Contents lists available at ScienceDirect

Journal of Pharmacological Sciences

journal homepage: www.elsevier.com/locate/jphs

Full paper

Ribonucleotide reductase is an effective target to overcome gemcitabine resistance in gemcitabine-resistant pancreatic cancer cells with dual resistant factors

CrossMark

Kentaro Minami [a, b, 1], Yoshinari Shinsato [b, c, 1], Masatatsu Yamamoto [b, c], Homare Takahashi [b, d], Shaoxuan Zhang [e], Yukihiko Nishizawa [a], Sho Tabata [a, b, f], Ryuji Ikeda [a], Kohich Kawahara [b, c], Kazutake Tsujikawa [g], Kazuo Chijiiwa [d], Katsushi Yamada [a, h], Shin-ichi Akiyama [b, i], Sandra Pérez-Torras [j], Marcal Pastor-Anglada [j], Tatsuhiko Furukawa [b, c, *], Takeda Yasuo [a]

[a] Department of Clinical Pharmacy and Pharmacology, Graduate School of Medical and Dental Sciences, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890-8544, Japan
[b] Department of Molecular Oncology, Graduate School of Medical and Dental Sciences, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890-8544, Japan
[c] Center for the Research of Advanced Diagnosis and Therapy of Cancer, Graduate School of Medical and Dental Sciences, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890-8544, Japan
[d] Department of Surgical Oncology and Regulation of Organ Function, Miyazaki University School of Medicine, 5200 Kihara, Kiyotake, Miyazaki 889-1692, Japan
[e] Laboratory of Molecular Genetics, Institute of Frontier Medical Sciences, Jilin University, 1163 Xinmin Street, Changchun 130021, China
[f] Institute for Advanced Biosciences, Keio University, Mizukami 246-2, Kakuganji, Tsuruoka, Yamagata 997-0052, Japan
[g] Graduate School of Pharmaceutical Science, Osaka University, Yamada-oka 1-6, Suita, Osaka 565-0817, Japan
[h] Department of Clinical Pharmacology, Faculty of Pharmaceutical Sciences, Nagasaki International University, Huis Ten Bosch Cho 2825-7, Sasebo, Nagasaki 859-3298, Japan
[i] Clinical Research Center, National Kyushu Cancer Center, Notame, Minami-ku, Fukuoka 811-1395, Japan
[j] Department of Biochemistry and Molecular Biology, University of Barcelona, Institute of Biomedicine and Oncology Programme, National Biomedical Research Institute of Liver and Gastrointestinal Diseases (CIBER EHD) Diagonal 643, 08028 Barcelona, Spain

ARTICLE INFO

ABSTRACT

Gemcitabine is widely used for pancreatic, lung, and bladder cancer. However, drug resistance against gemcitabine is a large obstacle to effective chemotherapy. Nucleoside transporters, nucleoside and nucleotide metabolic enzymes, and efflux transporters have been reported to be involved in gemcitabine resistance. Although most of the resistant factors are supposed to be related to each other, it is unclear how one factor can affect the other one. In this study, we established gemcitabine-resistant pancreatic cancer cell lines. Gemcitabine resistance in these cells is caused by two major processes: a decrease in gemcitabine uptake and overexpression of ribonucleotide reductase large subunit (RRM1). Knockdown of RRM1, but not the overexpression of concentrative nucleoside transporter 1 (CNT1), could completely overcome the gemcitabine resistance. RRM1 knockdown in gemcitabine-resistant cells could increase the intracellular accumulation of gemcitabine by increasing the nucleoside transporter expression. Furthermore, a synergistic effect was observed between hydroxyurea, a ribonucleotide reductase (RR) inhibitor, and gemcitabine on the gemcitabine-resistant cells. Here we indicate that RR is one of the most promising targets to overcome gemcitabine resistance in gemcitabine-resistant cells with dual resistant factors.

* Corresponding author. Department of Molecular Oncology and Center for the Research of Advanced Diagnosis and Therapy of Cancer, Graduate School of Medical and Dental Sciences, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890-8544, Japan. Tel.: +81 99 275 5490; fax: +81 99 265 9687.
E-mail address: furukawa@m3.kufm.kagoshima-u.ac.jp (T. Furukawa).
Peer review under responsibility of Japanese Pharmacological Society.
[1] These authors contributed equally to this work.

1. Introduction

Pancreatic cancer is one of the most difficult malignancies to treat successfully. Only 15%–20% of patients are eligible for a potentially curative resection at the diagnosis. Even if surgical resection is performed, the recurrence rate is high and the survival rate after surgery is poor (1). Therefore, effective chemotherapy is indispensable to improve the prognosis of patients with pancreatic cancer.

Gemcitabine (2',2'-difluoro deoxycytidine) is used for the treatment of not only pancreatic cancer but also lung and bladder cancers. However, the occurrence of drug-resistant cells greatly hinders successful cancer therapy. Gemcitabine is a unique antimetabolite in that its metabolites dFdCDP and dFdCTP can inhibit ribonucleotide reductase (RR) by binding to ribonucleotide reductase large subunit (RRM1) and can terminate DNA elongation processes by incorporating into DNA, respectively (2).

Multiple factors, including the attenuation of nucleoside transporters, the expression change of gemcitabine-activating or -degradation enzymes and target molecules, and the expression of efflux transporters, have been reported to cause gemcitabine resistance (3–14).

However, the relationship between each factor is not clear, although several of these factors are thought to influence each other in gemcitabine metabolism. This relationship needs to be elucidated to find a good strategy to overcome gemcitabine resistance. Therefore, we established gemcitabine-resistant cell lines from a pancreatic cancer cell line, MIA PaCa-2, and systematically examined the mechanisms underlying the gemcitabine resistance of the cells.

2. Materials and methods

2.1. Cells

The human pancreatic cancer cell line MIA PaCa-2 was provided by the Riken BioResource Center, Japan. MIA PaCa-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Nissui Seiyaku, Tokyo, Japan), containing 10% fetal calf serum (FCS; PAA Laboratories, Pasching, Austria), at 37 °C in an atmosphere containing 5% CO_2.

