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Enhanced transformation and chemosensitivity of NIH3T3 cells transduced with hepatoma up-regulated protein

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Abstract

Hepatoma up-regulated protein (HURP) is a recently identified novel cell-cycle-regulated gene. The HURP gene is overexpressed in human hepatocellular carcinoma and transitional cell carcinoma. The cellular function of HURP is not fully understood. In this study, the NIH3T3 cells transduced with the exogenous HURP gene manifested the general characteristics of tumor cells, which had higher growth rate in low-serum media and advanced ability of colony formation on agarose-based plates. Transduced HURP was capable of specifically enhancing the chemosensitivity of deoxycytosine analogs, such as gemcitabine, ARA-C, and 5-AZA-CdR, but neither had an effect on the response of DNA intercalating agents, such as cisplatin, carboplatin, and doxorubicin, nor on the response of microtubule stabilizers, such as paclitaxel, docetaxel, and vinblastine. These results indicate that the HURP gene might be a potential oncogenic gene and capable of enhancing the chemosensitivity of deoxycytosine analogs in NIH3T3 cells.

Keywords: Transformation; Chemosensitivity; HURP; Anticancer drugs

Cancer is the result of an accumulation of multiple molecular alterations in the same cell or its descendents [1–3]. Alterations in two groups of genes, proto-oncogenes and tumor suppressor genes, are particularly important for this process [4,5]. During the past decade, the number of proto-oncogenes and tumor suppressor genes has grown continuously. In spite of the sizable number of genes already described, new genes with oncogenic potential or tumor suppressing activity are still being identified.

Recently, a novel cell-cycle-regulated gene, hepatoma up-regulated protein (HURP) that was overexpressed in human hepatocellular carcinoma, was identified (GenBank Accession No. AB076695). HURP was localized to the spindle poles during mitosis, and the endogenous levels of HURP mRNA were elevated in the G2/M phase of synchronized HeLa cells and in regenerating mouse liver after partial hepatectomy [6]. We found that HURP positivity was specifically detected in nearly 90% of transitional cell carcinoma (TCC) samples, but not in benign urological diseases, and a higher level of tissue HURP was found in those TCC patients with recurrence[7,8].

The cellular function of the HURP gene has remained unclear up to now. In order to provide further understanding of the underlying molecular mechanism of the HURP function, we explored the effect of HURP gene expression in murine fibroblasts. In this study, NIH3T3 cells were transduced with the HURP gene, the stable clones NIH3T3-1-3 and NIH3T3-3-3 were selected, and

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their two characteristics as described below were compared with those of mock transfectants and parental NIH3T3 cells. The two characteristics were: one, the growth rate of cells seeding a low-serum medium and soft agar, and the other, the IC_{50} values (the drug sensitivity in terms of 50% inhibitory concentration) of three kinds of chemotherapeutic agents: (1) microtubule stabilizers, such as paclitaxel, docetaxel, and vinblastine, (2) DNA intercalating agents, such as carboplatin, cisplatin, and doxorubicin, and (3) deoxycytidine analogs, such as 2′, 2′-difluorodeoxycytidine (dFdC, gemcitabine), cytosine arabinoside (ARA-C), and 5-aza-2′-deoxycytidine (5-AZA-CdR) were determined.

Materials and methods

Cell lines and drug preparation. All cell lines were obtained from the American Type Culture Collection (Manassas, MD). Mouse NIH3T3 fibroblasts were propagated and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Gaithersburg, MD), and the RT4 and T24 cell lines were maintained in McCoy's 5a medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco BRL), 100 IU penicillin/ml, and 100 μg streptomycin/ml (Gibco BRL) at 37 °C in a 5% CO_2 incubator. The transfected clones were selected and maintained in the same medium containing 900 μg/ml geneticin (G418-sulfate, Gibco BRL). Gemcitabine (Gemzar, provided by Eli Lilly, Indianapolis, Indiana), doxorubicin (Adriamycin, provided by Pfizer Pharmaceuticals, Ann Arbor, MI), carboplatin, ARA-C, and 5-AZA-CdR (purchased from Sigma Chemical, St. Louis, MO) were prepared in water. Docetaxel (Taxotere, provided by Aventis Pharma S.A., France), paclitaxel (Sigma), and vinblastine (Sigma) were prepared in DMSO. Cisplatin (Sigma) was prepared in DMF.

