

Increased expression of the large subunit of ribonucleotide reductase is involved in resistance to gemcitabine in human mammary adenocarcinoma cells

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Abstract

Resistance to cytotoxic nucleoside analogues is a major problem in cancer treatment. The cellular mechanisms involved in this phenomenon have been studied for several years, and some factors have been identified. However, this resistance seems to be multifactorial and more studies are needed to gain better insight into this domain. For this purpose, we developed a gemcitabine-resistant cell line (MCF7 1K) from the human mammary adenocarcinoma MCF7 strain by prolonged exposure to gemcitabine *in vitro*. MCF7 1K cells are highly resistant to gemcitabine (533-fold) and cross-resistance is observed with araC (47-fold), triapine (14-fold), and hydroxyurea (6.7-fold). Quantitative real-time reverse transcription-PCR and Western blot analysis showed an increase in the gene and protein expression of the large subunit of ribonucleotide reductase, R1. Ribonucleotide reductase activity was also significantly increased in the gemcitabine-resistant cells. Study of genomic DNA showed 12-fold increase in R1 gene dosage in MCF7 1K cells. In contrast, the gene and protein expression of the small subunit of ribonucleotide reductase, R2, were not modified in this cell line. These results show that gemcitabine resistance can be associated with genetic modifications of target genes in malignant cells, and suggest that the large subunit of human ribonucleotide reductase is involved in the cellular response to gemcitabine. [Mol Cancer Ther 2005;4(8):1268–76]

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Introduction

Gemcitabine (2',2'-difluorodeoxycytidine, dFdC) is a nucleoside analogue that shows activity against solid tumors and hematologic malignancies both as a single agent (1, 2) and in combination with other chemotherapeutic agents (3, 4). The cellular metabolism of gemcitabine is similar to that of normal deoxyribonucleosides (5). Once inside the cell, it is phosphorylated by deoxycytidine kinase to its monophosphorylated form, gemcitabine monophosphate (dFdCMP), and further by other intracellular kinases (nucleoside monophosphate kinases and nucleoside diphosphate kinases) to the metabolically active forms, gemcitabine diphosphate (dFdCDP) and gemcitabine triphosphate (dFdCTP; refs. 6–8). Diphosphorylated gemcitabine is an inhibitor of ribonucleotide reductase (9), thereby causing a decrease in dCTP pools and a decreased feedback inhibition of deoxycytidine kinase leading to an enhanced phosphorylation of gemcitabine (10), a process designated as "self-potential." The triphosphorylated form is cytotoxic by incorporation into DNA, causing masked chain termination after the incorporation of an additional nucleotide after gemcitabine (11). Cytotoxicity of gemcitabine in nondividing cells has been attributed to the fact that dFdCTP can disturb RNA metabolism by inhibiting CTP synthetase (12) or by direct incorporation into newly synthesized RNA (13).

Resistance to gemcitabine may involve a variety of mechanisms (14). In humans, cellular influx of gemcitabine involves different membrane nucleoside transporters, such as hENT1, hENT2, hCNT1, and hCNT3 (15). It has been shown that nucleoside transporter-deficient cells display resistance to gemcitabine (16). Many authors including ourselves have shown that deoxycytidine kinase-deficient cell lines display a gemcitabine-resistant phenotype (17–19). Gemcitabine and its monophosphorylated form can be inactivated by intracellular enzymes such as cytidine deaminase and dCMP deaminase, producing difluorodeoxyuridine and difluorodeoxyuridine monophosphate from dFdC and dFdCMP, respectively (6, 20). Intracellular 5'-nucleotidases are also likely to be involved in modifying gemcitabine activity by dephosphorylating dFdCMP or by modifying pools of endogenous deoxyribonucleotides, although it has been shown that three of these (cytoplasmic 5'-nucleotidase II, cytoplasmic 5'-deoxynucleotidase, and mitochondrial 5'-deoxynucleotidase) do not dephosphorylate dFdCMP *in vitro* (21). Finally, cellular gemcitabine resistance can be due to increased ribonucleotide reductase expression (22).

Ribonucleotide reductase is the key enzyme in the synthesis of intracellular deoxynucleotide triphosphates,

catalyzing the reduction of the corresponding nucleoside diphosphate. Active ribonucleotide reductase is a heterotetrameric enzyme composed of two homodimers of nonidentical subunits. The large subunit, R1, contains the substrate binding and catalytic site as well as the allosteric effector sites. The small subunit, R2, contains a diiron site and a tyrosyl radical that is essential for catalysis. In terms of chemoresistance, an overexpression of R2 has been repeatedly reported in hydroxyurea-resistant cell lines (23–25), and more recently in gemcitabine- and fludarabine-resistant cells (26, 27). Overexpression of the large subunit of ribonucleotide reductase, R1, has been recently observed *in vitro* in gemcitabine-resistant cells (22) as well as in non-small-cell lung cancer patients who were poor responders to a combination of gemcitabine and cisplatin (28).

