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

Lars Petter Jordheim,[1] Olivier Guittet,[2] Michel Lepoivre,[2] Carlos M. Galmarini,[1] and Charles Dumontet[1]

[1]Institut National de la Sante et de la Recherche Medicale U590, Laboratoire de Cytologie Analytique, Faculté de Médecine Rockefeller, Lyon, France and [2]Unité Mixte de Recherche, Centre National de la Recherche Scientifique 8619, Institut de Biochimie et de Biophysique Moléculaire et Cellulaire, Université de Paris XI, Orsay, France

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]

Received 4/22/05; revised 6/9/05; accepted 6/14/05.

Grant support: Mjålands Stiftelse for Kreftforskning and EWS Stiftelsen, Association pour la Recherche sur le Cancer (L.P. Jordheim), Comité du Rhône de la Ligue Contre le Cancer, and Ligue Contre le Cancer Research Fellowship (O. Guittet).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Lars P. Jordheim, Laboratoire de Cytologie Analytique, 8, Avenue Rockefeller, 69008 Lyon, France.
E-mail: jordheim@yahoo.com

Copyright © 2005 American Association for Cancer Research.

doi:10.1158/1535-7163.MCT-05-0121

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-potentiation.'' 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 syntethase (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 monophosphoralyted 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,

Downloaded from http://aacrjournals.org/mct/article-pdf/4/8/1268/1870646/1268-1276.pdf by guest on 18 April 2024

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-fluorouracile (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% CO_2. 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/H_2O/HCl (90:9:1, v/v/v) to solubilize the formazan crystals. Spectrophotometric determination of absorbance was done using a microplate reader (Labsystem Multiskanner RC). The IC_{50} 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 IC_{50} of the gemcitabine-resistant MCF7 1K cell line and the IC_{50} of the sensitive parental cell line MCF7 wt. The statistical significance was determined by comparison of mean IC_{50} 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 $MgCl_2$, 2 mmol/L EGTA, 0.5% NP40, 1 mg/mL soybean trypsin inhibitor, 100 μg/mL leupeptin, 100 μg/mL aprotinin, 30 mg/mL benzamidine, 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

Downloaded from http://aacrjournals.org/mct/article-pdf/4/8/1268/1870646/1268-1276.pdf by guest on 18 April 2024

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

(Continued on the following page)

Downloaded from http://aacrjournals.org/mct/article-pdf/4/8/1268/1870646/1268-1276.pdf by guest on 18 April 2024

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
RNR R2	GAGTTCCTC ACTGAGGCC	TTAGAAGTCA GCATCCAAG	—	3	95°C/15 s 58°C/5 s 72°C/10 s
DNA Polymerase α2	AGCTTGACCT GATTGCTGTC	ATGACGGGAC AAAGACAAGG	—	4	95°C/15 s 60°C/5 s 72°C/10 s 83°C/2 s
dCMPd	GTCGCCTTGT TCCCTTGTAA	TCTTGCTGCA CTTCGGTATG	—	—	95°C/15 s 58°C/5 s 72°C/10 s
CTP-S	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 μL of buffer 1 (100 mmol/L HEPES, 15 mmol/L $Mg(CH_3COO)_2$, pH 7.6) and mechanistically disrupted. After centrifugation (100,000 × *g*, 20 minutes, 4°C), the proteins present in the supernatant were precipitated with 45% $(NH_4)_2SO_4$. Pellets were resuspended in 150 μL 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 μL of these extracts in a final volume of 50 μL containing 5 mmol/L ATP, 10 mmol/L DTT, 100 μmol/L CDP, and [5-^{3}H]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 μm ODB column (Interchrom, Montlucon, France), and detected by a Berthold radioactivity detector. The mobile phase was composed of 10 mmol/L CH_3COONa (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 μL containing forward (5′-AACAAGGTCGTGTCCGCAAA-3′) and reverse (5′-CATCTTTGCTGGTGTACTCC-3′) primers (300 nmol/L each), $MgCl_2$ (2 mmol/L), deoxynucleotide triphosphates (500 μmol/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

Downloaded from http://aacrjournals.org/mct/article-pdf/4/8/1268/1870646/1268-1276.pdf by guest on 18 April 2024

of this concentration every 2 to 3 weeks. The cells obtained (MCF7 1K) were viable in a media containing 1 μmol/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).