Transfection into NIH3T3 cells and clone selection. One hundred thousand NIH3T3 cells in DMEM supplemented with 10% FCS were plated in 60-mm culture dishes and grown at 37 °C in a 5% CO_2 atmosphere until they reached about 60% confluence. Then, the cells were washed with serum-free DMEM and overlaid with 2 ml serum-free OPTI-MEM medium containing 10 μl lipofectAMINE (Life Technologies, Gaithersburg, MD) and 2 μg pHURP-NHA. After 5 h incubation at 37 °C, the medium was replaced with 4 ml DMEM supplemented with 10% FCS, and the incubation was continued for an additional 48 h. Then, the medium was replaced with 10% FCS-DMEM containing 900 μg/ml G418, which was changed every 3–4 days. After 3 weeks, neomycin resistant clones were picked up and transferred separately into dishes containing the selection medium with 900 μg/ml G418. Several NIH3T3 clones transfected with HURP cDNA were established and analyzed for HURP expression by Western blots. NIH3T3-1-3 and NIH3T3-3-3 were chosen as the HURP-transduced clones, and NIH3T3-1-5 and NIH3T3-2-3 were chosen as the mock transfectants.

RNA isolation. An RNA isolation system (Biotecx Laboratories, Houston, Texas) was used as previously described [7]. The content of the RNA was assessed (1 OD of A_{260} equal to 40 μg/ml RNA) spectrophotometrically, and the purity of extraction was assessed using the $A_{260/280}$ ratio, which in all cases was above 1.7.

Reverse-transcription polymerase chain reaction (RT-PCR). Single-strand complementary DNA (cDNA) was synthesized using oligo-dT priming of 2 μg of total RNA with 1 μg Superscript II reverse transcriptase (Life Technologies, Gaithersburg, MD, USA) for 50 min at 42 °C. After heating at 70 °C for 15 min, a first amplification reaction was carried out with one-tenth of the reverse-transcribed RNA, *Taq* polymerase buffer containing 200 μmol/L dNTPs, 1 U DynAzyme II DNA Polymerase (Finnzymes, Finland), and 10 μM each of two HURP primers for human: 5′-CAACGAAAACAGATGCTC-3′ (forward) and 5′-TGAGTAGCTG ATCGAGTC-3′ (reverse); two HURP primers for murine: 5′-CAACGGA AGCAGCTCCTCCA-3′ (forward) and 5′-TGAGTAGCCGCTGATTC AGTC-3′ (reverse), with denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min for 30 cycles, followed by incubation at 72 °C for 5 min. Amplified products were separated using electrophoresis with 0.1 μg GeneRulerTM 100 bp DNA Ladder (MBI Fermentas, Lithuania) in 1.5% agarose gels and visualized using ethidium bromide staining.

Western blotting. Fifty micrograms of total protein from the cells was denatured in a sodium dodecyl sulfate (SDS) lysis buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, and 100 mM dithiothreitol) and loaded into duplicated 12% SDS–polyacrylamide gels. After electrophoresis at 100 V for 3 h in SDS–PAGE running buffer (25 mM Tris–HCl, pH 8.8, 250 mM glycine, and 0.1% SDS), one of the gels was stained with 0.5% colorimetric Coomassie brilliant blue (Sigma, USA) as a quantitative control, and the other was transferred to the polyvinylidine difluoride (PVDF) membrane (Stratagene) and blocked with 5% skimmed milk in PBST (100 mM sodium chloride, 80 mM disodium hydrogen phosphate, 20 mM sodium dihydrogen phosphate, and 0.2% Tween 20, pH 7.5) solution at 4 °C overnight. After washing with PBST and phosphate-buffered saline (PBS), the membrane was hybridized with the monoclonal anti-HURP primary antibody (kindly provided by Dr. Chou) at 37 °C for 1 h. The membrane was then washed and hybridized with monoclonal anti-mouse IgG conjugated with horseradish peroxidase at 25 °C for 1 h (KPL, USA). After washing, the membrane was incubated with the enhanced chemiluminescence (ECL) detection reagents (Amersham, USA) for 10 min and then exposed to radiographic film (Fuji, Japan).