As gemcitabine is a promising compound in the treatment of breast cancer, we developed a model of human breast cancer resistant to gemcitabine. Here we describe the development and the characterization of this line and identify cellular mechanisms likely to be involved in gemcitabine resistance.

Materials and Methods

Reagents

Compounds used for *in vitro* experiments were araC (cytarabine, Pharmacia & Upjohn, Saint-Quentin-en-Yvelines, France), cisplatin (Merck, Lyon, France), gemcitabine (Lilly, Indianapolis, IN), doxorubicin (Pharmacia & Upjohn), etoposide (Laboratoire Sandoz, Rueil-Malmaison, France), 5-fluorouracil (Produits Roche, Neuilly-sur-Seine, France), cyclopentenyl cytosine (a gift from V. Marquez, National Cancer Institute, Frederick, MD), triapine (a gift from Vion Pharmaceuticals, Inc., New Haven, CT), didox, and trimidox (gifts from Molecules for Health, Inc., Richmond, VA). Hydroxyurea, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, isopropanolol, and NaCl were purchased from Sigma-Aldrich (St. Quentin Fallavier, France) and HCl from Merck (Strasbourg, France). TRIzol RNA extraction kit, Moloney murine leukemia virus (MMLV) reverse transcriptase, Taq DNA Polymerase, and DMEM cell culture media were purchased from Invitrogen (Cergy Pontoise, France), L-glutamine and penicillin-streptomycin from Life Technologies, Inc. (Cergy Pontoise, France), and fetal bovine sera from PAN Biotech GmbH (Aidenbach, Germany).

Cell Culture

Human breast adenocarcinoma MCF7 cells were grown in DMEM media containing L-glutamine, penicillin (200 IU/mL), streptomycin (200 µg/mL), and 10% fetal bovine serum at 37°C in presence of 5% CO₂. The gemcitabine-resistant MCF7 1K cell line was obtained by stepwise increases in gemcitabine concentration *in vitro*, up to a concentration of 1 µmol/L, over a period of 6 months. MCF7 wild-type (MCF7 wt) designates the parental line which was cultivated in the absence of exposure to gemcitabine.

Cytotoxicity Studies

MCF7 cells were plated at 1,000 cells per well in 96-well plates (Becton Dickinson, San Jose, CA) in a volume of 100 µL and incubated for 24 hours at 37°C before drugs were added at different concentrations. After incubation at 37°C for 6 days, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (100 µg) was added and, after 2 hours of incubation at 37°C, the supernatant was replaced with 100 µL isopropanol/H₂O/HCl (90:9:1, v/v/v) to solubilize the formazan crystals. Spectrophotometric determination of absorbance was done using a microplate reader (Labsystem Multiskan RC). The IC₅₀ was defined as the concentration inhibiting proliferation to a level equal to 50% of that of controls and the resistance ratio was the ratio between the IC₅₀ of the gemcitabine-resistant MCF7 1K cell line and the IC₅₀ of the sensitive parental cell line MCF7 wt. The statistical significance was determined by comparison of mean IC₅₀ values for MCF7 wt and MCF7 1K cell lines using Student's *t* test and STATISTICA version 6 (StatSoft, Inc., Tulsa, OK). The value of resistance ratio expressed is the mean ± SE of the calculated resistance ratios for the three different experiments.

Quantitative Real-time Reverse Transcription-PCR

Total mRNA was extracted using TRIzol as previously described (29), then quantitative reverse transcription-PCR (RT-PCR) was done as described (18) using primers, probes, and PCR conditions as described in Table 1. Results were analyzed with RelQuant software (Roche, Mannheim, Germany) as indicated in the user manual, and expressed as mean values of three experiments in arbitrary units using one sample of the parental MCF7 wt cell line as reference. The statistical significance was determined by comparison of mean expression values in MCF7 wt and MCF7 1K cell lines using Student's *t* test and STATISTICA version 6 (StatSoft).

Western Blot

Protein expression was determined by Western blot analysis. Proteins were extracted from MCF7 wt and MCF7 1K cells with cold lysis buffer [20 mmol/L Tris-HCl (pH 6.8), 1 mmol/L MgCl₂, 2 mmol/L EGTA, 0.5% NP40, 1 mg/mL soybean trypsin inhibitor, 100 µg/mL leupeptin, 100 µg/mL aprotinin, 30 mg/mL benzamide, 1 mg/mL *N*-tosyl-L-phenylalanine chloromethyl, and 5 mg/mL phenylmethylsulfonyl potassium]. Protein concentrations were determined by the Bradford assay. Similar amounts of protein were separated by SDS-PAGE using 12% acrylamide and transferred onto a nitrocellulose membrane (Hybond-ECL, Amersham Corp. Buckinghamshire, United Kingdom). Membranes were incubated with primary antibodies directed against ribonucleotide reductase R1 or ribonucleotide reductase R2 (1:250 dilutions, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), then incubated with peroxidase-conjugated secondary antibodies. Protein signals were detected by enhanced chemiluminescence (Amersham) and by exposure to Kodak film (Eastman Kodak Company, New Haven, CT). The presence of equal amounts of proteins in each

