levels of 36.0, 2.7 and 51.9% of the level measured in the parental PC-9 cell line, respectively. The protein levels of ABCC1 and 2 in the plasma membrane were also decreased in the PC-9/CDDP cells compared with the parental cells. In western blot analysis, only faint bands corresponding to ABCC1 and 2 were obtained from the fractions derived from the PC-9/CDDP plasma membrane (Fig. 4).

Sensitivity of the cisplatin-resistant lung cancer PC-9/CDDP and PC-14/CDDP cell lines to ATO. As aforementioned, the cisplatin-resistant PC-9/CDDP cell line was revealed to demonstrate hypersensitivity to arsenite. Additionally, the cytotoxic effect of the arsenite-containing chemotherapeutic agent ATO was examined in the cisplatin-resistant NSCLC PC-9/CDDP and PC-14/CDDP cell lines.

The findings revealed that the PC-9/CDDP and PC-14/CDDP cell lines each demonstrated a 3-fold increase in sensitivity to ATO compared with the PC-9 and PC-14 parental cell lines (Table III).

Discussion

Cisplatin is a coordination compound of platinum. Certain cisplatin-resistant cell lines demonstrate cross-resistance to metals that include cadmium, antimony and arsenite (19). The identification of cross-resistant metals aids the evaluation of the mechanism underlying cisplatin resistance and metal transport systems. Previously, cisplatin-resistant cell lines were developed from metallothionein knockout (MT-KO) cells, and these cell lines demonstrated cross-resistance to arsenite and decreased arsenite accumulation (20). These results indicate the presence of a mechanism by which arsenite and cisplatin resistance in MT-KO cell lines depends on the same transport systems.

By contrast, an in vitro study has revealed that the combined administration of cisplatin and ATO demonstrates a synergistic inhibitory effect on the growth of various cancer cell lines, including squamous carcinoma, ovarian cancer and small cell lung cancer cells (7-9,21). In the present study, the cisplatin-resistant PC-9/CDDP cell line was revealed to be hypersensitive to arsenite. A marked decrease in the accumulation of arsenite was also observed in the PC-9/CDDP cells. These results indicate that the transport systems for cisplatin and arsenite are independent in PC-9 cells. Consequently, hypersensitivity to arsenite in PC-9/CDDP cells appears to be dependent on the cellular accumulation of arsenite.

It has previously been reported that the mechanisms of arsenite accumulation are mediated by transporter systems, influx transporters, including the aquaglyceroporins aquaporin (AQP)7 and AQP9 (22), and efflux transporters, such as ABCC1 and ABCC2 (11). Although the cisplatin uptake transporter was not identified in the present cell lines, the transporter of cisplatin in PC-9/CDDP cells may not contribute to the uptake of arsenite.
The present results clearly reveal that GSH and GS-X pump-associated factors, including GST activity and ABC transporter expression, were downregulated in PC-9/CDDP cells. GSH and GST are important for the formation of the arsenic-glutathione conjugate, which is a good substrate for ABCC1 and ABCC2.

In conclusion, ATO, an approved drug, potentially possesses sensitivity for cisplatin-resistant NSCLC cell lines. The mechanism of ATO hypersensitivity is dependent on the downregulation of GS-X pump systems, particularly GSH, GST, ABCC1 and ABCC2.

The aforementioned findings indicate that ATO has the potential to treat cisplatin resistance in NSCLC and that ABCC1 and ABCC2 are important biomarkers when considering a chemotherapeutic regimen containing ATO for the treatment of cisplatin-resistant NSCLC.

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References