Cell proliferation and cytotoxic assay. Drug toxicity was determined by colorimetric XTT assay as previously described [9]. Briefly, cells (2×10^3 cells/well) were seeded in 96-well plates and incubated in the medium for 24 h. Anticancer drugs were then added for 48 h. At the end of the drug treatment, 50 μl of XTT (1 mg/ml; sigma) was added and incubated for another 2 h at 37 °C. Reduced XTT was measured spectrophotometrically using a Dynatech microplate reader (Dynatech lab) at 450 nm. Absorbance values were normalized to the values obtained for the vehicle-treated cells to determine the percent of survival. Each assay was performed in triplicate.

Anchorage-independent proliferation assay. In the assay for anchorage-independent growth, 4×10^4 cells/well (6-well plate) were plated in 5 ml of 0.3% agarose in DMEM, and 10% fetal bovine serum was overlaid onto a solid layer of 0.6% agarose in DMEM with 10% fetal bovine serum. The cultures were maintained for 2 weeks, and colonies larger than 0.1 mm (small) or >0.5 mm (large) were counted. The plating efficiency in agar was determined by dividing the number of colonies obtained per plate by the number of cells plated.

Results and discussions

Generation of NIH3T3 clones with increased HURP expression

After transfection of the HURP gene into NIH3T3 fibroblasts, several stable G418 resistant clones were obtained and tested for HURP gene expression at both the mRNA (Fig. 1A) and protein levels (Fig. 1B). The mRNA transcripts of the HURP gene were determined by RT-PCR; a 370 bp PCR product was detected in NIH3T3-1-3 and NIH3T3-3-3, but not in parental NIH3T3 cells. Two human bladder cancer cell lines, RT4 and T24, which exhibited HURP-positive, were parallelly analyzed as a positive control (Fig. 1A). The protein level of HURP was examined by Western blots; the protein expression of HURP displayed a pattern similar to the

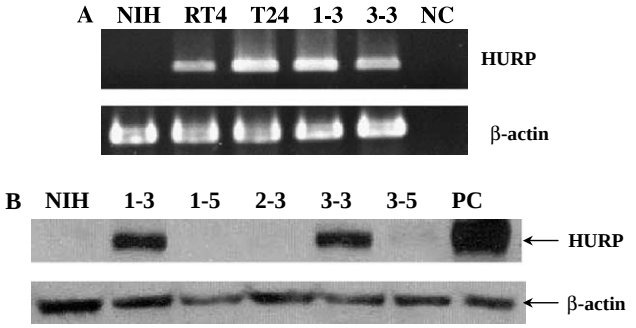

Fig. 1. The HURP expression level of the transfected NIH3T3 stable clones. (A) RT-PCR analysis of the expression level of HURP mRNA in NIH3T3, T24, NIH3T3-1-3, and NIH3T3-3-3. β-Actin was used as an internal control. NC: negative control. (B) Western blot analysis of the expression level of HURP protein in NIH3T3, T24, and transfected clones. Fifty micrograms of the total protein was fractionated on the 10% SDS–polyacrylamide gel and blotted onto the PVDF membrane. The expression of HURP was detected by anti-HURP antibodies, followed by enhanced chemiluminescence detection reagents. The cell lysates collected from NIH3T3 cells transiently transfected with the HURP gene for 48 h were analyzed as a positive control (PC).

