

Cross-resistance of platinum derivatives in H-1R, a cisplatin-resistant cell line

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Abstract. We previously established H-1R cells, a cisplatin (CDDP)-resistant cell line, from H-1 cells, a CDDP-sensitive oral carcinoma cell line. The aim of this study was to identify the molecular mechanism of cross-resistance to antitumor drugs containing a platinum agent in H-1R cells. The 3-(3,4-dimethyl-thiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) assay and clonogenicity assay indicated that H-1R cells showed strong cross-resistance to carboplatin, nedaplatin and oxaliplatin. The expression status of the copper transporter and organic cation transporters was confirmed by real-time quantitative reverse transcriptase-polymerase chain reaction. The transporters ATP7A, ATP7B, hCtr1, hOCT1 and hOCT2 were up-regulated, whereas hOCT3 was down-regulated. The cellular glutathione level was elevated 2-fold in H-1R cells compared with H-1 cells. Our results suggested that H-1 and H-1R cells may be useful in searching for candidate genes responsible for cross-resistance to platinum derivatives and for further studies to understand the mechanism of platinum resistance.

Introduction

A number of chemotherapeutic agents have been used to treat cancer. However, the survival rate has not improved

substantially as a result of treatment with current chemotherapeutic agents. One factor that contributes to this lack of chemotherapeutic success is resistance to anticancer drugs. Although many mechanisms of drug resistance have been reported (1-3), our knowledge is insufficient to achieve a satisfactory clinical outcome.

Cisplatin (CDDP) is a first-generation compound that is commonly used as chemotherapeutic agent effective alone or in combination with other drugs to treat a wide variety of malignant solid tumors, including testicular, ovarian, bladder, esophageal and head and neck squamous cell carcinomas (4). Resistance to CDDP is a major obstacle to effective cancer therapy because clinically relevant resistance levels emerge quickly after treatment. A number of analogues have been synthesized to enlarge the spectrum of activity, overcome resistance and reduce toxicity. Carboplatin (CBDCA) is less nephrotoxic, but its cross-resistance with CDDP limits its application in otherwise CDDP-treatable diseases (5). Nedaplatin (NDP) is a second-generation platinum complex with reduced nephrotoxicity. Phase I and II studies have revealed a spectrum of solid tumor activity similar to that of CDDP (6-8). Oxaliplatin (L-OHP) is a third-generation platinum compound with a 1,2-diaminocyclohexane (DACH) carrier ligand that has a wide spectrum of anticancer activity (9). L-OHP was included in treatment strategies against a variety of cancers including chemoradiation protocols for gastrointestinal, especially rectal cancer (10).

The main mechanism of action of platinum derivatives is the interaction of platinum with DNA, resulting in the inhibition of DNA synthesis (11). Therefore, the cellular kinetics of platinum is considered a relevant factor that determines sensitivity to platinum derivatives. Recent studies have suggested that some copper transporters, such as the uptake transporter hCtr1 and efflux transporters ATP7A and ATP7B, control the cellular kinetics of platinum derivatives, suggesting that elevated expression has been associated with CDDP resistance (12-15). The organic cation transporters, hOCT1, hOCT2, and hOCT3, also affect the cellular kinetics of platinum derivatives (16,17). Moreover, elevated glutathione

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has been associated with acquired resistance to a variety of anticancer agents (18,19).

In the current study, we evaluated and compared the growth inhibitory activity of platinum derivatives in a CDDP-resistant oral squamous cell carcinoma (OSCC) cell line and a parental cell line to clarify the molecular mechanism by which cells acquire platinum resistance.

Materials and methods

Chemicals. CDDP, CBDCA and L-OHP were purchased from Sigma-Aldrich Chemical Co., St. Louis, MO. NDP was purchased from LKT Laboratories, Inc., St. Paul, MN.