IC_{50} 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-fluorouracile, 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 α2 ($P < 0.05$). 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 synthethase 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 (μmol/L)	IC_{50} MCF7 1K (μmol/L)	Resistance ratio	*P*
Gemcitabine	0.00037 ± 0.00015	0.20 ± 0.10	533 ± 58	0.026
AraC	0.017 ± 0.012	0.80 ± 0.28	47 ± 19	0.011*
Triapine	0.33 ± 0.15	4.3 ± 0.58	14 ± 5.1	0.00032*
Hydroxyurea	133 ± 57	833 ± 153	6.7 ± 1.5	0.0018*
Didox	80 ± 17	87 ± 23	1.1 ± 0.064	0.71
Trimidox	67 ± 15	70 ± 10	1.1 ± 0.17	0.77
Cisplatin	3.7 ± 0.58	13 ± 5.8	3.6 ± 1.3	0.88
Doxorubicin	0.14 ± 0.05	0.15 ± 0.04	1.1 ± 0.12	0.045*
5-Fluorouracil	1.1 ± 0.76	1.3 ± 0.58	1.3 ± 0.34	0.73
Cyclopentenyl cytosine	0.011 ± 0.0081	0.012 ± 0.0068	1.2 ± 0.35	0.80
Etoposide	0.50 ± 0.20	0.53 ± 0.25	1.1 ± 0.033	0.87

NOTE: IC_{50} 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.

Downloaded from http://aacrjournals.org/mct/article-pdf/4/8/1268/1870646/1268-1276.pdf by guest on 18 April 2024

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

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

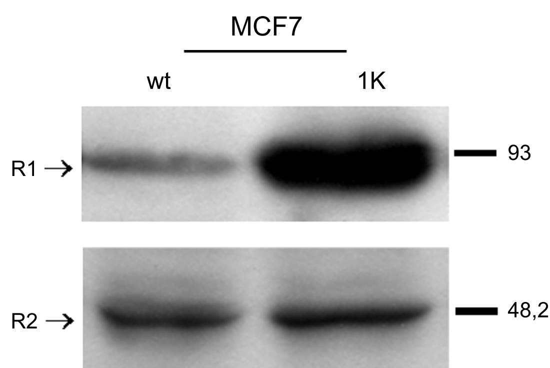

Figure 1. Expression of ribonucleotide reductase R1 and R2 in gemcitabine-resistant (*1K*) and sensitive (*wt*) MCF7 cells. Western blot analysis in MCF7 wt and MCF7 1K cells using anti – ribonucleotide reductase R1 and anti – ribonucleotide reductase R2 antibodies.

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

Downloaded from http://aacrjournals.org/mct/article-pdf/4/8/1268/1870646/1268-1276.pdf by guest on 18 April 2024

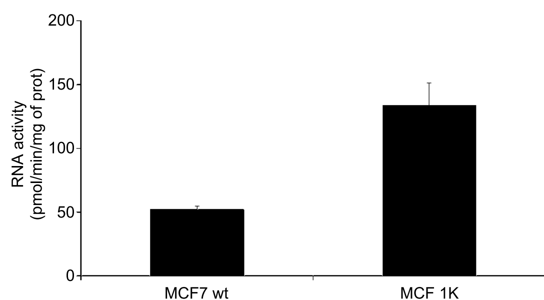

Figure 2. Ribonucleotide reductase activity in gemcitabine-sensitive (*wt*) and gemcitabine-resistant (*1K*) MCF7 cells. *Columns,* mean of three independent experiments. $P < 0.01$ (Student's t test).

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 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 oropharyngineal 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-G_2 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, nonidentified 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 CAAACTAC 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

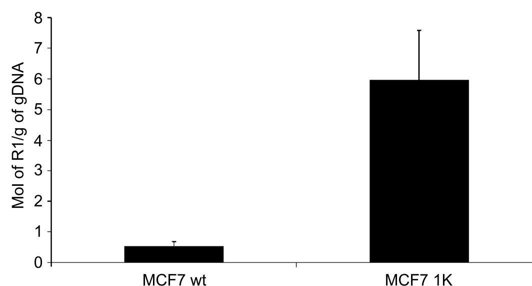

Figure 3. Quantification by quantitative PCR of ribonucleotide reductase R1 gene copies in genomic DNA in gemcitabine-sensitive (*wt*) and gemcitabine-resistant (*1K*) MCF7 cells. *Columns,* mean of three independent experiments. $P < 0.01$ (Student's t test).

Downloaded from http://aacrjournals.org/mct/article-pdf/4/8/1268/1870646/1268-1276.pdf by guest on 18 April 2024

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.

References

1. Vansteenkiste JF, Vandebroek JE, Nackaerts KL, et al. Clinical-benefit response in advanced non-small-cell lung cancer: A multicentre prospective randomised phase III study of single agent gemcitabine versus cisplatin-vindesine. Ann Oncol 2001;12:1221 – 30.