mRNA level, and a 118-kDa band was detected in NIH3T3-1-3 and NIH3T3-3-3, but not in NIH3T3-1-5 and NIH3T3-2-3, and parental NIH3T3 cells. The cell lysates collected from NIH3T3 cells transiently transfected with the HURP gene for 48 h were analyzed as a positive control (Fig. 1B). NIH3T3-1-3 and NIH3T3-3-3, which exhibited HURP-positive, were selected as HURP-transduced clones, and NIH3T3-1-5 and NIH3T3-2-3 were selected as the mock transfectants for further studies.

In vitro proliferation of NIH3T3 cells with increased HURP expression

To determine whether a high level of HURP could confer any growth advantage to the cells, the growth rate of the cells cultured in 1% and 10% serum was examined. All cells grew at steady state in the media containing 10% serum (Fig. 2A). The parental and mock-transfected cells grew very slowly in the media containing 1% serum during four days. However, the NIH3T3-1-3 and NIH3T3-3-3 cells proliferated at a steady rate in the 1% serum media from day 2 to day 4 (Fig. 2B). This result indicated that the HURP-transduced cells were more proliferative than the parental and mock-transfected cells in the 1% serum media. The ability to grow in low-serum media indicated that the NIH3T3 cells transduced with elevated HURP levels behaved like transformed cells, which can proliferate independently of the growth factors.

Anchorage-independent growth assay

Next, the growth rate of the parental and transfected cells was measured using the parameter of plating efficiency on an agarose-based plate, as shown in Table 1. The parental and mock-transfected NIH3T3 cells rarely

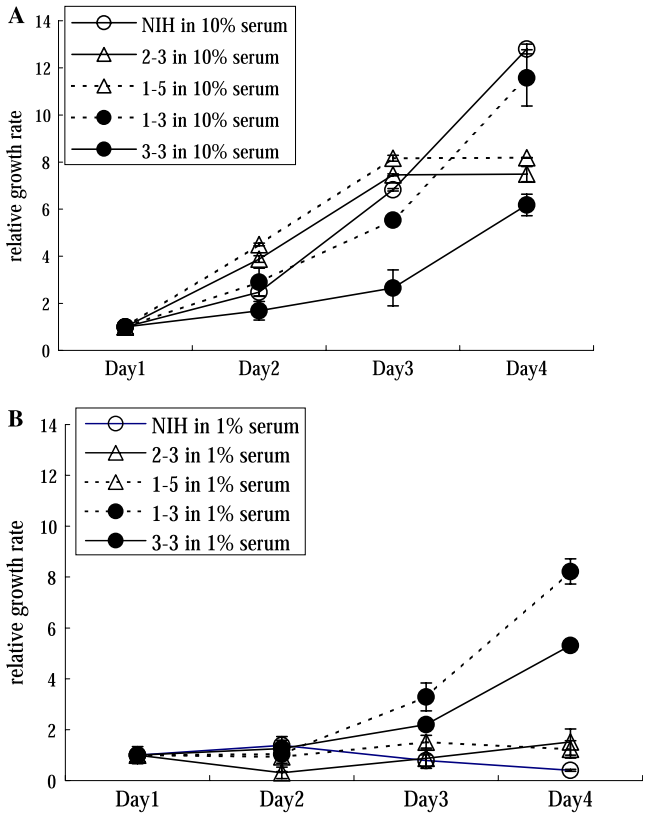

Fig. 2. Cell growth rate of the transfected NIH3T3 cells in DMEM with 10% serum (A) and 1% serum (B). Parental NIH3T3 cells (open circle), mock transfectants (open triangle), and HURP-transduced clones (closed circle) were seeded at a density of 2×10^3 cells/well into 96-well plates in 10% or 1% serum media for 4 days. The cell number was counted at a 24-hour interval for 4 days by the XTT microassay. Each assay was performed in triplicate and expressed as means ± SD (error bars).