Table 1. Primers, probes, and PCR conditions used in quantitative RT-PCR studies

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Probe (5'-3)	MgCl ₂ (mmol/L)	PCR conditions
<i>hENT-1</i>	GCTGGGTCTG ACCGTCGTAT	CGTTACAGGG TGCATGATGG	—	3	95°C/15 s 58°C/5 s 72°C/10 s
<i>hENT-2</i>	ATGAGAACGGG ATTCCCAGTAG	GCTCTGATTG CGGCTCCTT	CAGAAAGTAGCTCTG ACCCTGGATCTTGACCT	3	95°C/15 s 60°C/60 s
<i>hCNT-1</i>	TCTGTGGATT GCCAATTCAG	CGGAGCACTAT CTGGGAGAAGT	—	2	95°C/0 s 58°C/5 s 72°C/4 s
<i>MRP5</i>	CCCAGGCAAC AGAGTCTAACC	CGGTAATCA ATGCCCAAGTC	—	—	95°C/15 s 55°C/5 s 72°C/9 s
<i>dCK</i>	AAACCTGAACG ATGGTCTTTTACC	CTTTGAGCTTG CCATTCAGAGA	CAAACATATGCCTGTCT CAGTCGAATAAGAGCTC	2	95°C/15 s 60°C/60 s
<i>dGK</i>	ACCCAGAATG GCACGTAGCTA	CCTGATAGATA TGCCACTCGATG	—	3	95°C/0 s 62°C/4 s 72°C/14 s
<i>TK1</i>	GGGGCAGATC CAGGTGATTC	GCATACTTGAT CACAGGCACTT	—	2	95°C/0 s 58°C/5 s 72°C/4 s
<i>TK2</i>	TTACCTTCGG ACCAATCCTG	TGCTTCCGAT TCTCTGGAGT	—	2	95°C/0 s 60°C/10 s 72°C/12 s
<i>UMP-CMPK</i>	GGGCATATT CTTTGCTTCCA	TGCATTTCAA GGTTCCACTG	—	3	95°C/10 s 53°C/5 s 72°C/8 s
<i>NDPK-2</i>	ATGCAGTGCG GCCTGGTGGG	GACCCAGTCAT GAGCACAAGAC	—	3	95°C/15 s 63°C/5 s 72°C/16 s
<i>cN-II</i>	ACCTGCTGTATT ACCTTTCAGCTA	GCTCCACCG TTGATTCATGA	CTCTTCAGGGCTG CCCATGTCTTGA	2	95°C/15 s 60°C/60 s
<i>cN-III</i>	AATCGGCGA TGTACTAGAG	CATCTGCCAT TCTTAAGTCTC	—	2	95°C/15 s 58°C/5 s 72°C/10 s
<i>cdN</i>	GGACACGCAG GTCTTCATCTG	GCGGTACTTC TCACCCACACA	CCAGCCCCCTGCT GAAGTACCACC	2	95°C/15 s 60°C/60 s
<i>mdN</i>	CATCAGCATT TGGGAGTCAA	CGACACAATC TGCTCCAGAA	CGTCTTCATCTG CACAAGCCCCA	3	95°C/15 s 60°C/60 s
<i>RNR R1</i>	GCAGCTGAG AGAGGTGCTTT	CAGGATCCAC ACATCAGACA	—	3	95°C/15 s 58°C/5 s 72°C/10 s

(Continued on the following page)

Table 1. Primers, probes, and PCR conditions used in quantitative RT-PCR studies (Cont'd)

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Probe (5'-3')	MgCl ₂ (mmol/L)	PCR conditions
<i>RNR R2</i>	GAGTTCCTC ACTGAGGCC	TTAGAAGTCA GCATCCAAG	—	3	95°C/15 s 58°C/5 s 72°C/10 s
<i>DNA Polymerase α2</i>	AGCTTGACCT GATTGCTGTC	ATGACGGGAC AAAGACAAGG	—	4	95°C/15 s 60°C/5 s 72°C/10 s 83°C/2 s
<i>dCMPd</i>	GTCGCCTTGT TCCCTTGTA	TCTTGCTGCA CTTCGGTATG	—	—	95°C/15 s 58°C/5 s 72°C/10 s
<i>CTP-S</i>	GTGGCGAAAT ACACCGAGTT	TCCTCGAACA CCAAATCCTC	—	—	95°C/15 s 58°C/5 s 72°C/10 s

NOTE: SYBR Green was used for detection of DNA amplification when no probe is cited.