Cell lines and cell culture. We used the OSCC-derived cell lines, H-1 and H-1R, described in our previous study (20). H-1 cells were established from a biopsy specimen of moderately differentiated OSCC in the lower gingiva of a Japanese man and cells were grown in Delbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) and 50 U/ml of penicillin and streptomycin. According to our previous methods (21), CDDP-resistant sublines were established by repeated subcultures in the presence of increasing CDDP concentrations. The strongest CDDP-resistant subline is H-1R (20), which is fully resistant to CDDP and can grow exponentially in the presence of 0.5 μ g/ml of CDDP. CDDP-resistant sublines showed no loss of resistance after a 2-month culture in a drug-free medium. Total RNA was obtained from parental cell lines cultured without CDDP, whereas it was obtained from each resistant cell line cultured with 0.5 μ g/ml of CDDP.

Assessment of cell viability by MTT assay. To assess chemosensitivity to CDDP, CBDCA, NDP and L-OHP, we determined the proliferation rates using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Funakaosi, Tokyo, Japan). The cells were seeded in each well of 96-well plates at 2×10^3 cells/well with DMEM containing 10% FBS with various drug concentrations for 72 h. Thereafter, the number of cells was quantified with the MTT cell growth assay kit, as described previously (21). We also tested the sensitivity of H-1 cells and H-1R cells to CDDP; the sensitivity to CDDP did not differ from our previous study (20). Six wells were used for each concentration and the experiment was repeated three times. The 50% inhibitory concentration (IC_{50}) was calculated from the survival curve.

Clonogenicity assay. Cells were exposed to several concentrations of CDDP, CBDCA, NDP and L-OHP (1×10^{-4} – 1×10^{-10} M) for 24 h. The cells were trypsinized and seeded in 60-mm petri dishes at low density (100 cells/dish) for 14 days in an atmosphere containing 7% CO_2 in an incubator at 37°C. The cells were fixed, stained with crystal violet and the colonies counted using an inverted microscope. Colonies containing >50 cells were scored as survivors. When no colonies formed (zero survival), the experiments were repeated with cell numbers that were two to five times higher than previously. Each experiment was performed at least three times; each survival point was calculated from at least

Table I. Sequences of oligonucleotide primers designed for real-time PCR.

Gene	Primers	Size (base pair)
<i>ATP7A</i>	(F) 5'-ACTGCAAGGTGTTTCAGCGAAT-3' (R) 5'-GCTGTGACCCCTTCTGAGGATTT-3'	218
<i>ATP7B</i>	(F) 5'-TGCAAAGTCAGCAACGTGGA-3' (R) 5'-CATCTCGTGGTCTGTCATAGCG-3'	213
<i>hCtr1</i>	(F) 5'-GCACATCATCCAGGTGGTCAT-3' (R) 5'-CAACTTCCCACTGCAATCGAT-3'	205
<i>hOCT1</i>	(F) 5'-TCCTCCCTGTGTGACATAGGTG-3' (R) 5'-GCTTTGCTTTTCTCCCAAGGT-3'	202
<i>hOCT2</i>	(F) 5'-ATAGCAGACAGGTTTGGCCGTA-3' (R) 5'-CCACTGTTCTCCGATATCTCCG-3'	205
<i>hOCT3</i>	(F) 5'-ATGCAGCAGACAGGTATGGCA-3' (R) 5'-ACGATTCCCACAATCCTCCTTT-3'	227
<i>GAPDH</i>	(F) 5'-CATCTCTGCCCCCTCTGCTGA-3' (R) 5'-GGATGACCCTTGCCACAGCCT-3'	305

Table II. Sensitivity of the CDDP-resistant cell lines to platinum drugs.

Cell line	Drug	IC_{50} (μ mol/l)		
		Parent	Resistant	RI ^a
H-1R	CDDP	2.31 \pm 0.07	23.43 \pm 0.75	10.14 ^b
	CBDCA	59.26 \pm 4.18	307.46 \pm 19.02	5.18 ^c
	NDP	5.79 \pm 0.35	53.47 \pm 0.21	9.23 ^b
	L-OHP	1.33 \pm 0.31	35.26 \pm 2.33	26.51 ^b

^aThe resistance index (RI) is calculated as $RI = (IC_{50} \text{ H-1R cells}) / (IC_{50} \text{ H-1 cells})$. ^b $P < 0.01$. ^c $P < 0.05$.