Table 1
Anchorage-independent assay of the transfected NIH3T3 stable clones in DMEM with 10% serum

Cell type	Number of colonies developed in soft agar (mean ± SD)
Parental NIH3T3 cells	10.2 ± 1.5
Mock transfectants NIH3T3-1-5	13.3 ± 2.3
HURP-transduced clones NIH3T3-1-3	61.7 ± 4.5

Anchorage-independent assay was performed as described in Materials and methods. The colonies containing at least 20 cells were counted after 21 days. Each soft agar assay was performed in duplicate. The results are expressed as the mean of the number of colonies ± SD. Data were compared by Kruskal–Wallis H test, followed by Dunn's test for pairwise comparisons. Overall p value <0.001.

grew in the soft agar; about 10.2 and 13.3 colonies had formed as of day 21, respectively. The NIH3T3-1-3 cells were able to grow quickly; about 61.7 colonies were formed during the three weeks. These results showed that the murine fibroblasts with increased HURP expression lost contact inhibition and acquired the ability of anchorage-independent proliferation, allowing colony formation in soft agar.

Overexpression of HURP in 293T cells has been reported to enhance cell growth ability in a low-serum medium and in polyhema-based plates [6]. Consistent with this finding, we found that only the HURP-transduced cells, but not the parental and mock-transfected NIH3T3 cells, manifested the general characteristics of tumor cells, such as active cell proliferation in low-serum media and in agarose-based plates (Fig. 2 and Table 1). These results indicate that the HURP gene overexpressed in NIH3T3 cells is an important determinant of their rate of proliferation in vitro, their clonogenicity in soft agar. These results might explain the HURP overexpression found in some human tumor specimens compared to their adjacent normal tissues [6–8,10].

The chemosensitivity of NIH3T3 cells with increased HURP expression

To provide further understanding of the underlying molecular mechanism of the HURP function, the correlation between HURP levels and cell chemosensitivity to anticancer drugs was evaluated. We examined the cytotoxicity of the parental, mock-transfected, and HURP-transduced NIH3T3 cells responding to three kinds of drug treatment; the results are depicted in the following figures. First, Fig. 3 shows the chemoresponse curves of the microtubule stabilizers, such as paclitaxel (Fig. 3A), docetaxel (Fig. 3B), and vinblastine (Fig. 3C) in these cells. Second, the chemosensitive curves of DNA intercalating agents, such as carboplatin (Fig. 4A), cisplatin (Fig. 4B), and doxorubicin (Fig. 4C) were compared as well. Figs. 3 and 4 show that all cells displayed similar response curves to microtubule stabilizers and DNA intercalating agents. Finally, the cytotoxic curves of deoxycytidine analogs, such as gemcitabine (Fig. 5A), ARA-C (Fig. 5B), and 5-AZA-CdR (Fig. 5C), were examined. The HURP-transduced NIH3T3 cells exhibited higher sensitivity to deoxycytidine analogs than the parental and mock-transfected cells (Fig. 5). Furthermore, Table 2 summarizes the IC_{50} values of these cells responding to six chemosensitive drugs. Notably, the IC_{50} values of gemcitabine in the NIH3T3-1-3 cells (29.37 nM) and NIH3T3-3-3 cells (4.92 nM) were lower than in the NIH3T3 (110.67 nM), NIH3T3-2-3 (104.54 nM), and NIH3T3-1-5 cells (102.00 nM). Similarly, the IC_{50} values of ARA-C and 5-AZA-CdR in the HURP-transduced cells were lower than in the parental and mock-transfected cells (Table 2). These results indicate that HURP-transduced NIH3T3 cells exhibited a higher sensitivity to deoxycytidine analogs, but not to other kinds of anticancer drugs.