Abbreviations: *hENT-1*, human equilibrative nucleoside transporter 1; *hENT-2*, human equilibrative nucleoside transporter 2; *hCNT-1*, human concentrative nucleoside transporter 1; *MRP5*, multidrug resistance protein 5; *dCK*, deoxycytidine kinase; *dGK*, deoxyguanosine kinase; *TK1*, thymidine kinase 1; *TK2*, thymidine kinase 2; *UMP-CMPK*, uridylate-cytidylate kinase; *NDPK-2*, nucleoside diphosphate kinase 2 or nm23-B; *cN-II*, cytoplasmic 5'-nucleotidase II; *cN-III*, cytoplasmic 5'-nucleotidase III; *cdN*, cytoplasmic 5'-deoxynucleotidase; *mdN*, mitochondrial 5'-deoxynucleotidase; *RNR R1*, large subunit of ribonucleotide reductase; *RNR R2*, small subunit of ribonucleotide reductase; *dCMPd*, dCMP deaminase; *CTP-S*, CTP synthetase.

sample was checked by Ponceau red staining of the membrane. Quantification of the intensity of bands corresponding to R1 and R2 was done using Kodak Digital Science 1D (Eastman Kodak Company).

Ribonucleotide Reductase Activity

Ribonucleotide reductase activity was measured as described elsewhere (30). Briefly, exponentially growing MCF7 wt and MCF7 1K cells (100×10^6) were harvested and resuspended in 700 μ L of buffer 1 (100 mmol/L HEPES, 15 mmol/L Mg(CH₃COO)₂, pH 7.6) and mechanically disrupted. After centrifugation ($100,000 \times g$, 20 minutes, 4°C), the proteins present in the supernatant were precipitated with 45% (NH₄)₂SO₄. Pellets were resuspended in 150 μ L of buffer 2 (buffer 1 plus DTT 10 mmol/L). Equilibrated Bio-Spin 6 columns (Bio-Rad, Hercules, CA) were used to desalt these solutions and ribonucleotide reductase activity was measured using 20, 30, and 40 μ L of these extracts in a final volume of 50 μ L containing 5 mmol/L ATP, 10 mmol/L DTT, 100 μ mol/L CDP, and [5-³H]CDP (specific activity, 659 GBq/mmol). After incubation for 50 minutes at 33°C, the nucleotides were dephosphorylated by *C. adamanteus* venom at 37°C for 2 hours. Reaction products (deoxycytidine and deoxyuridine) were separated from reaction substrates (cytidine and uracil) by high performance liquid chromatography using an Uptisphere 5 μ m ODB column (Interchrom, Montlucon, France), and detected by a Berthold radioactivity detector. The mobile phase was composed of 10 mmol/L CH₃COONa (pH 4.8), methanol 4.25%, and low UV Pic-B5 1% (Waters, Saint-Quentin en Yvelines, France). Under these conditions, retention times

were 10.15 minutes for cytidine, 10.42 minutes for uracil, 15.22 for deoxycytidine, and 15.17 for deoxyuridine. The statistical significance was determined using Student's *t* test and STATISTICA version 6 (StatSoft).

Analysis of Ribonucleotide Reductase R1 Gene Copy Number

Genomic DNA was prepared from MCF7 wt and MCF7 1K cells with the phenol/chloroform extraction method. Exon 12 of ribonucleotide reductase R1 was amplified using a Lightcycler thermal cycler (Roche, Mannheim, Germany) in a final reaction volume of 6.67 μ L containing forward (5'-AACAAAGGTCGTGCCGAAA-3') and reverse (5'-CATCTTTGCTGGTGACTCC-3') primers (300 nmol/L each), MgCl₂ (2 mmol/L), deoxynucleotide triphosphates (500 μ mol/L), LC-FastStart DNA Master SYBR Green, and genomic DNA (50.1 ng) or an R1-containing pET3a vector (0.015-1.5 amol; a generous gift from Prof. L. Thelander, Umeå University, Sweden; ref. 31). Cycling conditions were 15 seconds at 95°C followed by 5 seconds at 58°C and 10 seconds at 72°C. Results were analyzed with RelQuant software (Roche, Mannheim, Germany) as indicated in the user manual. The statistical significance was determined using Student's *t* test and STATISTICA version 6 (StatSoft).

Results

Development and Characterization of Gemcitabine-Resistant MCF7 1K Cells

Gemcitabine-resistant MCF7 1K cells were developed by continuous exposure to increasing concentrations of gemcitabine over a period of 6 months. The initial concentration for selection of resistant cells was 2 nmol/L, with a doubling

of this concentration every 2 to 3 weeks. The cells obtained (MCF7 1K) were viable in a media containing 1 $\mu\text{mol/L}$ of gemcitabine and showed no apparent morphologic differences or differences in growth rate in comparison with the parental MCF7 wt cells grown in the absence of gemcitabine (data not shown).