12 single results. Surviving fractions were calculated, each referring to its specific control.

Determination of cellular reduced glutathione content. Total glutathione (GSH) contents in the H-1 and H-1R cells were measured using the total glutathione quantification kit (Dojindo Molecular Technologies, Kumamoto, Japan) according to the manufacturer's instructions. Briefly, dissected lenses were lysed by the addition of 80 μ l of 10 mM HCl and by freezing and thawing twice. Twenty microliters of 5% salicylsalicylic acid was added to the homogenate and the mixture was centrifuged at 8,000 \times g for 10 min. GSH levels in the supernatant were determined according to the manufacturer's protocol by measuring absorbance at 405 nm with a microtiter plate ELISA reader.

Isolation of RNA. Total RNA was extracted from the H-1 and H-1R cells using the FastPure™ RNA kit (Takara Bio, Shiga, Japan). The quality of the total RNA was determined using

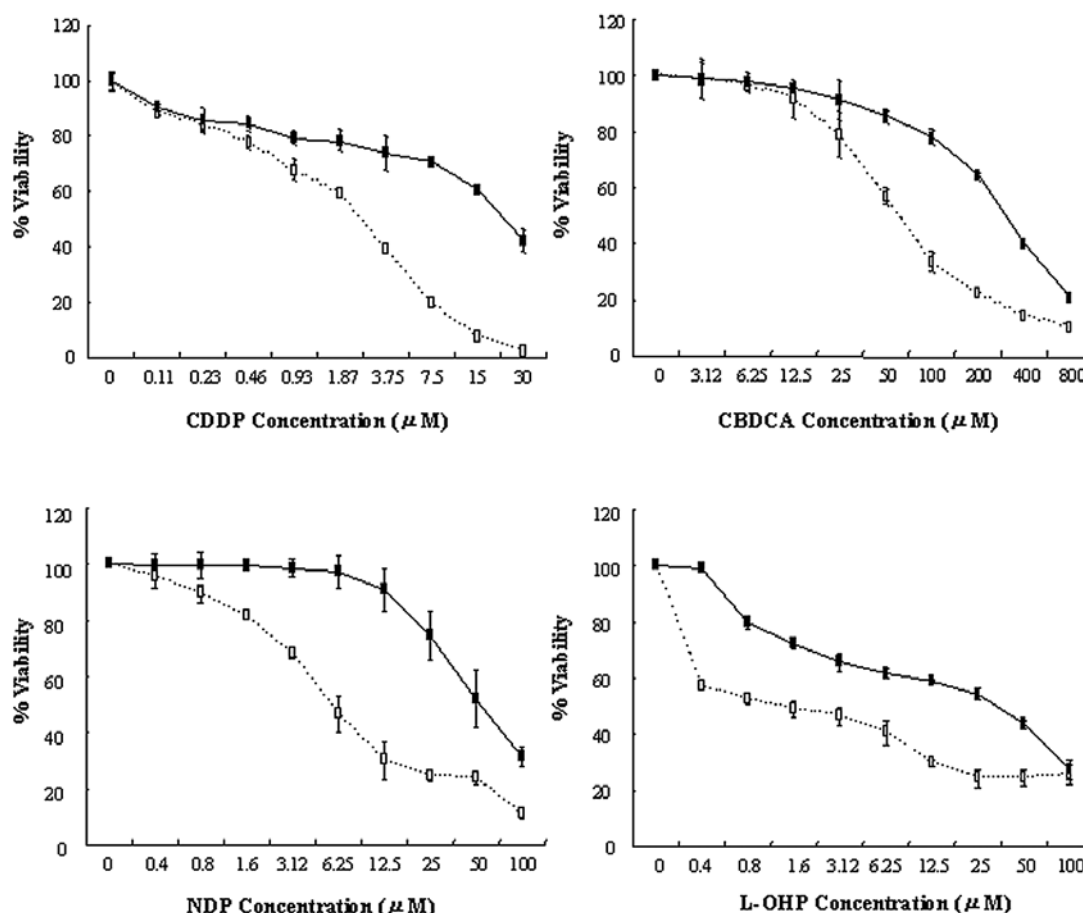


Figure 1. Sensitivity of H-1 and H-1R cells to platinum agents. Solid symbols, resistant sublines and open symbols, parental cells. Viability, expressed as a percentage of the control, was measured 72 h after the platinum drug-containing medium had been replaced with drug-free medium. The data are expressed the mean \pm SD of three independent experiments.

