Mefenamic acid enhances anticancer drug sensitivity via inhibition of aldo-keto reductase 1C enzyme activity

MASASHI SHIIBA[1,2], HITOMI YAMAGAMI[1], AYUMI YAMAMOTO[1], YASUYUKI MINAKAWA[1], ATSUSHI OKAMOTO[1], ATSUSHI KASAMATSU[1], YOSUKE SAKAMOTO[1], KATSUHIRO UZAWA[1], YUICHI TAKIGUCHI[2] and HIDEKI TANZAWA[1]

Departments of [1]Oral Science, [2]Medical Oncology, Graduate School of Medicine,
Chiba University, Chuo-ku, Chiba 260-8670, Japan

Abstract. Resistance to anticancer medications often leads to poor outcomes. The present study explored an effective approach for enhancing chemotherapy targeted against human cancer cells. Real-time quantitative real-time polymerase chain reaction (qRT-PCR) analysis revealed overexpression of members of aldo-keto reductase (AKR) 1C family, AKR1C1, AKR1C2, AKR1C3, and AKR1C4, in cisplatin, cis-diamminedichloroplatinum (II) (CDDP)-resistant human cancer cell lines, HeLa (cervical cancer cells) and Sa3 (oral squamous cell carcinoma cells). The genes were downregulated using small-interfering RNA (siRNA) transfection, and the sensitivity to CDDP or 5-fluorouracil (5-FU) was investigated. When the genes were knocked down, sensitivity to CDDP and 5-FU was restored. Furthermore, we found that administration of mefenamic acid, a widely used non-steroidal anti-inflammatory drug (NSAID) and a known inhibitor of AKR1Cs, enhanced sensitivity to CDDP and 5-FU. The present study suggests that AKR1C family is closely associated with drug resistance to CDDP and 5-FU, and mefenamic acid enhances their sensitivity through its inhibitory activity in drug-resistant human cancer cells. Thus, the use of mefenamic acid to control biological function of AKR1C may lead to effective clinical outcomes by overcoming anticancer drug resistance.

Introduction

Resistance to chemotherapy is a major issue in the treatment of cancer. Cancer cells exhibit intrinsic and acquired resistance to anticancer agents, both resulting from various genetic and epigenetic changes in the cells (1). Recent studies have revealed that resistance to cancer chemotherapy occurs not only with conventional chemotherapy, but also with targeted therapies such as gefitinib (2) and imatinib (3). Thus, it is crucial to identify agents that regulate chemotherapy resistance to promote effective clinical outcomes. Cisplatin, cis-diamminedichloroplatinum (II) (CDDP), is a vital anticancer agent commonly used in chemotherapy for various human cancers. Previous studies have revealed that CDDP resistance is correlated with reduced drug accumulation in the cells (4,5), increased DNA repair 6), higher level of intracellular thiols such as glutathione and metallothioneins (7,8), and anti-apoptotic activity (9). These findings suggest that a series of events contribute to acquired CDDP resistance.

In the present study, we focused on members of aldo-keto reductase (AKR) 1C family, AKR1C1, AKR1C2, AKR1C3, and AKR1C4, as putative genes, which may be associated with CDDP resistance. AKR1C is one of the AKR superfamily members and has 4 isoforms; AKR1C1, AKR1C2, AKR1C3, and AKR1C4. These are mapped on chromosome 10p15-14 and share high sequence homology with each other (10). These enzymes catalyze steroids (11), prostaglandins (12), and lipid aldehydes (13); however, altered expression profiles of AKR1C family members have been reported in some malignant tumors. Upregulated AKR1C3 expression has been demonstrated in breast cancer (14), prostate cancer (15), adenocarcinoma and squamous cell carcinoma of the lung (16), and squamous cell carcinoma of the head and neck (17). Previous studies suggested that overexpression of AKR1C1 and AKR1C2 was closely associated with platinum drug resistance in human cancers (18,19). Thus, it is reasonable to conclude that AKR1Cs may regulate chemotherapy resistance to anticancer agents and that controlling AKR1C activity using its inhibitors may lead to favorable therapeutic outcomes. The present study aimed to determine an approach that suppresses the mechanism of anticancer drug resistance by controlling AKR1C enzyme activities.

Materials and methods

Cell lines and cell culture. The cervical cancer cell line, HeLa and the OSCC-derived cell line, Sa3 were used in the present study. CDDP-resistant cells established from these cell lines,

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Correspondence to: Dr Masashi Shiiba, Department of Oral Science, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan
E-mail: m.shiiba@faculty.chiba-u.jp

HeLa-R and Sa3-R, were used (20). Cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and 50 U/ml of penicillin and streptomycin.