IC₅₀ values and resistance ratios for the gemcitabine-resistant MCF7 1K cell line and the sensitive parental cell line MCF7 wt are listed in Table 2. MCF7 1K cells were 533-fold less sensitive to gemcitabine than the parental cell line MCF7 wt. Significant cross-resistance was observed for the nucleoside analogue araC (resistance ratio, 47) and the ribonucleotide reductase inhibitors triapine (resistance ratio, 14) and hydroxyurea (resistance ratio, 6.7). In addition, a weak cross-resistance was observed for doxorubicin (resistance ratio, 1.1). Conversely, MCF7 1K cells showed no decreased sensitivity to the ribonucleotide reductase inhibitors didox and trimidox, nor to cyclopentenyl cytosine, cisplatin, 5-fluorouracil, and etoposide.

Identification of Genes Involved in Resistance by Quantitative RT-PCR

To identify cellular mechanisms responsible for the resistance to nucleoside analogues observed in MCF7 1K cells, we quantified the expression of genes known to be involved in the metabolism and the cytotoxicity of nucleoside analogues by real-time quantitative RT-PCR (Table 3). The major difference between the two cell lines concerned the large subunit of ribonucleotide reductase, R1. We observed that the level of ribonucleotide reductase R1 mRNA was increased 17-fold in the gemcitabine-resistant cell line ($P < 0.01$). Additional significant modifications in the expression pattern of genes involved in metabolism of nucleoside analogues included a 2.1-fold increase in expression of the membrane efflux pump multidrug resistance protein 5 ($P < 0.01$), a 1.8-fold increase in expression of mitochondrial 5'-deoxynucleotidase (5'-3'-deoxynucleotidase 2; $P < 0.05$), and a 1.5-fold increase in expression of DNA polymerase $\alpha 2$ ($P < 0.05$). As for the membrane

nucleoside transporters studied (hENT-1, hENT-2, and hCNT-1), no significant difference was found. However, the expression of all three of these transporters was slightly lower in MCF7 1K cells, suggesting that the overall nucleoside transport might be altered in this cell line.

The expression levels of the nucleoside and nucleotide kinases deoxycytidine kinase, deoxyguanosine kinase, thymidine kinase 1, thymidine kinase 2, uridylate-cytidylate-kinase, and nucleoside diphosphate kinase 2 were not modified. Finally, no difference was found in the expression levels of the 5'-nucleotidases cytoplasmic 5'-nucleotidase II, cytoplasmic 5'-nucleotidase III (pyrimidine 5'-nucleotidase I), and cytoplasmic 5'-deoxynucleotidase (5'-3'-deoxynucleotidase 1 and pyrimidine 5'-nucleotidase I), the small subunit of ribonucleotide reductase (R2), and various intracellular gemcitabine targets such as CTP synthase and the dFdCMP-inactivating protein dCMP deaminase. Thus, the quantitative RT-PCR study suggested that the nucleoside analogue resistance observed in MCF7 1K cells could be due to increased ribonucleotide reductase R1 expression.

Ribonucleotide Reductase Protein Levels

A high expression of ribonucleotide reductase R1 in MCF7 1K cells was confirmed at the protein level by Western blot (Fig. 1). The quantification of the bands corresponding to R1 and R2 on a scanned film showed a 5.2-fold increase in R1 protein in MCF7 1K cells, whereas R2 expression was only increased 1.3-fold in these cells.

Ribonucleotide Reductase Activity

We compared the overall ribonucleotide reductase activity in the two cell lines by a specific CDP reductase assay. A significant 2.8-fold increase in ribonucleotide reductase activity was observed in the gemcitabine-resistant MCF7 cell line (51 ± 2.7 pmol/min/mg protein in MCF7 wt versus 134 ± 17 pmol/min/mg protein in MCF7 1K; $P < 0.01$; Fig. 2). The increase of R1 expression thus seems to be sufficient to increase ribonucleotide reductase activity in MCF7 cells.

Table 2. Sensitivity of gemcitabine-resistant MCF7 cells towards various anticancer drugs