the Bioanalyzer (Agilent Technologies, Palo Alto, CA). The extracted RNA samples were stored separately at -80°C until use.

cDNA preparation. Total RNA was extracted from cells using FastPureTM RNA kit from cells. Five micrograms of total RNA from each sample was reverse transcribed to cDNA using Ready-To-Go You-Primer First-Standard Beads (GE Healthcare, Little Chalfort, Buckinghamshire, UK) and oligo (dT) primer (Sigma Genosys, Ishikari, Japan), according to the manufacturer's protocol.

Analysis of mRNA expression by real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). qRT-PCR was performed to evaluate the expression levels of ATP7A, ATP7B, OCT1, OCT2, OCT3 and hCtr1 mRNA in the H-1 and H-1R cells. qRT-PCR was performed with a single method using a LightCycler FastStart DNA Master SYBR-Green 1 kit (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's procedure. To prepare the standard curve, 5 μg of total RNA from normal oral tissue was reverse-transcribed with Superscript RT (Invitrogen, Life Technologies, Carlsbad, CA, USA) and oligo-d(T)12-18 primer, after which serial dilutions were made corresponding to cDNA transcribed from 300, 30, 3.0 and 0.3 ng of total RNA. The primers were designed using the

Primer Express[®] program (Applied Biosystems, CA, USA). The sequence of specific primers also was checked before use to avoid amplification of genomic DNA or pseudogenes by Primer3 program (available at http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Amplified products were analyzed by 3% agarose gel electrophoresis for size and purity. The sequences are shown in Table I. The PCR reactions using the LightCycler (Roche) apparatus were carried out in a final volume of 20 μl of a reaction mixture comprised of 2 μl of FirstStart DNA Master SYBR-Green I mix, 3 mM MgCl_2 and 0.2 μl of the primers, according to the manufacturer's instructions. The reaction mixture then was loaded into glass capillary tubes and subjected to an initial denaturation at 95°C for 10 min, followed by 45 rounds of amplification at 95°C (10 sec) for denaturation, 63°C (10 sec) for annealing and 72°C for extension, with a temperature slope of $20^{\circ}\text{C}/\text{sec}$, performed in the LightCycler. The transcript amounts for ATP7A, ATP7B, OCT1, OCT2, OCT3 and hCtr1 were estimated from the respective standard curves and normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward 5'-CATCTCTGCCCCCTCTGCTGA-3' and reverse 5'-GGATGACCTTGCCACAGCCT-3') transcript amount determined in corresponding samples.

Statistical analysis. Comparisons were made using the Student's t-test with the assumption of unequal variance and