MGEM6 cells were transfected with pDNA3.1-hCNT1 using electroporation (Gene Pulser, Bio Rad). The cells were selected with G418 and MGEM6/CNT1#17 cells were used for further analysis. MGEM6 and MGEM6/CNT1#17 cells were transfected with pLKO.1-shRRM1 (Sigma–Aldrich Corp., St. Louis, MO, USA) as described above. The cells were selected by puromycin and MGEM6/KD5-6 and MGEM6/CNT1#17KD5-7 cells were used for further analysis.

2.2. Plasmids

The concentrative nucleoside transporter 1 (CNT1) plasmid pDNA3.1-hCNT1is described elsewhere (15). Mission shRNA expression plasmids against RRM1 mRNA (NM_001033_2471S) were purchased from Sigma–Aldrich Corp. (St. Louis, MO, USA).

2.3. Chemicals, anticancer agents, and antibodies

DMEM was purchased from Nissui Seiyaku (Tokyo, Japan), FCS was purchased from PAA Laboratories (Pasching, Austria), (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), G418, puromycin, cytosine arabinoside (Ara-C), 2-chlorodeoxyadenosine (cladribine), N-methyl-D-glucamine, nitrobenzylthioinosine (NBTI), dipyridamole, and hydroxyurea (HU) were purchased from Sigma–Aldrich Corp (St. Louis, MO, USA), p-amidinophenyl methanesulfonyl fluoride (p-APMSF) was purchased from Wako (Osaka, Japan), and [^{3}H]

gemcitabine was purchased from Moravek Biochemicals and Radio-chemicals (Brea, CA, USA).

Gemcitabine was obtained as a gift from Ely Lilly and Co. Anti-hCNT1 was generated as described previously (16). Other antibodies were purchased from the indicated companies: hENT1 (Abgent, San Diego, CA, USA: AP1086c), hENT2 (Abcam, Cambridge, UK: ab48595), RRM1 (Cell Signaling Technology, Danvers, MA, USA: #3388), RRM2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA: sc10846), p53R2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA: sc10840), GAPDH (Cell Signaling Technology, Danvers, MA, USA) sodium/potassium-transporting ATPase α3 (Na^+/K^+ ATPase α3) (Abcam, MA, USA, XVIF9-G10).

2.4. Cell viability assay

The sensitivity to the anticancer agents was determined by the MTT assay using 96-well plates seeded 5×10^3 cells/well as described previously (17). The IC_{50} was determined as the concentration of the agents that reduced the number of cells to 50% of that of cells cultured in control medium.

2.5. Gemcitabine accumulation under normal and inhibitory conditions with nucleoside transporters

To measure the gemcitabine accumulation, subconfluent cells cultured in 12-well plates were incubated with 1 μM hot (^{3}[H]-labeled) and cold gemcitabine in the medium at 37 °C for 1 h. After removal of the media, the cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed with 0.5 mL ice-cold PBS containing 1% Triton X-100 and 0.2% sodium dodecyl sulfate (SDS). Five milliliters scintillation solution was added to the lysate and the radioactivity was measured with a liquid scintillation counter (18).

For the prewash and the 1-hr incubation with gemcitabine under inhibitory conditions for one or more nucleoside transporter(s), we used 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered Ringer's solution for the control conditions, consisting of 135 mM NaCl, 5 mM KCl, 3.33 mM NaH_2PO_4, 0.83 mM Na_2HPO_4, 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10 mM glucose, and 5 mM HEPES (pH 7.4); Na-free buffer for the CNT1 inhibition condition, containing 140 mM N-methyl-D-glucamine, 5 mM HEPES, 5 mM KH_2PO_4, 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 10 mM glucose (pH 7.4); Na-free buffer containing 100 nM NBTI for the CNT1 and ENT1 inhibition condition; and Na-free buffer containing 10 μM dipyridamole for the CNT1, equilibrative nucleoside transporter 1 (ENT1), and ENT2 inhibition condition (19).

To determine the efflux rate of gemcitabine from the cells, subconfluent cells cultured in 12-well plates were incubated with 1 μM hot and cold gemcitabine in DMEM, containing 10% FCS, at 37 °C for 1 h, and were washed with PBS at 37 °C. After adding fresh, warm medium without gemcitabine until each indicated time, the cells were washed and solubilized. The radioactivity of the lysates was determined as described above.

2.6. Cell fractionation and immunoblotting

Cell membrane fractions were isolated as described previously (17). Total cell lysate was isolated from the cells with lysis buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1% aprotinin, and 1 mM p-APMSF). After lysis, the cell debris was removed by centrifugation at $14,000 \times g$ for 15 min at 4 °C.

Immunoblotting was performed as described previously (17) and the blotted membranes were developed using the enhanced chemiluminescence immunoblotting detection system (GE healthcare Bio-Sciences, Piscataway, NJ, USA) and were exposed to X-ray film. The chemiluminescence intensities of the bands were

quantified using Fluor Chem FC2 Imager (Alpha Innotech, San Leandro, CA) and the normalized numbers by the intensity of respective loading control Na^+/K^+ ATPase α3 for Figs. 2A and 3E or GAPDH for Fig. 3A were indicated.

2.7. Reverse transcription PCR

Total RNA from the cultured cells was isolated using TRIzol (Life Science Technology, Carlsbad, CA, USA), and RNA (2 μg) was reverse-transcribed using a ReverTra Ace kit (Toyobo, Osaka, Japan), according to the manufacturer's instructions.

2.8. Quantitative reverse transcription PCR

Quantitative reverse transcription PCR was performed as described previously (20). The respective forward and reverse primers' sequences are provided in Supplementary Table 1.

2.9. Analysis of synergistic effects

To evaluate a potential synergistic effect between hydroxyurea (HU) and gemcitabine, we used the median effect analysis method of Chou and Talalay (21). Molar ratios of gemcitabine and HU in the evaluation were determined as 1:25000 and 1:200 with IC_{50} of each agent to MIA PaCa-2 and MGEM6. The combination index (CI) was calculated for each combination ratio by using the formula: $CI = (D)1/(Da)1 + (D)2/(Da)2$, in which (Da)1 and (Da)2 are the concentrations required for single agents to achieve a drug effect (a = 0.1–0.9), that means growth inhibition rate and (D)1 and (D)2 are the concentrations of gemcitabine and HU, respectively, used to achieve the same effect. The CI value determines the effect of drug combinations with <1, 1, and >1 indicating a synergistic, additive, and antagonistic effect, respectively, since synergism and antagonism are defined as greater (synergism) and less (antagonism) than the expected additive effect, respectively (21).