	IC ₅₀ MCF7 wt ($\mu\text{mol/L}$)	IC ₅₀ MCF7 1K ($\mu\text{mol/L}$)	Resistance ratio	<i>P</i>
Gemcitabine	0.00037 \pm 0.00015	0.20 \pm 0.10	533 \pm 58	0.026
AraC	0.017 \pm 0.012	0.80 \pm 0.28	47 \pm 19	0.011*
Triapine	0.33 \pm 0.15	4.3 \pm 0.58	14 \pm 5.1	0.00032*
Hydroxyurea	133 \pm 57	833 \pm 153	6.7 \pm 1.5	0.0018*
Didox	80 \pm 17	87 \pm 23	1.1 \pm 0.064	0.71
Trimidox	67 \pm 15	70 \pm 10	1.1 \pm 0.17	0.77
Cisplatin	3.7 \pm 0.58	13 \pm 5.8	3.6 \pm 1.3	0.88
Doxorubicin	0.14 \pm 0.05	0.15 \pm 0.04	1.1 \pm 0.12	0.045*
5-Fluorouracil	1.1 \pm 0.76	1.3 \pm 0.58	1.3 \pm 0.34	0.73
Cyclopentenyl cytosine	0.011 \pm 0.0081	0.012 \pm 0.0068	1.2 \pm 0.35	0.80
Etoposide	0.50 \pm 0.20	0.53 \pm 0.25	1.1 \pm 0.033	0.87

NOTE: IC₅₀ values were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and resistant ratios were calculated for MCF7 wt and MCF7 1K cells as described in Materials and Methods. All experiments were done thrice. *P* values were obtained with Student's *t* test.

*Statistically significant.

Table 3. Gene expression of nucleoside analogue – related genes in gemcitabine-resistant MCF7 cells

	Gene	MCF7 wt	MCF7 1K	P
Transporters	<i>hENT-1</i>	0.85 ± 0.17	0.73 ± 0.031	0.27
	<i>hENT-2</i>	0.91 ± 0.081	0.83 ± 0.050	0.22
	<i>hCNT-1</i>	1.3 ± 0.38	0.85 ± 0.21	0.15
	<i>MRP5</i>	1.1 ± 0.14	2.4 ± 0.41	0.0078*
Kinases	<i>dCK</i>	0.65 ± 0.38	0.90 ± 0.035	0.32
	<i>dGK</i>	0.76 ± 0.31	1.01 ± 0.084	0.17
	<i>TK1</i>	1.3 ± 0.69	0.78 ± 0.24	0.28
	<i>TK2</i>	0.65 ± 0.30	1.0 ± 0.41	0.26
	<i>UMP-CMPK</i>	1.2 ± 0.19	1.5 ± 0.47	0.34
	<i>NDPK-2</i>	0.90 ± 0.089	1.2 ± 0.22	0.12
5'-Nucleotidases	<i>cN-II</i>	0.92 ± 0.22	0.78 ± 0.19	0.45
	<i>cN-III</i>	1.1 ± 0.26	1.6 ± 0.33	0.11
	<i>cdN</i>	0.81 ± 0.29	1.1 ± 0.21	0.26
	<i>mdN</i>	0.79 ± 0.30	1.4 ± 0.050	0.023*
Other	<i>RNR R1</i>	1.1 ± 0.057	18 ± 5.8	0.0070*
	<i>RNR R2</i>	1.1 ± 0.10	0.91 ± 0.33	0.37
	<i>DNA polymerase α2</i>	1.1 ± 0.18	0.72 ± 0.087	0.042*
	<i>dCMP d</i>	0.85 ± 0.13	0.79 ± 0.16	0.68
	<i>CTP-S</i>	0.97 ± 0.11	1.1 ± 0.24	0.48

NOTE: mRNA levels are expressed as arbitrary PCR units in MCF7 wt and MCF7 1K. All experiments were done thrice.

Abbreviations: *hENT-1*, human equilibrative nucleoside transporter 1; *hENT-2*, human equilibrative nucleoside transporter 2; *hCNT-1*, human concentrative nucleoside transporter 1; *MRP5*, multidrug resistance protein 5; *dCK*, deoxycytidine kinase; *dGK*, deoxyguanosine kinase; *TK1*, thymidine kinase 1; *TK2*, thymidine kinase 2; *UMP-CMPK*, uridylylate-cytidylylate kinase; *NDPK-2*, nucleoside diphosphate kinase 2; *cN-II*, cytoplasmic 5'-nucleotidase II; *cN-III*, cytoplasmic 5'-nucleotidase III; *cdN*, cytoplasmic 5'-deoxynucleotidase; *mdN*, mitochondrial 5'-deoxynucleotidase; *RNR R1*, large subunit of ribonucleotide reductase; *RNR R2*, small subunit of ribonucleotide reductase; *dCMPd*, dCMP deaminase; *CTP-S*, CTP synthetase.

* $P < 0.05$ with Student's *t* test.

Ribonucleotide Reductase R1 Gene Copy Number

We continued the study of ribonucleotide reductase R1 in MCF7 1K cells by a quantitative determination of *R1* gene copies in genomic DNA. Using an R1-containing pET3a vector as a standard and quantitative real-time PCR, we found that MCF7 1K cells contain 12 times more ribonucleotide reductase *R1* gene copies than MCF7 wt cells (6.0 ± 1.6 mol *R1*/g of genomic DNA in MCF7 1K versus 0.50 ± 0.17 mol *R1*/g of genomic DNA in MCF7 wt; $P < 0.01$; Fig. 3). This result indicates that gemcitabine resistance is associated with a high level of amplification of *R1* gene in resistant MCF7 cells.