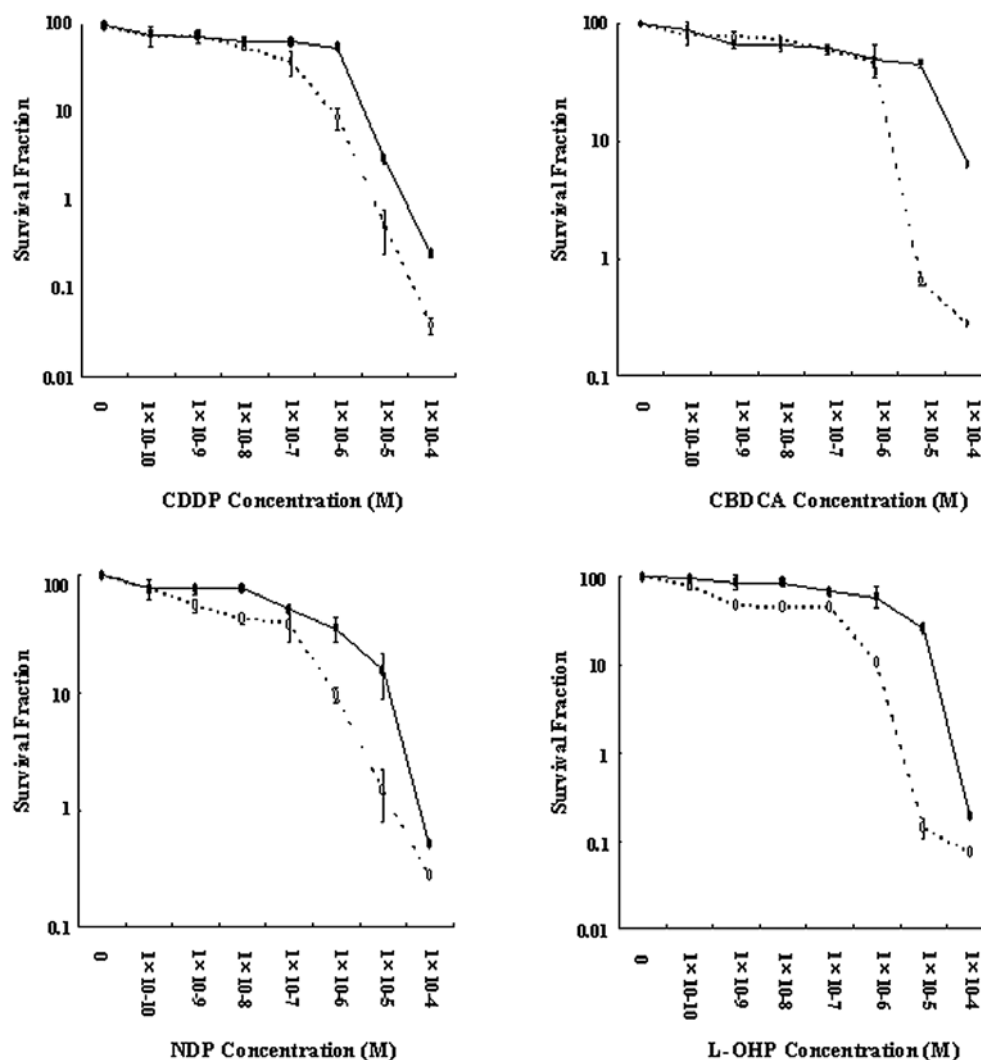


Figure 2. Clonogenicity assay of H-1 and H-1R cells exposed to several platinum agents. Solid symbols, resistant sublines and open symbols, parental cells. Cells were exposed to different concentrations of several platinum drugs for 24 h and clonogenicity assays were performed to estimate the surviving fraction. The data are expressed as the mean \pm SD of three independent experiments.

by determining the 95% confidence interval with an α of 0.05.

Results

Assessment of cellular sensitivity of CDDP-resistant cells to drugs. To evaluate the specificity of the resistance to platinum derivatives, we tested the sensitivity of the CDDP-resistant cells and parental cells to several drugs. Table II shows the IC_{50} and the relative resistance calculated from the ratio of the IC_{50} of the CDDP-resistant cells to those of the parental cells. The IC_{50} values of platinum drugs in resistant cells were all significantly ($P < 0.05$ or $P < 0.01$) higher than those in the parental cells (Table II). Fig. 1 shows cytotoxicity curves using four platinum compounds in the two sets of parental and CDDP-resistant cell lines. H-1R cells were resistant to the selecting platinum agent, with high cross-resistance to L-OHP. The IC_{50} values of both CDDP and CBDCA indicated similar resistance index values close to unity (Table II). With the more sensitive clonogenicity assay, the reduced cell survival induced by platinum drugs was concentration-dependent

(Fig. 2). In parental cells, susceptibility to platinum derivatives increased by an order of magnitude.

Cellular GSH in CDDP-resistant cell lines. Previous studies have shown a correlation between intracellular GSH levels and platinum drugs resistance (22,23). To determine whether H-1R cell resistance resulted from altered GSH levels, cellular GSH was measured in the H-1 and H-1R cells. The GSH level was significantly elevated by 2-fold in the H-1R cells compared with the H-1 cells (Fig. 3).