Ethics statement. The present study was approved by the Ethics Committee of the Graduate School of Medicine, Chiba University (approval number, 236) and performed according to the tenets of the Declaration of Helsinki. All the animal experiments were performed in accordance with the ethical standards of Canadian Council on Animal Care (CCAC) and institutional guidelines.

RNA extraction and reverse transcription. Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The quality of the total RNA was determined using the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). cDNA was synthesized from total RNA using Ready-to-Go You-Prime first-strand beads (GE Healthcare, Buckinghamshire, UK) and oligo (dT) primer (Sigma Genosys, Ishikari, Japan) according to the manufacturer's protocol.

Real-time qRT-PCR analysis. Real-time qRT-PCR was performed using LightCycler® 480 Probes Master kit (Roche Diagnostics GmbH, Mannheim, Germany) according to manufacturer's instructions. PCR reactions were performed in the Light Cycler (Roche Diagnostics GmbH) apparatus. Transcript amount was estimated using standard curves and normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts determined in corresponding samples. Nucleotide sequences of the specific primers for *AKR1C1, AKR1C2, AKR1C3, AKR1C4,* and *GAPDH* are shown in Table I.

Small-interfering RNA and transfection reagents. SMARTpool siRNA targeting AKR1Cs (siAKR1C1, siAKR1C2, siAKR1C3, and siAKR1C4) (Dharmacon, Lafayette, CO, USA) were used in gene silencing. Vehicle control and siCONTROL nontargeting siRNA pool (D-001206-13-20; siNT) were used as negative controls. cyclophilin B (siCONTROL cyclophilin B; siCyclo) was used as a positive silencing control to ascertain the transfection efficiency in each experiment. Cells were transfected with siRNAs using DharmaFECT 1 siRNA transfection reagent (Dharmacon).

3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assay (MTS assay). Cells (2000 cells/well) were seeded in triplicate in a 96-well plate and cultured for 72 h. MTS reagent (Promega) was then added and incubated for 4 h, and relative cell viability was determined by recording the absorbance at 490 nm. Assays were performed with or without anticancer agent [CDDP, 5-fluorouracil (5-FU)] or non-steroidal anti-inflammatory drugs (NSAIDs). The experiment was repeated 3 times.

Western blotting analysis. Expression of AKR1C family proteins was detected using western blot analysis. Cells were pelleted and resuspended to a concentration of 10^8 cells/ml in lysis buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.4, containing 1% Triton X-100, 10% glycerol, 5 mM EDTA, 10 mM NaF, 1 mM sodium orthovanadate, 100 U/ml
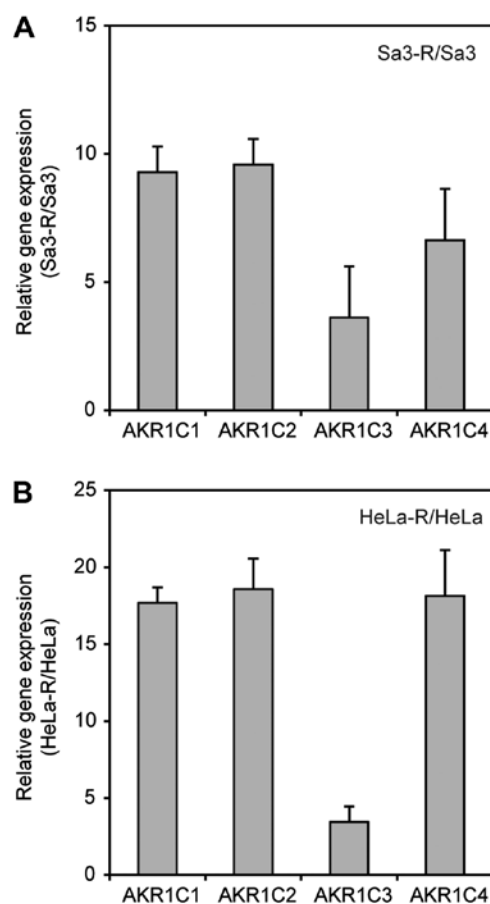

Figure 1. mRNA expression of AKR1Cs in human cancer cell lines as determined by real-time qRT-PCR. mRNA expression levels of AKR1Cs in human cancer cells were validated by real-time qRT-PCR analysis. Average values of relative expression (resistant vs. parent cells) in Sa3 and Sa3-R (A), HeLa and HeLa-R (B) are shown. Error bars represent standard deviation.

aprotinin, 10 mM iodoacetamide, and 25 µg/ml p-nitrophenyl-p'-guanidinobenzoate) with a cocktail of proteinase inhibitors (Roche Diagnostics GmbH). After centrifugation at 15,000 x g for 20 min, supernatants (cell lysates) were collected and subjected to SDS-PAGE (4-12%) and transferred to nitrocellulose membranes (Invitrogen). After blocking with Blocking One (Nacalai Tesque, Kyoto, Japan), the membrane was incubated with each antibody against AKR1C1, AKR1C2, AKR1C3, AKR1C4 (Sigma-Aldrich, St. Louis, MO, USA), respectively. Then, the membrane was incubated with the horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibody (Promega, Madison, WI, USA). The signals were detected using SuperSignal West Pico Chemiluminescent substrate (Thermo) and were visualized using ATTO Light-Capture II (Atto, Tokyo, Japan). Protein expression profiles of AKR1Cs were normalized with the internal control, β-actin.