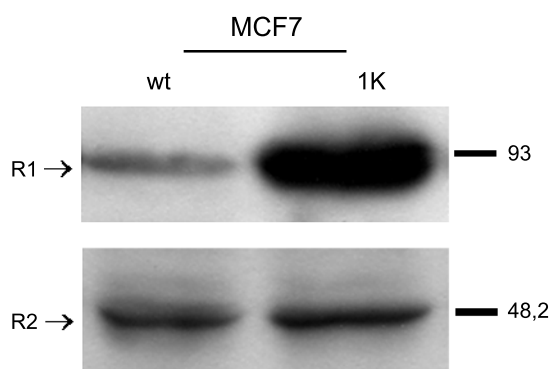


Figure 1. Expression of ribonucleotide reductase R1 and R2 in gemcitabine-resistant (1K) and sensitive (wt) MCF7 cells. Western blot analysis in MCF7 wt and MCF7 1K cells using anti-ribonucleotide reductase R1 and anti-ribonucleotide reductase R2 antibodies.

Discussion

Gemcitabine is the first member of the nucleoside analogue family used for the treatment of solid tumors. It has shown activity against malignant cells from a variety of tumors, and is currently indicated in the treatment of lung, pancreatic, and bladder cancer. Several studies have suggested that gemcitabine is also active against breast cancer. To provide insight on the mechanisms of gemcitabine resistance likely to occur in breast cancer patients treated with this compound, we have developed a gemcitabine-resistant human mammary adenocarcinoma cell line derived from MCF7 cells. The resistant cell line MCF7 1K is 533-fold less sensitive to the cytotoxic action of gemcitabine than the parental cell line MCF7 wt, and displays cross-resistance to the pyrimidine analogue araC and, to a smaller extent, the ribonucleotide reductase inhibitors triapine and hydroxyurea. In contrast, no modification in the sensitivity to the two recent ribonucleotide reductase inhibitors didox and trimidox was observed.

The search for potential mechanisms of resistance in the MCF7 1K cells showed significantly increased levels of subunit R1 of the target enzyme ribonucleotide reductase, the membrane efflux protein multidrug resistance protein 5, and mitochondrial 5'-deoxynucleotidase, as well as a significant decrease in the expression level of DNA polymerase. The major difference observed was a 17-fold increase in the expression of the R1 subunit of ribonucleotide reductase, which was found to be due to gene amplification and was confirmed at the protein level and by

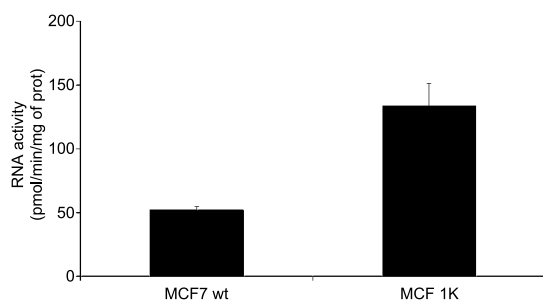


Figure 2. Ribonucleotide reductase activity in gemcitabine-sensitive (*wt*) and gemcitabine-resistant (*1K*) MCF7 cells. Columns, mean of three independent experiments. $P < 0.01$ (Student's *t* test).

enzymatic assays. Because some of the cytotoxic activity of gemcitabine is due to ribonucleotide reductase inhibition by the diphosphorylated metabolite (9), this increased activity could explain the resistance to this molecule as well as to the ribonucleotide reductase inhibitors.

The other differences observed between parental MCF7 and resistant MCF7 cells might also play a role in the resistance to gemcitabine in the MCF7 1K cells. Increased efflux of gemcitabine by multidrug resistance protein 5, increased degradation of dFdCMP by mitochondrial 5'-deoxynucleotidase, and decreased incorporation of dFdCTP into DNA by DNA polymerases could reduce the cytotoxic activity of this compound. Resistance to gemcitabine in MCF7 cells is thus likely to be multifactorial, and the prominent mechanism seems to be overexpression of ribonucleotide reductase. This gene expression study can explain the cross-resistance phenotype of the MCF7 1K cells we observed. As cited above, the ribonucleotide reductase R1 overexpression would be likely to be responsible for the cross-resistance towards the ribonucleotide reductase inhibitors triapine and hydroxyurea. In addition, the cross-resistance towards araC can be explained by eventual modifications in endogenous deoxyribonucleotides due to ribonucleotide reductase overexpression as well as to decreased DNA polymerase expression level.