Expression of copper transporter and organic cation transporter mRNAs. The expression levels of the copper transporters ATP7A, ATP7B and hCtr1 and the organic cation transporters hOCT1, hOCT2 and hOCT3 were examined (Fig. 4). The copper transporter mRNAs were detected in both cell lines and the levels were higher in the CDDP-resistant cell line than in the parental cell line. The mRNAs of hOCT1 and hOCT2 also were higher in the CDDP-resistant cell lines. In contrast, high expression levels of hOCT3 were observed in the parental cell line.

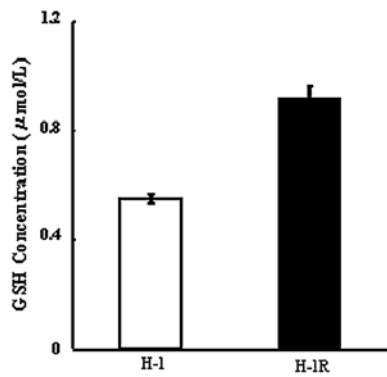


Figure 3. Cellular GSH content in H-1 and H-1R cells. The amount of cellular GSH in the H-1 and H-1R cells was determined using total glutathione quantification kit as described in Materials and methods. The data are expressed as the mean \pm SD of three independent experiments.

Discussion

Platinum analogues are key anticancer agents used in the chemotherapy of solid tumors, such as ovarian (24), advanced bladder carcinoma (25), testicular (26), head and neck (27-29) and lung carcinomas (30).

However, acquired and intrinsic resistance limits application to a relatively narrow range of tumor types. To broaden the anticancer spectrum of CDDP, thousands of structural analogues have been tested. CDDP analogues with two amine ligands, such as CBDCA and NDP, are cross-resistant with CDDP (31). Analogues with different ligands display more diverse activity profiles (32). Notably, L-OHP, with DACH in place of the two amine ligands, exhibits antitumor activity against several cell lines with acquired CDDP resistance as well as clinical tumors that are intrinsically resistant to platinum derivatives (32,33). Although other preclinical studies have suggested that L-OHP was preferentially active in cell lines with very high levels of CDDP resistance and less in cell lines with clinical levels of CDDP resistance, in clinical trials, the response to L-OHP was much lower in patients with CDDP-refractory or resistant cancers compared to platinum-sensitive cancers (34). In the current study, a CDDP-resistant subline, H-1R, exhibited substantial cross-resistance to CBDCA, NDP, and L-OHP, indicating that at least some of the mechanisms engaged to protect H-1R cells against H-1 cells also provides protection against these other drugs.

Previous studies have shown that expression of ATP7A, ATP7B and hCtr1 can render cells resistant to platinum