2.10. Statistical analysis

The differences between the groups were analyzed using the Student's t-test. A p value of <0.05 was considered significant.

3. Results

3.1. Establishment of gemcitabine-resistant cells and the estimation of drug resistance

After exposure to 1 mg/mL ethyl methanesulfonate for 24 h, MIA PaCa-2 cells were cultured in normal medium for 1 week. Thereafter, cells were selected using media containing subsequently 10 and 100 nM gemcitabine for 1 week and 2 weeks, respectively. Twelve colonies were isolated after the selection process. Two clones, named MGEM6 and MGEM8, were cultured in 100 nM gemcitabine

containing DMEM with 10% FCS and used for further analyses, since these clones showed a growth comparative with that of the parental cells. We performed a survival assay in the presence of gemcitabine and found that compared to the parental cells, MGEM6 and MGEM8 cells were 67.8- and 33.0-fold more resistant to gemcitabine, respectively (Table 1). MGEM6 and MGEM8 cells were also resistant to Ara-C and cladribine, although to a lesser extent.

3.2. Gemcitabine accumulation was decreased in gemcitabine-resistant cells

First, we examined gemcitabine and its phosphorylated forms accumulation in the gemcitabine-resistant and parental cells. The concentration of gemcitabine in the resistant cells was about one third of that in the parental cells (Fig. 1A).

To understand the mechanism underlying the low gemcitabine and its derivatives accumulation in the resistant cells, we evaluated the efflux gemcitabine and its probable phosphorylated derivatives from gemcitabine efflux of these cells and found that it was comparable with that of the parental cells (Fig. 1B). From these data, we expected that the gemcitabine uptake would be decreased in MGEM6 and MGEM8 cells.

3.3. Gemcitabine accumulation was decreased owing to the attenuation of CNT1 expression

Cellular uptake of gemcitabine has been reported to be mainly mediated by CNT1, ENT1, and ENT2 (22). To identify the transporters responsible for the low gemcitabine uptake, we evaluated the gemcitabine accumulation under inhibitory conditions for the nucleoside transporter(s) CNT1, ENT1, and/or ENT2. In the absence of Na ions, the gemcitabine accumulation dramatically decreased in the parental cells but not in the MGEM6 and MGEM8 cells compared with that in the presence of these ions (Fig. 1C). These data indicate that the gemcitabine uptake by the sodium-dependent uptake transporter CNT1 was decreased in MGEM6 and MGEM8 cells. The uptake of gemcitabine by ENT2 was lower in the gemcitabine-resistant cells than in the parental cells. As shown by immunoblotting, the expression of CNT was clearly decreased in MGEM6 and MGEM8 cells; the expression of ENT2, however, was comparable with that of the parental cells (Fig. 2).

3.4. CNT1 overexpression sensitized the gemcitabine-resistant cells to gemcitabine

To determine whether the decrease in gemcitabine uptake was correlated with the gemcitabine resistance, we transfected MGEM6 cells with CNT1. The expression of CNT1 was highly increased in CNT1-transfected, MGEM6/CNT1#17, and MGEM6/CNT1#19 cells compared with that in MIA PaCa-2 and MGEM6 cells (Fig. 2A). Gemcitabine sensitivity was 3–6-fold higher in CNT1-transfected MGEM6 cells than in MGEM6 cells (Table 1). Although the level of gemcitabine

Table 1
The sensitivity of gemcitabine-resistant cells to the anticancer agents tested. The IC_{50} values are means ± SD from triplicate determinations by using the MTT assay. RR (relative resistance) is the ratio that IC_{50} for the cells divided by IC_{50} for MIA PaCa-2 cells. * indicates statistical significance ($p < 0.05$).

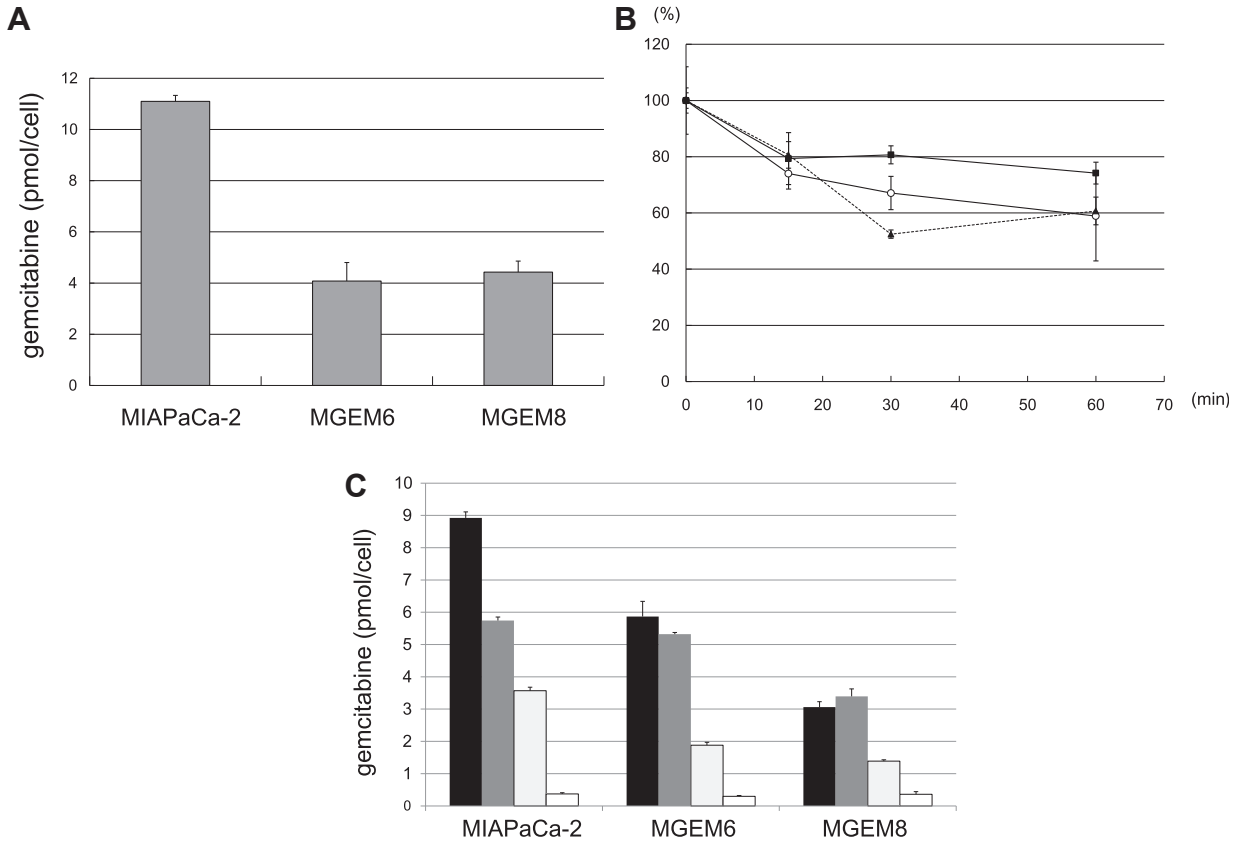| Agents	Gemcitabine		Ara-C		Cladribine	
Cells	IC_{50}	RR	IC_{50}	RR	IC_{50}	RR
MIAPaCa-2	6.9 ± 0.7		0.93 ± 0.09		12.65 ± 2.75	
MGEM6	465.9 ± 26.7	67.8*	1.39 ± 0.09	1.50*	48.73 ± 1.01	3.85*
MGEM8	230.5 ± 12.0	33.0*	7.37 ± 0.68	7.96*	59.90 ± 1.24	4.73*
MGEM6/CNT1#17	171.0 ± 16.6	24.9*	1.90 ± 0.27	2.05*	29.10 ± 1.75	2.30*
MGEM6/CNT1#19	80.1 ± 4.4	11.7*	ND		ND	
MGEM6/KD5-6	3.7 ± 0.6	0.5	0.32 ± 0.06	0.34	1.60 ± 0.04	0.13
MGEM6/CNT1#17KD5-7	3.1 ± 0.3	0.5	0.20 ± 0.03	0.22	1.27 ± 0.32	0.10