The mechanism of HURP biological activity in normal and malignant cells is not yet fully understood. Previous study has reported that the HURP mRNA transcripts were elevated in the G2/M phase of synchronized HeLa cells and localized to the spindle poles during mitosis[6]. In this study, we found that the NIH3T3 cells transduced with the exogenous HURP gene were capable of enhancing

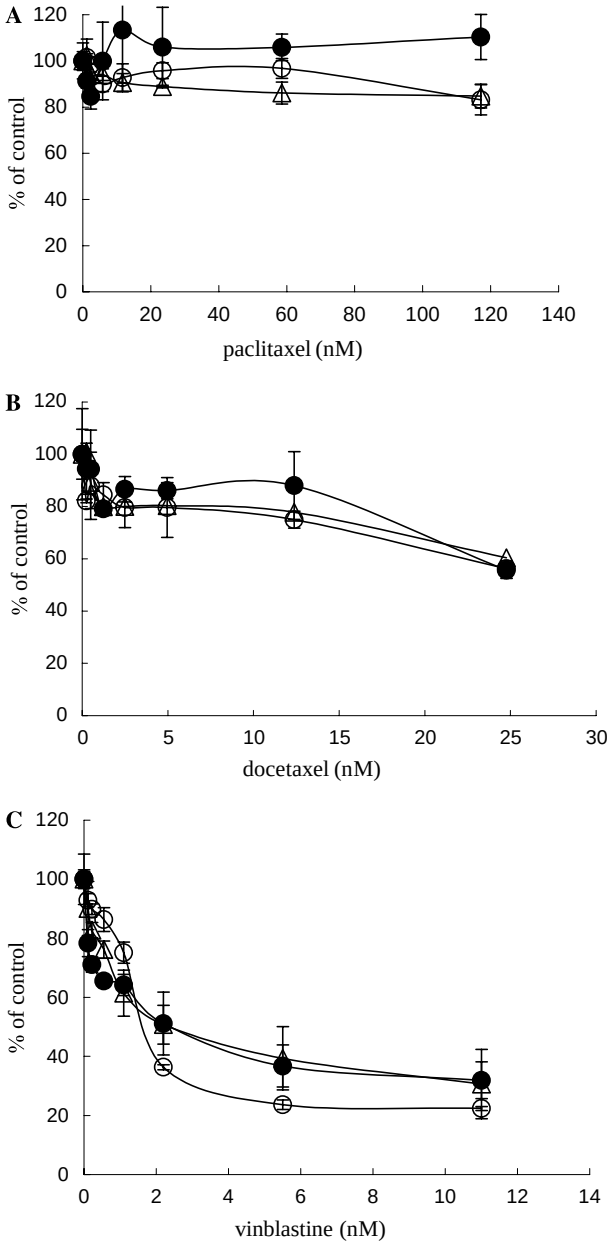

Fig. 3. The dose–response curves of paclitaxel, docetaxel, and vinblastine in the transfected NIH3T3 cells. Parental NIH3T3 cells (open circles), mock transfectants NIH3T3-1-5 (open triangle), and HURP-transduced NIH3T3-1-3 (closed circles) cells were seeded into 96-well plates at a density of 2×10^3 cells/well and incubated for 24 h, then incubated with various concentrations of paclitaxel (A), docetaxel (B), and vinblastine (C) for 48 h. Absorbance values were normalized to the values obtained for the vehicle-treated cells to determine the percent of cell viability. Each assay was performed in triplicate and expressed as the means ± SD (error bars).

the chemosensitivity of cells responding to deoxycytidine analogs (Fig. 5 and Table 2). Reports have shown that gemcitabine is one hundred times more cytotoxic than ARA-C in different kinds of leukemia cell lines, indicating that the kinetics of the induction of this process for these two anti-metabolites are different [11,12]. This phenomenon is also presented in this study (Fig. 5 and Table 2).