Xenograft. HeLa and HeLa-R cells were used in the xenograft experiment. Cells (1×10^7) were dissolved in 200 µl of phosphate buffered saline (PBS) and injected subcutaneously into the backs of 6-week-old female athymic nude mice; BALB/cAnNcrj-nu/nu (Charles River Japan Inc., Kanagawa, Japan). When the transplanted tumor volume reached 100 mm³, the mice were divided into 4 groups; control (n=5), anticancer agent (CDDP or 5-FU) alone (n=5), mefenamic acid alone
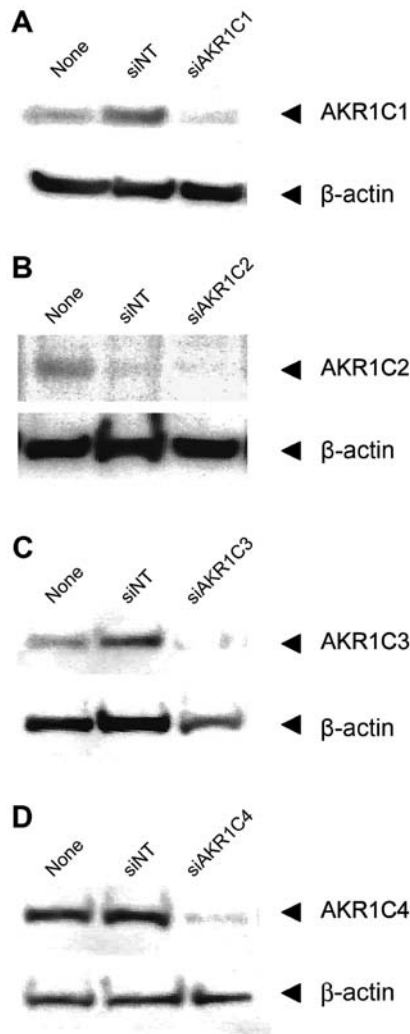
Figure 2. Validation of decreased AKR1Cs protein expression induced by siRNA transfection. the AKR1C protein expression was examined by western blot analysis using anti-AKR1C antibodies and anti-β-actin antibody in HeLa-R cells 120 h after transfection with siRNAs. AKR1C1 (A), AKR1C2 (B), AKR1C3 (C) and AKR1C4 (D) protein levels reduced significantly compared to the siNT-transfected cells.
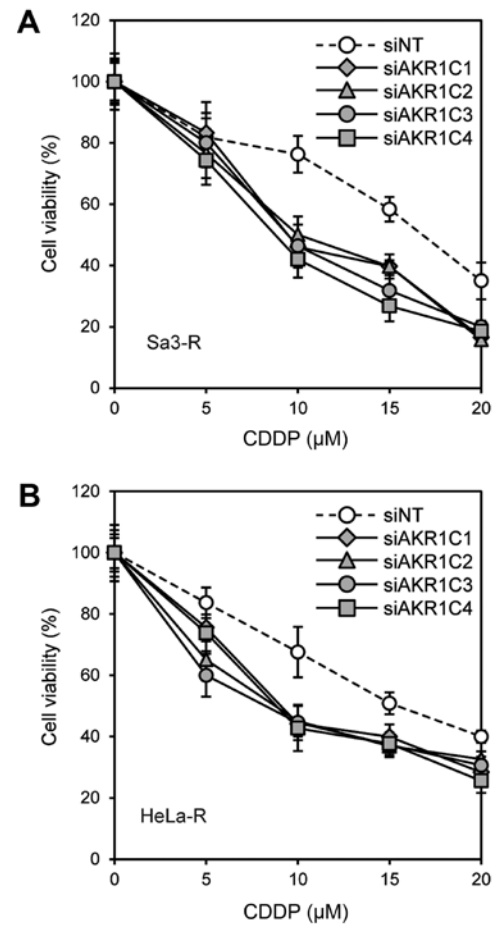

Figure 3. Effect of AKR1C gene silencing on CDDP sensitivity in human cancer cell lines. AKR1Cs were knocked down in Sa-3R (A) and HeLa-R cells (B), and sensitivity to CDDP was determined by MTS assay. Cells treated with non-targeting gene (siNT) were used as a control. Averages of 5 samples are shown. Error bars represent standard deviation.