A

B

C

Fig. 1. The intracellular gemcitabine concentration and the efflux of gemcitabine from gemcitabine-resistant cells. The intracellular concentrations of gemcitabine were evaluated using [^{3}H] gemcitabine. A. Gemcitabine accumulation was lower in the gemcitabine-resistant cells than in the parental cells. The intracellular accumulation of gemcitabine in each cell line is shown (pmol/cell). B. Gemcitabine efflux from the gemcitabine-resistant cells was comparable with that of the parental cells. After incubation with gemcitabine, the time-dependent change in the intracellular gemcitabine concentration was estimated as the efflux of gemcitabine. The x-axis represents time (min) and the y-axis represents the ratio of the intracellular gemcitabine accumulation at the indicated time to that at time zero (%). The open circles denote MIA PaCa-2 cells and the closed square and the closed triangle denote MGEM6 and MGEM8 cells, respectively. C. Gemcitabine accumulation of each cell line under normal and inhibitory conditions. Black box: incubation with HEPES-buffered Ringer's solution, dark gray box: Na-free CNT1 transport inhibiting condition, light grey box: Na-free buffer containing 100 nM NBTI for the CNT1 and ENT1 transport inhibiting condition, white box: Na-free buffer containing 10 μM dipyridamole for the CNT1, ENT1, and ENT2 transport inhibiting conditions. Data are means ± SD from triplicate studies.
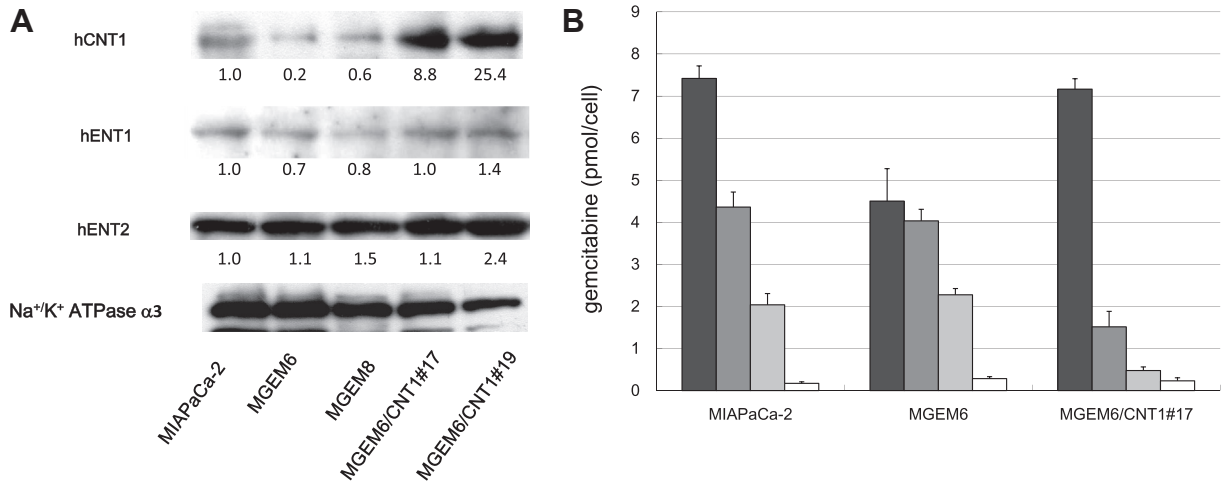
A

B

Fig. 2. CNT1 overexpression increased gemcitabine accumulation of gemcitabine resistant cells. A. Immunoblotting of nucleoside transporters. Membrane fractions (100 μg) of MIA PaCa-2, MGEM6, MGEM8, MGEM6CNT1#17, and MGEM6CNT1#19 cells were separated by 7.5% SDS-PAGE and transferred to polyvinyl difluoride (PVD) membranes. The hCNT1, hENT1, hENT2 and Na$^+$/K$^+$ ATPase α3 proteins were detected with specific antibodies, and the immunoreactive bands were visualized using chemiluminescence and were exposed to X-ray film. The quantified intensities were indicated below the bands as the values normalized by the intensity of Na$^+$/K$^+$ ATPase α3 bands. B. Gemcitabine accumulation under normal and inhibitory conditions. A. The intracellular gemcitabine accumulation in MIA PaCa-2, MGEM6 and MGEM6CNT1#17 was determined as described in the Materials and Methods using [^{3}H] gemcitabine.

accumulation in MGEM6/*CNT1*#17 cells was similar to that in the parental cells with an increase in CNT1-dependent uptake (Fig. 2B), MGEM6/*CNT1*#17 cells were still highly resistant to gemcitabine (Table 1). These data suggest that other mechanisms could have contributed to the gemcitabine-resistant phenotype of these cells.