2. Markman M, Webster K, Zanotti K, Kulp B, Peterson G, Belinson J. Phase 2 trial of single-agent gemcitabine in platinum-paclitaxel refractory ovarian cancer. Gynecol Oncol 2003;90:593 – 6.

3. Heinemann V. Role of gemcitabine in the treatment of advanced and metastatic breast cancer. Oncology 2003;64:191 – 206.

4. Hussain SA, James ND. The systemic treatment of advanced and metastatic bladder cancer. Lancet Oncol 2003;4:489 – 97.

5. Jordheim L, Galmarini CM, Dumontet C. Drug resistance to cytotoxic nucleoside analogues. Curr Drug Targets 2003;4:443 – 60.

6. Bouffard DY, Laliberte J, Momparler RL. Kinetic studies on 2′,2′-difluorodeoxycytidine (gemcitabine) with purified human deoxycytidine kinase and cytidine deaminase. Biochem Pharmacol 1993;45:1857 – 61.

7. Krishnan P, Fu Q, Lam W, Liou JY, Dutschman G, Cheng YC. Phosphorylation of pyrimidine deoxynucleoside analog diphosphates: selective phosphorylation of L-nucleoside analog diphosphates by 3-phosphoglycerate kinase. J Biol Chem 2002;277:5453 – 9.

8. Liou JY, Dutschman GE, Lam W, Jiang Z, Cheng YC. Characterization of human UMP/CMP kinase and its phosphorylation of D- and L-form deoxycytidine analogue monophosphates. Cancer Res 2002;62:1624 – 31.

9. Heinemann V, Xu YZ, Chubb S, et al. Inhibition of ribonucleotide reduction in CCRF-CEM cells by 2′,2′-difluorodeoxycytidine. Mol Pharmacol 1990;38:567 – 72.

10. Kim MY, Ives DH. Human deoxycytidine kinase: kinetic mechanism and end product regulation. Biochemistry 1989;28:9043 – 7.

11. Huang P, Chubb S, Hertel LW, Grindey GB, Plunkett W. Action of 2′,2′-difluorodeoxycytidine on DNA synthesis. Cancer Res 1991;51:6110 – 7.

12. Heinemann V, Schulz L, Issels RD, Plunkett W. Gemcitabine: a modulator of intracellular nucleotide and deoxynucleotide metabolism. Semin Oncol 1995;22:11 – 8.

13. Ruiz van Haperen VW, Veerman G, Vermorken JB, Peters GJ. 2′,2′-Difluoro-deoxycytidine (gemcitabine) incorporation into RNA and DNA of tumour cell lines. Biochem Pharmacol 1993;46:762 – 6.

14. Bergman AM, Pinedo HM, Peters GJ. Determinants of resistance to 2′,2′-difluorodeoxycytidine (gemcitabine). Drug Resist Updat 2002;5:19 – 33.

15. Mackey J, Baldwin SA, Young JD, Cass CE. Nucleoside transport and its significance for anticancer drug resistance. Drug Resist Updat 1998;1:310 – 24.

16. Mackey JR, Mani RS, Selner M, et al. Functional nucleoside transporters are required for gemcitabine influx and manifestation of toxicity in cancer cell lines. Cancer Res 1998;58:4349 – 57.

17. Galmarini CM, Clarke ML, Santos CL, et al. Sensitization of ara-C-resistant lymphoma cells by a pronucleotide analogue. Int J Cancer 2003;107:149 – 54.

18. Jordheim LP, Cros E, Gouy MH, et al. Characterization of a gemcitabine-resistant murine leukemic cell line: reversion of *in vitro* resistance by a mononucleotide prodrug. Clin Cancer Res 2004;10:5614 – 21.

19. Dumontet C, Fabianowska-Majewska K, Mantincic D, et al. Common resistance mechanisms to deoxynucleoside analogues in variants of the human erythroleukaemic line K562. Br J Haematol 1999;106:78 – 85.

20. Heinemann V, Xu YZ, Chubb S, et al. Cellular elimination of 2′,2′-difluorodeoxycytidine 5′-triphosphate: a mechanism of self-potentiation. Cancer Res 1992;52:533 – 9.

21. Mazzon C, Rampazzo C, Scaini MC, et al. Cytosolic and mitochondrial deoxyribonucleotidases: activity with substrate analogs, inhibitors and implications for therapy. Biochem Pharmacol 2003;66:471 – 9.

22. Davidson JD, Ma L, Flagella M, Geeganage S, Gelbert LM, Slapak CA. An increase in the expression of ribonucleotide reductase large subunit 1 is associated with gemcitabine resistance in non-small cell lung cancer cell lines. Cancer Res 2004;64:3761 – 6.