(n=5), and anticancer agent plus mefenamic acid (n=5). CDDP (2 mg/kg) was administered intraperitoneally once weekly for 4 weeks. 5-FU (7.5 mg/kg) was administered intraperitoneally five times a week for 3 weeks. The freeze-dried diet containing 0.0125% of mefenamic acid was prepared and fed to the mice. In addition to tumor volume, body weight of mice was monitored throughout the experiment period. Six weeks after medication was initiated, tumor tissues were resected and analyzed.

Statistical analysis. The average values and standard deviation were analysed, and P-value was calculated using two-tailed, Student's t-test in MTS assay and in xenograft experiment. For all tests, α-level was 5% and the criterion for statistical significance was $P < 0.05$.

Results

Characterization of CDDP-resistant cell lines. The cervical cancer cell line, HeLa (Japanese Collection of Research Biosources Cell Bank, Osaka, Japan) and the OSCC-derived cell line, Sa3 (RIKEN BioResource Center, Ibaraki, Japan) were used in the present study. Moreover, CDDP-resistant cells established from these cell lines, HeLa-R and Sa3-R, were used (20). The sensitivity of these cells to various concentrations of CDDP was determined using MTS assay. Sa3-R and HeLa-R cells showed significantly higher viable cell rates than the parent clones with the same concentration of CDDP. The 50% inhibitory concentration (IC_{50}) values (μM) in the Sa3, Sa3-R, HeLa, and HeLa-R cells were 1.4, 18.4, 1.0, and 12.9, respectively. CDDP-resistant cell lines also showed drug resistance to 5-FU, suggesting that the cell lines potentially had cross-resistance to anticancer reagents. The IC_{50} values ($\mu g/ml$) in the Sa3, Sa3-R, HeLa, and HeLa-R cells were 1.1, 38.3, 51.5, and 212.6, respectively. No morphologic difference was observed between CDDP-resistant cell lines and the parent cell lines. The CDDP-resistant cells as well as the parent cell lines had proliferative ability.

Analysis of gene expression in CDDP-resistant cells. Real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis revealed that *aldo-keto reductase (AKR) 1C1, AKR1C2, AKR1C3*, and *AKR1C4* were significantly upregulated in the CDDP-resistant cell lines (Fig. 1).
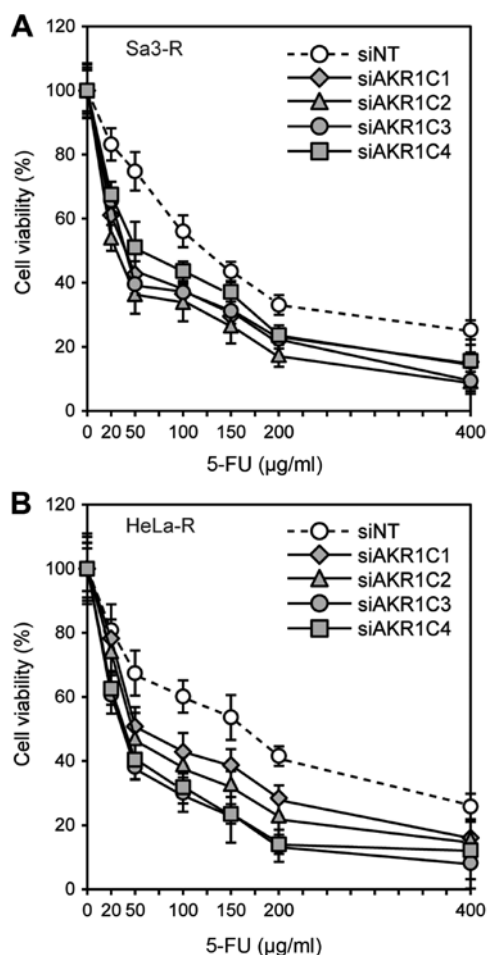
Figure 4. Effect of AKR1C gene silencing on 5-FU sensitivity in human cancer cell lines. AKR1Cs were knocked down in Sa-3R (A) and HeLa-R cells (B), and sensitivity to 5-FU was determined by MTS assay. Cells treated with non-targeting gene (siNT) were used as a control. Averages of 5 samples are shown. Error bars represent standard deviation.
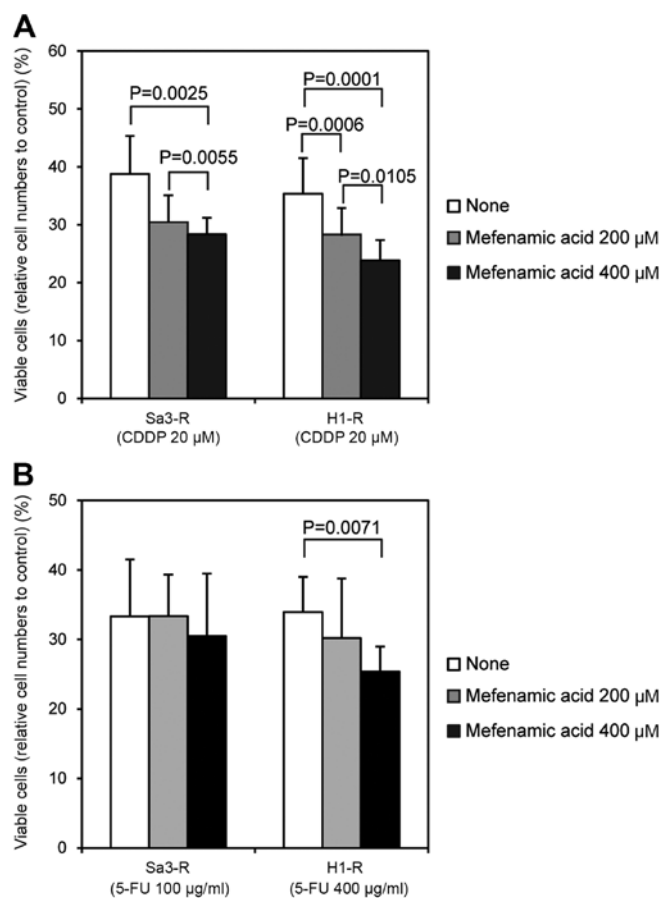