3.5. RRM1 overexpression was associated with gemcitabine resistance

A high expression of RRM1 and/or ribonucleotide reductase small subunit (RRM2) have/has been reported to be related to gemcitabine resistance (12). We therefore examined RRM1, RRM2, and p53R2 expression in our cell lines. RRM1 expression was more than 10-fold higher in MGEM6 and MGEM8 cells than in the parental cells (Fig. 3A); the expression of RRM2 decreased slightly and p53R2 was comparable (Fig. 3A).

To assess whether the high expression of RRM1 was associated with gemcitabine resistance, *RRM1* expression in MGEM6 and MGEM6/*CNT1*#17 cells was knocked-down using shRNA and was largely reduced in MGEM6/KD5-6 and MGEM6/*CNT1*#17KD5-7 cells compared with that in MGEM6 cells, as shown by immunoblotting (Fig. 3B). Furthermore, *RRM1*-silenced MGEM6, MGEM6/KD5-6, and MGEM6/*CNT1*#17KD5-7 cells, and *RRM1*-silenced and CNT1-overexpressing MGEM6 cells were more sensitive to gemcitabine than MGEM6 cells were (Table 1). MGEM6/KD5-6 cells were as

sensitive to gemcitabine as the parental and MGEM6/*CNT1*#17KD5-7 cells were, although CNT1 expression in MGEM6/KD5-6 cells was expected to be lower than that in the parental cells and similar to that in MGEM6, since we didn't manipulate CNT1 expression of MGEM6/KD5-6 cells directly (Table 1).

To clarify the *RRM1* knockdown effect on gemcitabine accumulation, we estimated the intracellular gemcitabine concentration and the expression of nucleoside transporters. We found that the gemcitabine concentration was much higher in MGEM6/KD5-6 than in MGEM6 cells (Fig. 3C). Furthermore, the expression of *CNT1, ENT1*, and *ENT2* mRNA was increased compared with that in MGEM6 cells (Fig. 3D). As shown by immunoblotting, the expression of CNT1 was clearly increased in MGEM6/KD5-6 cells (Fig. 3E). These data indicate that the knockdown of RRM1 could not only reduce RRM1 expression but also increase gemcitabine accumulation by increasing the expression of uptake transporters. In addition, the knockdown of *RRM1* made MGEM6 cells more sensitive to Ara-C and cladribine than the parental cells were (Table 1).

3.6. The inhibition of RRM1 activity can overcome gemcitabine resistance

To understand whether the inhibition of RR activity could enhance the gemcitabine cytotoxic activity, we examined the sensitivity of the cells to gemcitabine in the presence of a non-toxic
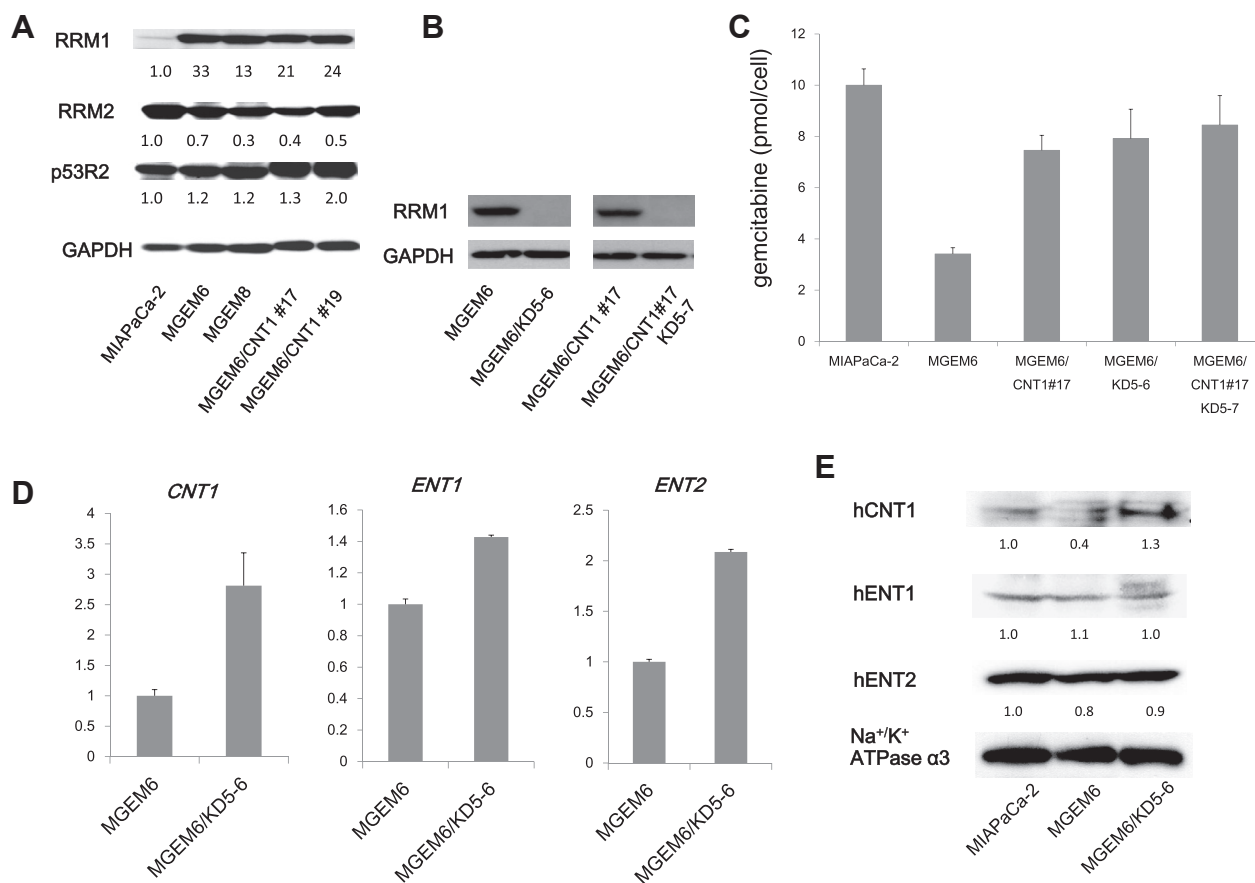