23. Hurta RA, Wright JA. Amplification of the genes for both components of ribonucleotide reductase in hydroxyurea resistant mammalian cells. Biochem Biophys Res Commun 1990;167:258 – 64.

24. Wright JA, Alam TG, McClarty GA, Tagger AY, Thelander L. Altered expression of ribonucleotide reductase and role of M2 gene amplification in hydroxyurea-resistant hamster, mouse, rat, and human cell lines. Somat Cell Mol Genet 1987;13:155 – 65.

25. Yen Y, Grill SP, Dutschman GE, Chang CN, Zhou BS, Cheng YC. Characterization of a hydroxyurea-resistant human KB cell line with supersensitivity to 6-thioguanine. Cancer Res 1994;54:3686 – 91.

26. 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 – 7.

27. Mansson E, Flordal E, Liliemark J, et al. Down-regulation of deoxycytidine kinase in human leukemic cell lines resistant to cladribine and clofarabine and increased ribonucleotide reductase activity contributes to fludarabine resistance. Biochem Pharmacol 2003;65:237 – 47.

28. Rosell R, Danenberg KD, Alberola V, et al. Ribonucleotide reductase messenger RNA expression and survival in gemcitabine/cisplatin-treated advanced non-small cell lung cancer patients. Clin Cancer Res 2004;10:1318 – 25.

29. Galmarini CM, Thomas X, Calvo F, et al. Potential mechanisms of resistance to cytarabine in AML patients. Leuk Res 2002;26:621 – 9.

30. Lepoivre M, Chenais B, Yapo A, Lemaire G, Thelander L, Tenu JP. Alterations of ribonucleotide reductase activity following induction of the nitrite-generating pathway in adenocarcinoma cells. J Biol Chem 1990;265:14143 – 9.

31. Guittet O, Hakansson P, Voevodskaya N, et al. Mammalian p53R2 protein forms an active ribonucleotide reductase *in vitro* with the R1 protein, which is expressed both in resting cells in response to DNA damage and in proliferating cells. J Biol Chem 2001;276:40647 – 51.

32. Zhou BS, Hsu NY, Pan BC, Doroshow JH, Yen Y. Overexpression of ribonucleotide reductase in transfected human KB cells increases their resistance to hydroxyurea: M2 but not M1 is sufficient to increase

Downloaded from http://aacrjournals.org/mct/article-pdf/4/8/1268/1870646/1268-1276.pdf by guest on 18 April 2024

resistance to hydroxyurea in transfected cells. Cancer Res 1995;55: 1328 – 33.

33. Mann GJ, Musgrove EA, Fox RM, Thelander L. Ribonucleotide reductase M1 subunit in cellular proliferation, quiescence, and differentiation. Cancer Res 1988;48:5151 – 6.

34. Kuo ML, Kinsella TJ. Expression of ribonucleotide reductase after ionizing radiation in human cervical carcinoma cells. Cancer Res 1998; 58:2245 – 52.

35. Tay DL, Bhathal PS, Fox RM. Quantitation of G_0 and G_1 phase cells in primary carcinomas. Antibody to M1 subunit of ribonucleotide reductase shows G_1 phase restriction point block. J Clin Invest 1991;87:519 – 27.

36. Rubin EH, Cory JG. Differential turnover of the subunits of ribonucleotide reductase in synchronized leukemia L1210 cells. Cancer Res 1986;46:6165 – 8.

37. Engstrom Y, Eriksson S, Jildevik I, Skog S, Thelander L, Tribukait B. Cell cycle-dependent expression of mammalian ribonucleotide reductase. Differential regulation of the two subunits. J Biol Chem 1985;260: 9114 – 6.

38. Chen FY, Amara FM, Wright JA. Defining a novel ribonucleotide reductase r1 mRNA cis element that binds to an unique cytoplasmic trans-acting protein. Nucleic Acids Res 1994;22:4796 – 7.

39. Schuetz JD, Connelly MC, Sun D, et al. MRP4: a previously unidentified factor in resistance to nucleoside-based antiviral drugs. Nat Med 1999;5:1048 – 51.

40. Zhou B, Mo X, Liu X, Qiu W, Yen Y. Human ribonucleotide reductase M2 subunit gene amplification and transcriptional regulation in a homogeneous staining chromosome region responsible for the mechanism of drug resistance. Cytogenet Cell Genet 2001;95:34 – 42.

Downloaded from http://aacrjournals.org/mct/article-pdf/4/8/1268/1870646/1268-1276.pdf by guest on 18 April 2024