Figure 5. Effect of mefenamic acid on chemosensitivity in cancer cell lines. Mefenamic acid was added to Sa3-R and HeLa-R cells cultured with CDDP (A) or 5-FU (B), and relative cell viability was compared. Mefenamic acid induced dose-dependent and greater chemosensitivity. Averages of 5 samples are shown. Error bars represent standard deviation.

Table I. Specific primers used in real-time qRT-PCR.

Gene	Forward	Reverse
AKR1C1	5'-catgcctgtcctgggattt-3'	5'-agaatcaatatggcggaagc-3'
AKR1C2	5'-ttccatacagaaacttcttttccac-3'	5'-ggttaaccaatggcatgtga-3'
AKR1C3	5'-cattggggtgtcaaacttca-3'	5'-ccggttgaaatacggatgac-3'
AKR1C4	5'-tcggggtgtcaaacttcaa-3'	5'-gctctggttgaggtaaggatga-3'
GAPDH	5'-catctctgccccctctgctga-3'	5'-ggatgaccttgcccacagcct-3'

Data are expressed as the average ± standard deviation (SD) of two independent experiments with samples in triplicate. The results indicated that AKR1C family members were associated with resistance to CDDP; thus, the AKR1C family was subjected to further investigation.

Functional analysis in small-interfering RNA (siRNA)-transfected cells. To determine whether AKR1C gene silencing contributes to CDDP sensitivity, cells were transfected with siRNAs. The AKR1C protein expression was examined by western blot analysis in Sa3-R and HeLa-R cells 120 h after transfection with siRNAs. AKR1C protein levels in non-targeting siRNA (siNT)-transfected cells were comparable to those of AKR1Cs in non-treated cells. AKR1C protein levels reduced significantly compared to the siNT-transfected cells (Fig. 2). These transfected cells were subjected to functional analysis to determine the effect of AKR1C gene silencing on CDDP sensitivity. The sensitivities of these cells to CDDP were determined by MTS assay. Increased sensitivity to CDDP and 5-FU was observed in Sa3-R cells transfected with siAKR1C1, siAKR1C2, siAKR1C3, and siAKR1C4, compared to that observed in the corresponding cells treated with siNT (Figs. 3 and 4).

AKR1C inhibitor-induced enhancement of CDDP sensitivity in vitro. Since mefenamic acid is an effective inhibitor of AKR1Cs (21), mefenamic acid was added, and the alteration of CDDP sensitivity in tumor cell lines was determined by MTS assay. CDDP-resistant cells treated with mefenamic acid (Sa3-R and HeLa-R) demonstrated restored sensitivity to CDDP and 5-FU in a dose-dependent manner (Fig. 5). As a result, mefenamic acid was used for further experiments *in vivo*.