Fig. 3. The gemcitabine accumulation and the expression of nucleoside transporters increased in *RRM1*-silenced cells. A. Immunoblotting of RRM1, RRM2, and p53R2. Total cell lysates (20 μg) of the indicated cell lines were separated by 7.5% SDS-PAGE, and each protein was detected with specific antibodies. The quantified intensities were indicated below the bands as the values normalized by the intensity of GAPDH. B. Immunoblotting of RRM1 in *RRM1*-silenced cells. ShRNA effectively knocked down RRM1 protein expression in MGEM6/KD5-6 and MGEM6/*CNT1*#17KD5-7 cells. C. Gemcitabine accumulation in *RRM1*-silenced cells. Intracellular gemcitabine concentrations of the indicated cells in RRM1-silenced cells were determined as described in Materials and Methods using [^{3}H] gemcitabine. D. q-PCR of nucleoside transporter expression in MGEM6 and MGEM6/KD5-6 cells. The mRNA expressions of nucleoside transporters were evaluated of the indicated cells. Their expressions were indicated with the arbitrary unit as standardized with the expression in MGEM6 cells. E. Immunoblotting of nucleoside transporters in MIA PaCa-2, MGEM6, and MGEM6/KD5-6 cells. The expression of nucleoside transporters were examined by immunoblotting as described above. The quantified intensities were indicated below the bands with the values normalized by the intensity of Na$^+$/K$^+$ ATPase α3 band.

Table 2
The sensitivity of the cells to gemcitabine in the presence of nontoxic dose of HU. IC_{50} values are means $\pm$ SDs from triplicate determination with MTT assay in the absence and presence of HU (100 μM). RR means relative resistance. RR is the ratio of IC_{50} for each cell divided by IC_{50} for MIAPaCa-2 cells. * indicates statistically significant ($P < 0.05$).

Agents	Gemcitabine		Gemcitabine + HU	
Cells	IC_{50}	RR	IC_{50}	RR
MIAPaCa-2	6.1 ± 0.2		5.7 ± 0.1	
MGEM6	644.7 ± 33.5	105.8*	338.9 ± 54.0	59.6*
MGEM8	178.3 ± 3.0	29.2*	111.4 ± 2.0	19.6*

concentration (100 μM) of the RRM2 inhibitor HU. HU enhanced the gemcitabine cytotoxicity against MGEM6 and MGEM8 cells about 1.5 fold (Table 2). Next, we evaluated whether HU synergistically enhanced the effect of gemcitabine. The CI values for MGEM6 cells were less than 1.0 in any of the affected fractions (Fig. 4), which indicates cell survival ratios, while those for MIA PaCa-2 cells were 1.0. We therefore showed a synergistic effect of the combination of gemcitabine and HU only on the gemcitabine-resistant cells (Fig. 4).

4. Discussion

Although gemcitabine is a valuable anticancer agent, drug resistance proves to be a large obstacle for successful cancer chemotherapy. Several molecular changes, the attenuation of the expression of the nucleoside uptake transporters CNT1, ENT1, and ENT2, a change in the expression of the cytidine metabolic enzymes deoxycytidine kinase (DCK) and cytidine deaminase, the overexpression of the nucleotide metabolic enzymes RRM1 and RRM2, and an increase in the expression of the efflux transporters ABCC5 and ABCC10 have been reported to underlie gemcitabine resistance (3−14). At least some of these proteins are thought to influence each other, since almost all of them are involved in nucleic acid homeostasis.

In the present study, we established gemcitabine-resistant cells and found that the cells have two mechanisms underlying their gemcitabine resistance. The accumulation data obtained from the nucleoside transporter function inhibition experiments showed that gemcitabine uptake by CNT1 and ENT2 was decreased in the gemcitabine-resistant cells compared with that in the parental cells. In contrast, ENT2 expression in MGEM6 and MGEM8 cells was comparable with that in the parental cells as shown by immunoblotting. ENT2-dependent substrate uptake might be regulated at a functional level since disrupted plasma membrane localization of

ENT2 was previously reported to be related to gemcitabine resistance (5).

Not only CNT1 but also CNT3 has been reported to transport gemcitabine (23,24). The effect of CNT3 on gemcitabine resistance in the cells was thought to be marginal since we could not detect *CNT3* mRNA by using RT-PCR (data not shown); however, we found a clear decrease in CNT1 protein expression. *RRM1* has been recognized as a tumor suppressor gene from a study on the involvement of chromosome 11 in non-small cell lung cancer and Wilms' tumor (25,26). However, it has been controversial whether RRM1 is a favorable or poor prognosis factor (27−29). The gemcitabine metabolite dFdCDP can inhibit RRM1 (30) and RRM1 overexpression has been reported to play a role in gemcitabine resistance (10,11). Indeed, we found that RRM1 overexpression is one of the mechanisms underlying gemcitabine resistance in MGEM6 cells. *CNT1*-transfected MGEM6 cells in which the gemcitabine accumulation was similar to that of the parental cells were 24-fold more resistant to gemcitabine than the parental cells were, maybe because high amout of RRM1 can trap dFdCDP and block its inhibitory activity of RR and dFdCTP synthesis. Only knockdown of *RRM1* sufficiently suppressed the gemcitabine resistance phenotype of MGEM6 cells. Although *RRM1* kockdown induced mRNA of nucleoside transports, only CNT1 protein increased (Fig. 3D, E). These results suggested that ENT1 and ENT2 protein might be regulated with unidentified posttranscriptional regulations. Since in the cells with high RRM1 expression RRM1 can capture dFdCDP and sustain RR activity, the nucleotide salvage pathway that activates gemcitabine is dispensable. Reversely, when RRM1 expression is decreased, dFdCDP can suppress RR activity and the *de novo* pathway of dNTP synthesis (11). Under this condition, dFdCTP concentration increases and effectively induces cell death since the cancer cells are dependent on the salvage pathway for nucleotide supply.