Gemcitabine resistance has been associated with gene amplification and increased expression of the small ribonucleotide reductase subunit R2 (26). In our human mammary adenocarcinoma cell model of gemcitabine resistance, we found such modifications for the large subunit R1. Our results are strengthened by those obtained in a gemcitabine-resistant non-small-cell lung cancer cells (22). These authors reported that ribonucleotide reductase R1 mRNA was enhanced 125-fold in 62-fold resistant cells. This was associated with an increased protein content and yet a statistically nonsignificant 2.6-fold increase in ribonucleotide reductase activity. In addition, a 1.4-fold increase in ribonucleotide reductase activity was observed in R1-transfected oropharyngeal carcinoma cells, showing that an overexpression of this subunit might be sufficient to increase enzyme activity (32).

Active ribonucleotide reductase is a tetrameric enzyme with two R1 molecules associated with two R2 molecules.

The expression of R1 protein is constant throughout the cell cycle (33) whereas R2 is expressed only during S-G₂ phases (34). It is generally considered that R2 is the limiting subunit for activity (33, 35–37). However, R1 expression has been proposed to regulate ribonucleotide reductase activity in a mouse leukemia cell line (35). In agreement with this, we observed an increase in ribonucleotide reductase activity associated only with ribonucleotide reductase R1 overexpression in MCF7 1K cells, suggesting that MCF7 wt cells contain some free R2 protein. Alternatively, unidentified posttranslational modifications of R2 protein might have occurred in MCF7 1K cells resulting in increased protein activity.

Ribonucleotide reductase R1 overexpression might be involved in gemcitabine resistance through various mechanisms. First, dFdCDP has been shown to bind to the R1 subunit (9). Increased R1 protein content could contribute to the sequestration of dFdCDP, thereby reducing its further activation into the main metabolite, dFdCTP. Alternatively, an increase in R1 protein, one of the targets of gemcitabine, might explain reduced inhibition of ribonucleotide reductase activity, one of the potential mechanisms of cytotoxicity of gemcitabine in tumor cells. Finally, increased ribonucleotide reductase activity could induce higher production and accumulation of intracellular dCTP. Gemcitabine DNA incorporation would subsequently decrease because of more intense competition with this endogenous compound. Increased intracellular dCTP concentration will also inhibit deoxycytidine kinase and thereby decrease the phosphorylation of gemcitabine inside cells.

Ribonucleotide reductase R1 gene amplification seems to be the mechanism underlying R1 overexpression observed in MCF7 1K cells (Fig. 3). However, other possible explanations were studied. A regulatory region of mRNA stability has been identified in the 3' untranslated region of R1 mRNA (38). The interaction between the sequence CAACTAC located 37 bp after the stop codon and a yet unidentified protein (R1 binding protein) seems to decrease R1 mRNA stability. Therefore, any modification of this sequence could be involved in the increased stability of R1 transcript. Although no difference was observed in the 3' untranslated sequence between MCF7 wt and MCF7 1K

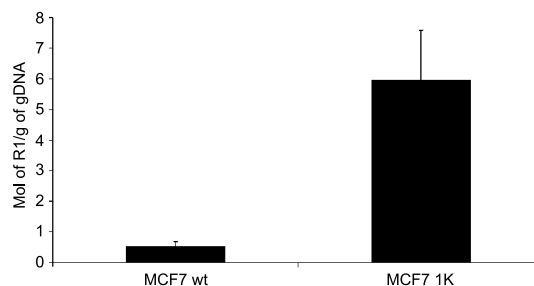


Figure 3. Quantification by quantitative PCR of ribonucleotide reductase R1 gene copies in genomic DNA in gemcitabine-sensitive (*wt*) and gemcitabine-resistant (*1K*) MCF7 cells. Columns, mean of three independent experiments. $P < 0.01$ (Student's *t* test).

cells (data not shown), a modification in R1 binding protein could be involved in increased R1 expression. We have also sequenced portions of the R1 promoter, but no modification was observed in the gemcitabine-resistant cells (data not shown). Similarly, modifications in transcription factors could be involved in altered R1 expression in MCF7 1K cells.

The amplification of the R1 gene in the gemcitabine-resistant cells could be compared with the results of Schuetz et al. (39). They found an 800- to 1,000-fold amplification of *multidrug resistance protein 4* gene in CCRF-CEM cells resistant to the antiviral nucleoside analogue 9-(2-phosphonylmethoxyethyl)adenine. Others have observed an amplification of the R2 gene in gemcitabine- or hydroxyurea-resistant cells (25, 40). The multiplication of genes during induction of resistance is therefore a well-known phenomenon.

In conclusion, we have developed a gemcitabine-resistant human mammary adenocarcinoma cell model with increased gene and protein expression of the large subunit of ribonucleotide reductase and increased ribonucleotide reductase activity. This study underscores the implication of ribonucleotide reductase R1 in gemcitabine activity and resistance mechanisms. Ongoing studies on R1 inhibition should show whether its overproduction in MCF7 1K cells is responsible for the chemoresistant phenotype.

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