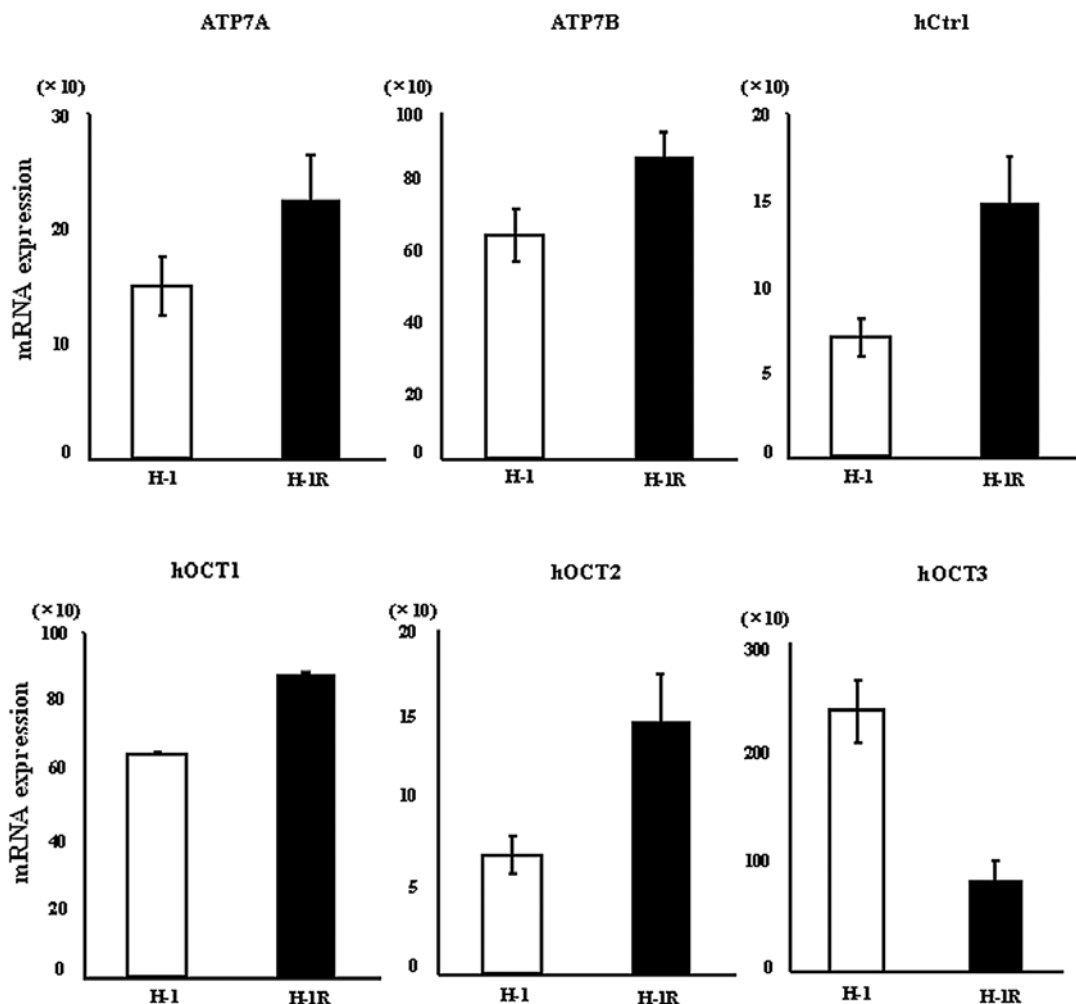


Figure 4. Levels of copper and organic cation transporter mRNAs in H-1 and H-1R cells. GAPDH is amplified as an internal control. The data are expressed as the mean \pm SD of three independent experiments.

agents (12-15). Thus, it was of interest to determine whether H-1R cells had altered expression of these copper transporters. Real-time PCR showed up-regulated expression of copper transporter genes (ATP7A, ATP7B and hCtr1) and confirmed part of the mechanism of platinum drug resistance in H-1R cells (Fig. 4). To study other mechanisms of drug resistance, such as organic cation transporters (hOCT1, hOCT2 and hOCT3), which likely play an important role in anticancer drug resistance (35), the expression of hOCT1 and hOCT2 mRNA was up-regulated in H-1R cells compared with H-1 cells. However, H-1R cells exhibited lower hOCT3 mRNA expression levels than the parental cell line.

Our data suggested that the expression of hOCT1 and hOCT2 may play a role in platinum resistance. Increased intracellular GSH had been associated previously with platinum resistance (23,36). We also determined GSH content in resistant and parental cell lines. The results showed that the level of GSH in H-1R cells was 2-fold higher than in parental H-1 cells (Fig. 3), suggesting that modulation of platinum resistance in H-1R cells may occur through pathways relevant to GSH content.

In conclusion, our study showed that the mechanisms responsible for platinum resistance in H-1R cells are overexpression of the copper transporters (ATP7A, ATP7B and hCtr1) and organic cation transporters genes (hOCT1 and hOCT2). In addition, GSH levels also contribute to platinum resistance in H-1R cells. H-1 and H-1R cells are useful for elucidating the candidates responsible for platinum resistance. Collectively, these findings could pave the way for further efforts to elucidate platinum resistance.

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