Our data support that suppression of RRM1 expression and/or RR activity is a promising approach to overcome gemcitabine resistance in RRM1-overexpressing tumors. However, since a much higher concentration of a RRM1 inhibitor is needed to inhibit RR activity for RRM1-overexpressing cells than for sensitive cells, such a concentration will induce strong adverse effects to normal cells. From this point of view, RRM2-specific or RR holoenzyme formation inhibitors might be practical and promising agents to enhance the effect of gemcitabine. Although the effects of HU on RRM2 were reported to be small (31), in the present study we showed a synergistic effect between HU and gemcitabine only on the gemcitabine resistant cells. Unfortunately, we were unable to obtain some other RR inhibitors than used in this study as they will be more promising to overcome gemcitabine resistance (32, 33).
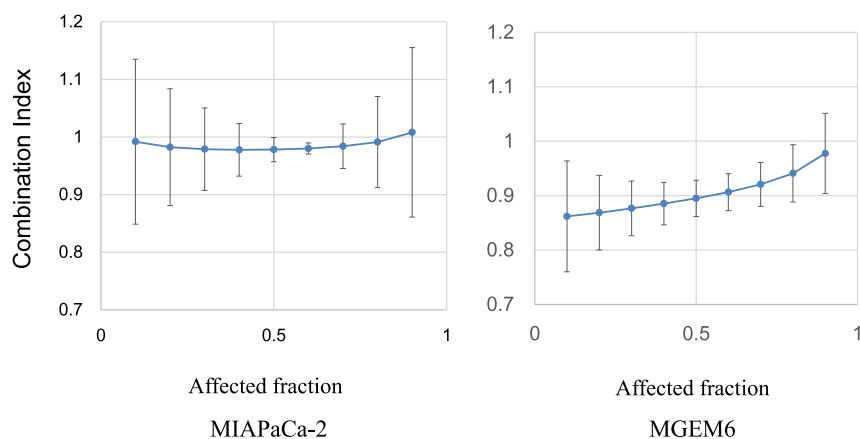

Fig. 4. CI values of gemcitabine and HU. The graphs show the CI values within a range of 0.1−0.9, with increments of 0.1, of every affected fraction of MIA PaCa-2 and MGEM6 cells.

An increase in cytidine deaminase and a decrease in DCK are two other mechanisms underlying gemcitabine resistance, although their respective increase and decrease limit the nucleotide supply from the salvage pathway. Interestingly, however, when RR activity is effectively inhibited, cancer cell growth is blocked because of this low nucleotide supply. Whether the relationship between gemcitabine and nucleotide metabolism in the gemcitabine-resistant cells is correlated with the expression of efflux transporters remains to be elucidated.

Here, we established gemcitabine-resistant cells with dual resistance mechanisms and showed that the inhibition of RR is one of the most logical and promising approaches to overcome gemcitabine resistance.

Conflict of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.jphs.2015.01.006.

References

(1) Carpelan-Holmstrom M, Nordling S, Pukkala E, Sankila R, Luttges J, Kloppel G, et al. Does anyone survive pancreatic ductal adenocarcinoma? A nationwide study re-evaluating the data of the Finnish Cancer Registry. Gut. 2005;54: 385—387.

(2) Huang P, Chubb S, Hertel LW, Grindey GB, Plunkett W. Action of 2',2'-difluorodeoxycytidine on DNA synthesis. Cancer Res. 1991;51:6110—6117.

(3) Rauchwerger DR, Firby PS, Hedley DW, Moore MJ. Equilibrative-sensitive nucleoside transporter and its role in gemcitabine sensitivity. Cancer Res. 2000;60:6075—6079.

(4) Spratlin J, Sangha R, Glubrecht D, Dabbagh L, Young JD, Dumontet C, et al. The absence of human equilibrative nucleoside transporter 1 is associated with reduced survival in patients with gemcitabine-treated pancreas adenocarcinoma. Clin Cancer Res. 2004;10:6956—6961.

(5) Nishio R, Tsuchiya H, Yasui T, Matsuura S, Kanki K, Kurimasa A, et al. Disrupted plasma membrane localization of equilibrative nucleoside transporter 2 in the chemoresistance of human pancreatic cells to gemcitabine (dFdCyd). Cancer Sci. 2011;102:622—629.

(6) Bhutia YD, Hung SW, Patel B, Lovin D, Govindarajan R. CNT1 expression influences proliferation and chemosensitivity in drug-resistant pancreatic cancer cells. Cancer Res. 2011;71:1825—1835.

(7) Ruiz van Haperen VW, Veerman G, Eriksson S, Boven E, Stegmann AP, Hermsen M, et al. Development and molecular characterization of a 2',2'-difluorodeoxycytidine-resistant variant of the human ovarian carcinoma cell line A2780. Cancer Res. 1994;54:4138—4143.

(8) Miwa M, Eda H, Ura M, Ouchi KF, Keith DD, Foley LH, et al. High susceptibility of human cancer xenografts with higher levels of cytidine deaminase to a 2'-deoxycytidine antimetabolite, 2'-deoxy-2'-methylidenecytidine. Clin Cancer Res. 1998;4:493—497.

(9) Weizman N, Krelin Y, Shabtay-Orbach A, Amit M, Binenbaum Y, Wong RJ, et al. Macrophages mediate gemcitabine resistance of pancreatic adenocarcinoma by upregulating cytidine deaminase. Oncogene. 2014;33:3812—3819.

(10) Rosell R, Felip E, Taron M, Majo J, Mendez P, Sanchez-Ronco M, et al. Gene expression as a predictive marker of outcome in stage IIB-IIIA-IIIB non-small cell lung cancer after induction gemcitabine-based chemotherapy followed by resectional surgery. Clin Cancer Res. 2004;10:4215s—4219s.