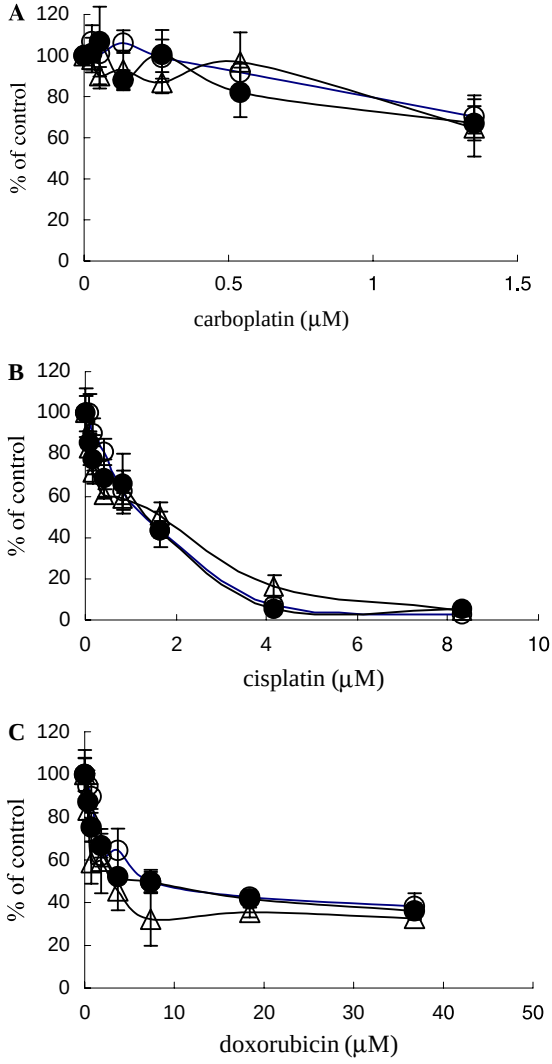

Fig. 4. The dose–response curves of carboplatin, cisplatin, and doxorubicin in the transfected NIH3T3 cells. Parental NIH3T3 (open circles), mock transfectants NIH3T3-1-5 (open triangle), and HURP-transduced NIH3T3-1-3 (closed circles) cells were seeded into 96-well plates at a density of 2×10^3 cells/well and incubated for 24 h, then incubated with various concentrations of carboplatin (A), cisplatin (B), and doxorubicin (C) for 48 h. Absorbance values were normalized to the values obtained for the vehicle-treated cells to determine the percent of cell viability. Each assay was performed in triplicate and expressed as the means ± SD (error bars).