Effect of mefenamic acid on CDDP sensitivity in vivo. Mouse tumor xenografts were established to investigate whether an AKR1C inhibitor enhances sensitivity to CDDP. HeLa and
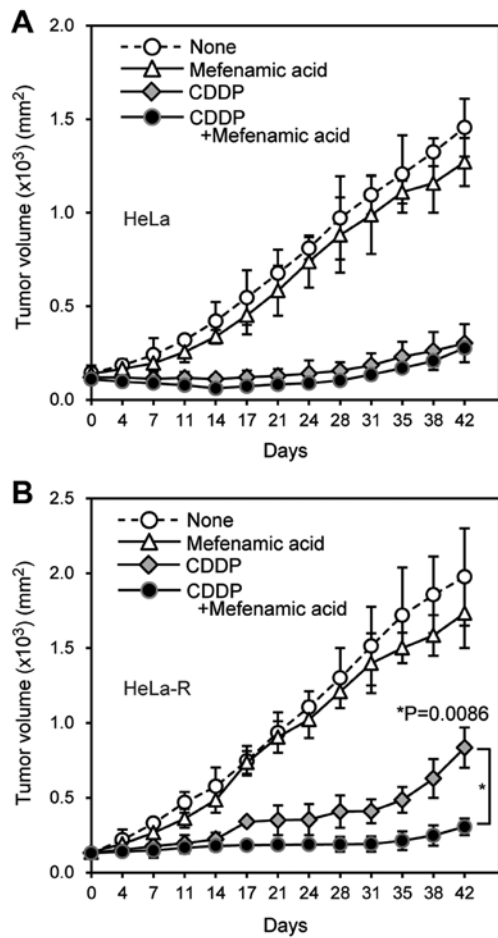
Figure 6. Effect of mefenamic acid on chemosensitivity in xenograft. Tumor volume was monitored after HeLa (A) or HeLa-R (B) cells were transplanted in mice. In HeLa-R transplanted mice, sensitivity to CDDP significantly increased when mefenamic acid was administered (P=0.0086, Student's t-test). Averages of 5 samples are shown. Error bars represent standard deviation.
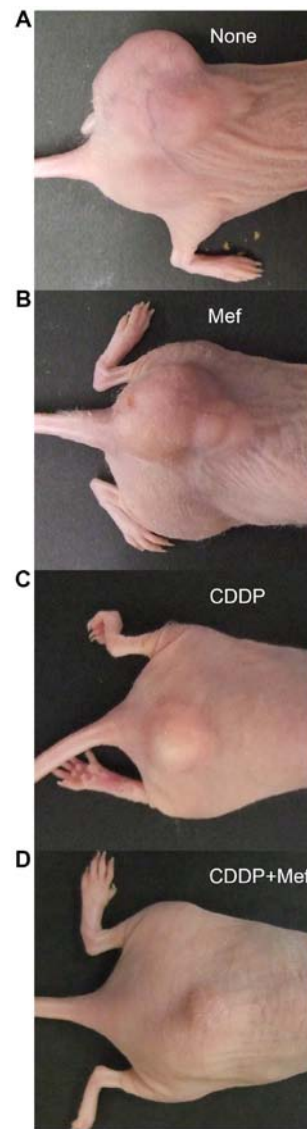

Figure 7. Tumor xenografts in mice. Images of HeLa-R transplanted mice were taken 4 weeks after transplantation. Representative data in 4 experimental groups; none (A), mefenamic acid (B), CDDP (C), CDDP plus mefenamic acid (D) are shown.

HeLa-R cells were subcutaneously implanted into female athymic nude mice, and the tumor volume was measured regularly until day 42. When CDDP was administered, tumor growth was suppressed in HeLa cell transplanted mice, however, tumor volume gradually increased in HeLa-R cell transplanted mice probably due to the CDDP-resistant characteristic (Fig. 6). The increased tumor volume significantly reduced in HeLa-R cell xenografts when mefenamic acid was added to CDDP (Fig. 7). Similarly, in the 5-FU administered mice, tumor volume in the mice treated with mefenamic acid was significantly smaller than without it (Fig. 8). Body weights of the mice were also measured to examine the systemic effect induced by these chemical reagents. The data revealed that the systemic effects observed after combined administration of mefenamic acid and anticancer agents were not different from that observed with individual administration (Figs. 9 and 10).

Discussion

In the present study, we identified AKR1C1, AKR1C2, AKR1C3, and AKR1C4 as putative genes, which may be associated with anticancer drug resistance. Knockdown of AKR1Cs genes apparently increased the cytotoxic effect of CDDP in CDDP-resistant cells, suggesting that inhibition of AKR1Cs' activity can induce enhanced CDDP sensitivity. Furthermore, administration of mefenamic acid, a known inhibitor of AKR1C, increased sensitivity to CDDP and 5-FU, *in vitro* and *in vivo* without mefenamic acid-induced adverse effects.