(11) Nakahira S, Nakamori S, Tsujie M, Takahashi Y, Okami J, Yoshioka S, et al. Involvement of ribonucleotide reductase M1 subunit overexpression in gemcitabine resistance of human pancreatic cancer. Int J Cancer. 2007;120: 1355—1363.

(12) Goan YG, Zhou B, Hu E, Mi S, Yen Y. Overexpression of ribonucleotide reductase as a mechanism of resistance to 2,2-difluorodeoxycytidine in the human KB cancer cell line. Cancer Res. 1999;59:4204—4207.

(13) Oguri T, Achiwa H, Sato S, Bessho Y, Takano Y, Miyazaki M, et al. The determinants of sensitivity and acquired resistance to gemcitabine differ in non-small cell lung cancer: a role of ABCC5 in gemcitabine sensitivity. Mol Cancer Ther. 2006;5:1800—1806.

(14) Hopper-Borge E, Xu X, Shen T, Shi Z, Chen ZS, Kruh GD. Human multidrug resistance protein 7 (ABCC10) is a resistance factor for nucleoside analogues and epothilone B. Cancer Res. 2009;69:178—184.

(15) Garcia-Manteiga J, Molina-Arcas M, Casado FJ, Mazo A, Pastor-Anglada M. Nucleoside transporter profiles in human pancreatic cancer cells: role of hCNT1 in 2',2'-difluorodeoxycytidine- induced cytotoxicity. Clin Cancer Res. 2003;9:5000—5008.

(16) Mata JF, Garcia-Manteiga JM, Lostao MP, Fernandez-Veledo S, Guillen-Gomez E, Larrayoz IM, et al. Role of the human concentrative nucleoside transporter (hCNT1) in the cytotoxic action of 5[Prime]-deoxy-5-fluorouridine, an active intermediate metabolite of capecitabine, a novel oral anti-cancer drug. Mol Pharmacol. 2001;59:1542—1548.

(17) Komatsu M, Sumizawa T, Mutoh M, Chen ZS, Terada K, Furukawa T, et al. Copper-transporting P-type adenosine triphosphatase (ATP7B) is associated with cisplatin resistance. Cancer Res. 2000;60:1312—1316.

(18) Owatari S, Akune S, Komatsu M, Ikeda R, Firth SD, Che XF, et al. Copper-transporting P-type ATPase, ATP7A, confers multidrug resistance and its expression is related to resistance to SN-38 in clinical colon cancer. Cancer Res. 2007;67:4860—4868.

(19) Ward JL, Sherali A, Mo ZP, Tse CM. Kinetic and pharmacological properties of cloned human equilibrative nucleoside transporters, ENT1 and ENT2, stably expressed in nucleoside transporter-deficient PK15 cells. Ent2 exhibits a low affinity for guanosine and cytidine but a high affinity for inosine. J Biol Chem. 2000;275:8375—8381.

(20) Shinsato Y, Furukawa T, Yunoue S, Yonezawa H, Minami K, Nishizawa Y, et al. Reduction of MLH1 and PMS2 confers temozolomide resistance and is associated with recurrence of glioblastoma. Oncotarget. 2013;4:2261—2270.

(21) Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul. 1984;22:27—55.

(22) Mackey JR, Yao SY, Smith KM, Karpinski E, Baldwin SA, Cass CE, et al. Gemcitabine transport in xenopus oocytes expressing recombinant plasma membrane mammalian nucleoside transporters. J Natl Cancer Inst. 1999;91: 1876—1881.

(23) Errasti-Murugarren E, Pastor-Anglada M, Casado FJ. Role of CNT3 in the transepithelial flux of nucleosides and nucleoside-derived drugs. J Physiol. 2007;582:1249—1260.

(24) Loewen SK, Yao SY, Slugoski MD, Mohabir NN, Turner RJ, Mackey JR, et al. Transport of physiological nucleosides and anti-viral and anti-neoplastic nucleoside drugs by recombinant *Escherichia coli* nucleoside-H(+) cotransporter (NupC) produced in *Xenopus laevis* oocytes. Mol Membr Biol. 2004;21:1—10.

(25) Zhao B, Bepler G. Transcript map and complete genomic sequence for the 310 kb region of minimal allele loss on chromosome segment 11p15.5 in non-small-cell lung cancer. Oncogene. 2001;20:8154—8164.

(26) Byrne JA, Little MH, Smith PJ. The M1 subunit of ribonucleotide reductase refines mapping of genetic rearrangements at chromosome 11p15. Cancer Genet Cytogenet. 1992;59:206—209.

(27) Zheng Z, Chen T, Li X, Haura E, Sharma A, Bepler G. DNA synthesis and repair genes RRM1 and ERCC1 in lung cancer. N Engl J Med. 2007;356:800—808.

(28) Bepler G, Sharma S, Cantor A, Gautam A, Haura E, Simon G, et al. RRM1 and PTEN as prognostic parameters for overall and disease-free survival in patients with non-small-cell lung cancer. J Clin Oncol. 2004;22:1878—1885.

(29) Wang Q, Liu X, Zhou J, Huang Y, Zhang S, Shen J, et al. Ribonucleotide reductase large subunit M1 predicts poor survival due to modulation of proliferative and invasive ability of gastric cancer. PLoS One. 2013;8:e70191.

(30) Heinemann V, Xu YZ, Chubb S, Sen A, Hertel LW, Grindey GB, et al. Inhibition of ribonucleotide reduction in CCRF-CEM cells by 2',2'-difluorodeoxycytidine. Mol Pharmacol. 1990;38:567—572.

(31) Shao J, Zhou B, Chu B, Yen Y. Ribonucleotide reductase inhibitors and future drug design. Curr Cancer Drug Targets. 2006;6:409—431.

(32) Sigmond J, Kamphuis JA, Laan AC, Hoebe EK, Bergman AM, Peters GJ. The synergistic interaction of gemcitabine and cytosine arabinoside with the ribonucleotide reductase inhibitor triapine is schedule dependent. Biochem Pharmacol. 2007;73:1548—1557.

(33) Zhou B, Su L, Hu S, Hu W, Yip ML, Wu J, et al. A small-molecule blocking ribonucleotide reductase holoenzyme formation inhibits cancer cell growth and overcomes drug resistance. Cancer Res. 2013;73:6484—6493.