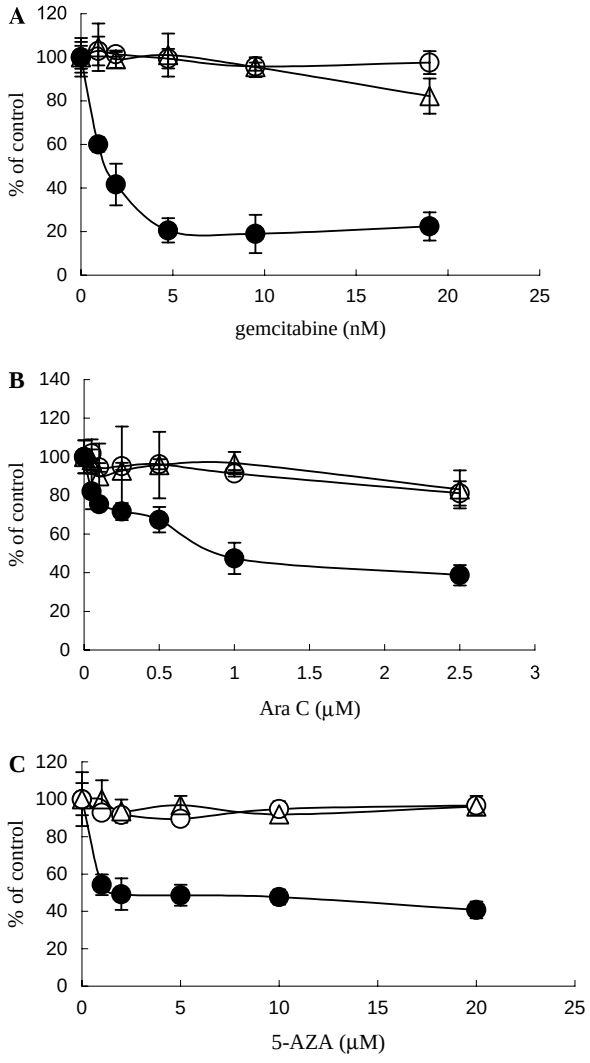

Fig. 5. The dose–response curves of gemcitabine, ARA-C, and 5-AZA in the transfected NIH3T3 cells. Parental NIH3T3 (open circles), mock transfectants NIH3T3-1-5 (open triangle), and HURP-transduced NIH3T3-1-3 (closed circles) cells were seeded into 96-well plates at a density of 2×10^3 cells/well and incubated for 24 h, then incubated with various concentrations of gemcitabine (A), ARA-C (B), and 5-AZA (C) for 48 h. Absorbance values were normalized to the values obtained for the vehicle-treated cells to determine the percent of cell viability. Each assay was performed in triplicate and expressed as the means ± SD (error bars).

The mechanism of action of gemcitabine appears to be related to its incorporation into RNA [13] and DNA [14], also to its inhibition of the enzymes ribonucleotide reductase [15] and deoxycytidine monophosphate deaminase [16].

The relationship between the HURP gene expression and the sensitivity of deoxycytidine analogs is worthy of further investigation. Deoxycytidine nucleoside analogs have to be phosphorylated to become active with deoxycytidine kinase (dCK). Overexpression of the dCK enzyme enhanced sensitivity to the antitumor activity of ARA-C [17,18]. Conversely, cytidine deaminase (CDA) catalyzed the degradation of deoxycytidine analogs [19,20]. The enzyme has been associated with sensitivity and resistance to ARA-C [20–22]. Transfection of the gene resulted in resistance to nucleoside analogs [23]. The expression level of dCK and CDA will be analyzed in the HURP-transduced NIH3T3 cells in the future.

Furthermore, we are planning to examine the differential mRNA expression of deoxycytidine analog metabolizing enzymes, including dCK, CDA, deoxycytidylate deaminase, pyrimidine nucleotidase, and human equilibrative nucleoside transporter 1 [24], in the HURP-transduced NIH3T3 cells using real-time quantitative RT-PCR.

Table 2
The chemosensitivity of the transfected NIH3T3 stable clones with increased HURP expression

Drug	IC_{50} value	Cell type					Overall p value
		NIH3T3	NIH3T3-2-3	NIH3T3-1-5	NIH3T3-1-3	NIH3T3-3-3	
Vinblastine (nM)	Mean	1.18	2.22	1.94	2.35	2.63	0.402
	SD	1.07	1.52	0.24	0.68	0.90	
Cisplatin (μM)	Mean	1.30	2.29	2.68	1.70	1.25	0.316
	SD	1.11	0.91	0.91	0.67	0.56	
Doxorubicin (μM)	Mean	7.31	7.53	6.45	6.67	5.95	0.934
	SD	0.66	1.32	2.36	3.00	2.79	
Gemcitabine (nM)	Mean	110.67	104.54	102.00	29.37	4.92	0.028
	SD	19.06	21.80	10.21	9.50	4.63	
AraC (μM)	Mean	7.45	8.25	8.14	0.67	0.55	0.036
	SD	1.34	2.07	3.22	0.49	0.21	
5-AZA (μM)	Mean	51.92	44.30	65.21	1.86	2.28	0.028
	SD	2.50	1.33	4.55	0.77	0.44	

Data were taken from the average of three independent assays and expressed as the means ± SD. Data were compared by Kruskal–Wallis H test, followed by Dunn's test for pairwise comparisons.

Acknowledgments

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