The causes of resistance to anticancer medications are multifactorial and involve numerous genetic and epigenetic changes (14,18,22). Cisplatin is converted to its active form by intracellular aquation of one of two chloride-leaving groups and covalently binding to purine DNA base leading to the formation of intrastrand or interstrand crosslink adducts, which lead to cellular apoptosis (23-25). This process of cisplatin activation may be inhibited by the enzyme activity of AKR1C; however, the mechanism by which this occurs is still unknown. Wang *et al* (18) demonstrated that interleukin-6, a pro-inflammatory cytokine, is crucial for overexpression of AKR1C1 and AKR1C2 and for resistance to anticancer drugs in NSCLC cells. Matsunaga *et al* reported that knockdown of
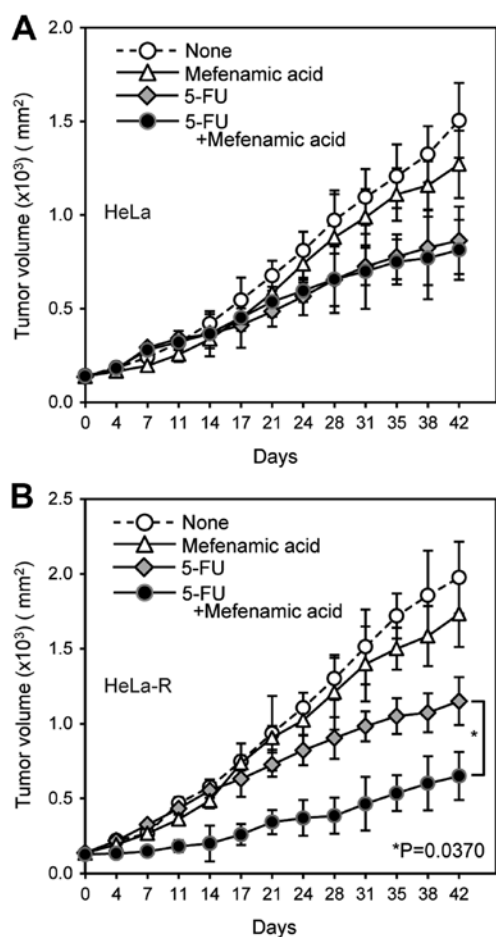
Figure 8. Effect of mefenamic acid on chemosensitivity in xenograft. Tumor volume was monitored after HeLa (A) or HeLa-R (B) cells were transplanted in mice. In HeLa-R transplanted mice, sensitivity to 5-FU significantly increased when mefenamic acid was administered (P=0.0370, Student's t-test). Averages of 5 samples are shown. Error bars represent standard deviation.

Figure 9. Monitoring of body weight of mice administered with CDDP and mefenamic acid. Body weight was monitored at various time points in mice transplanted with HeLa (A) or HeLa-R (B) cells. Averages of 5 samples are shown in the graph. Error bars represent standard deviation. Significant differences were not observed even after drugs were administered.

AKR1C1 and AKR1C3 and the use of their specific inhibitors improved sensitivity to CDDP in human colon cancer cells, and suggested that the underlying mechanism for CDDP resistance is most likely due to aldehyde detoxification, resulting from enhanced oxidative stress (26). Moreover, blockade of proteasome leads to a compensatory upregulation of AKR1C1 and AKR1C3 in CDDP-resistant cells (26). Novotna *et al* reported that human AKR1C3 might mediate deactivation of the anticancer drugs, oracin and doxorubicin, via carbonyl reduction in hormone-dependent malignancies such as prostate and breast cancers (27).

Previous studies suggested that formation of reactive oxygen species (ROS) induces the CDDP toxicity (24,28,29). Ebert *et al* reported that some proteasome inhibitors produce mild oxidative stress, which activates nuclear factor-erythroid 2 related factor 2 (Nrf2)-related genes leading to AKR1C induction, suggesting that proteasome inhibitors may elicit a protective effect (30).

Noteworthy, the present study revealed that overexpression of AKR1Cs is also associated with 5-FU drug resistance. 5-FU has been used clinically since the late 1950s for the treatment of various cancers (31). Both CDDP and 5-FU have specific pharmacokinetics; the mech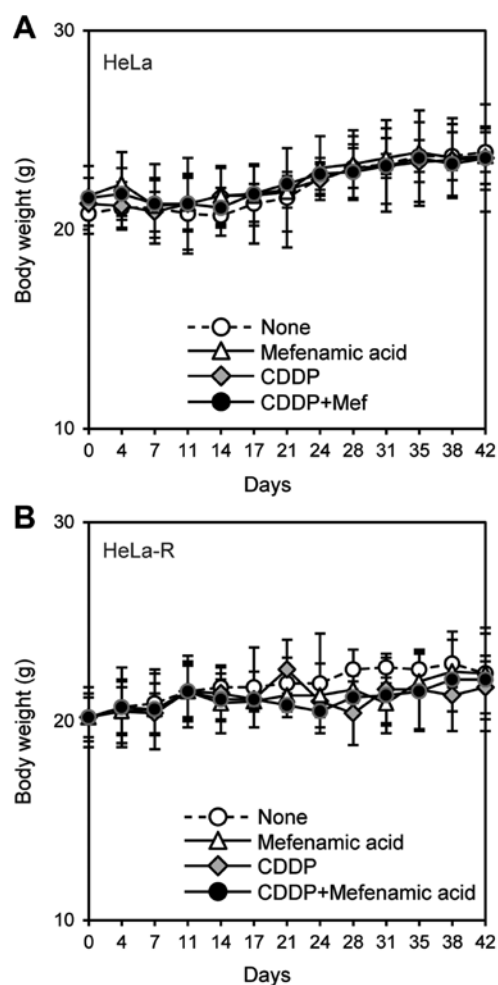anism of CDDP involves covalent binding to purine DNA bases, which primarily leads to cellular apoptosis (22), whereas 5-FU is enzymatically converted to the main active metabolites fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP), and fluorouridine triphosphate (FUTP). FUTP and FdUTP lead to RNA and DNA damage, respectively, and FdUTP induces DNA damage via inhibition of thymidylate synthase (TS) (32). This suggests that AKR1Cs may have various roles in anticancer drug resistance mechanisms, such as drug uptake, DNA-damage recognition and repair, and apoptosis.

Byrns *et al* reported that NSAIDs, sulindac, mefenamic acid, arylpropionic acids, and indomethacin, inhibit AKR1C enzyme activity (21,33). We investigated the inhibitory activity of these NSAIDs and determined mefenamic acid to be the most effective drug for this purpose, and we could not find the significant altered expression of AKR1Cs induced by mefenamic acid (data not shown). Both drug safety and effectiveness are equally important parameters for clinical use of a drug. Mefenamic acid has long been used as a medicinal agent and is deemed safe. Thus, the use of mefenamic acid as an AKR1C inhibitor to enhance the effect of chemotherapy is plausible.
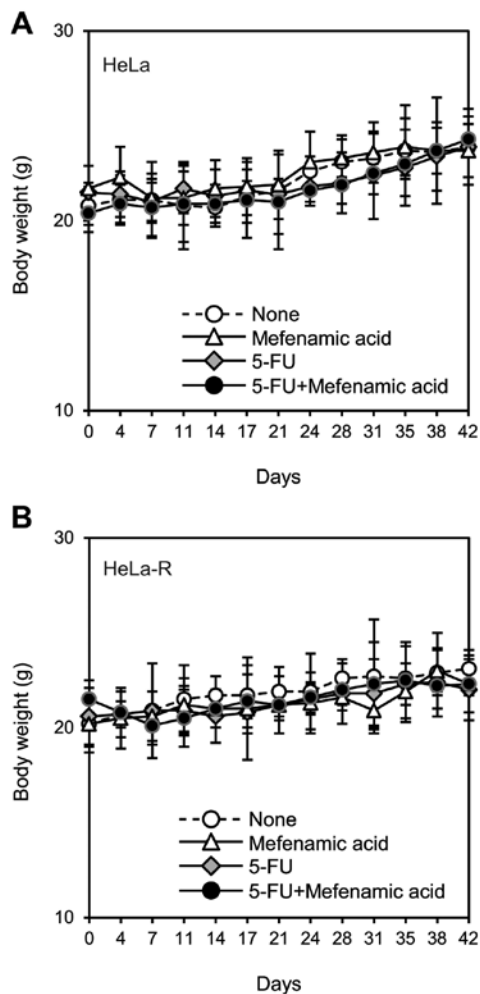
Figure 10. Monitoring of body weight of mice administered with 5-FU and mefenamic acid. Body weight was monitored at various time points in mice transplanted with HeLa (A) or HeLa-R (B) cells. Averages of 5 samples are shown in the graph. Error bars represent standard deviation. Significant differences were not observed even after drugs were administered.

In conclusion, the present study suggests that AKR1C1, AKR1C2, AKR1C3, and AKR1C4 are closely associated with drug resistance to both CDDP and 5FU, and that mefenamic acid, an inhibitor of AKR1C, restores sensitivity through inhibition of drug-resistance in human cancer cells. This implies that inhibition of the AKR1C biological function may lead to an effective clinical outcome by either overcoming anticancer drug resistance or reducing adverse effects of concomitant medications.

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