

Cancer Research

***In vivo* Induction of Resistance to Gemcitabine Results in Increased Expression of Ribonucleotide Reductase Subunit M1 as the Major Determinant**

Andries M. Bergman, Paul P. Eijk, Veronique W.T. Ruiz van Haperen, et al.

Cancer Res 2005;65:9510-9516. Published online October 17, 2005.

Updated Version Access the most recent version of this article at:
doi:[10.1158/0008-5472.CAN-05-0989](https://doi.org/10.1158/0008-5472.CAN-05-0989)

Cited Articles This article cites 33 articles, 15 of which you can access for free at:
<http://cancerres.aacrjournals.org/content/65/20/9510.full.html#ref-list-1>

Citing Articles This article has been cited by 12 HighWire-hosted articles. Access the articles at:
<http://cancerres.aacrjournals.org/content/65/20/9510.full.html#related-urls>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.

***In vivo* Induction of Resistance to Gemcitabine Results in Increased Expression of Ribonucleotide Reductase Subunit M1 as the Major Determinant**

Andries M. Bergman,¹ Paul P. Eijk,³ Veronique W.T. Ruiz van Haperen,¹ Kees Smid,¹ Gijsbert Veerman,¹ Isabelle Hubeek,² Paul van den IJssel,³ Bauke Ylstra,³ and Godefridus J. Peters¹

Departments of ¹Medical Oncology, ²Pediatric Hematology/Oncology, and ³Microarray Facility, VU University Medical Center, Amsterdam, the Netherlands

Abstract

Gemcitabine is a deoxycytidine (dCyd) analogue with activity against several solid cancers. Gemcitabine is activated by dCyd kinase (dCK) and interferes, as its triphosphate dFdCTP, with tumor growth through incorporation into DNA. Alternatively, the metabolite gemcitabine diphosphate (dFdCDP) can interfere with DNA synthesis and thus tumor growth through inhibition of ribonucleotide reductase. Gemcitabine can be inactivated by the enzyme dCyd deaminase (dCDA). In most *in vitro* models, resistance to gemcitabine was associated with a decreased dCK activity. In all these models, resistance was established using continuous exposure to gemcitabine with increasing concentrations; however, these *in vitro* models have limited clinical relevance. To develop *in vivo* resistance to gemcitabine, we treated mice bearing a moderately sensitive tumor Colon 26-A (T/C = 0.25) with a clinically relevant schedule (120 mg/kg every 3 days). By repeated transplant of the most resistant tumor and continuation of gemcitabine treatment for >1 year, the completely resistant tumor Colon 26-G (T/C = 0.96) was created. Initial studies focused on resistance mechanisms known from *in vitro* studies. In Colon 26-G, dCK activity was 1.7-fold decreased; dCDA and DNA polymerase were not changed; and Colon 26-G accumulated 1.5-fold less dFdCTP, 6 hours after a gemcitabine injection, than the parental tumor. Based on *in vitro* studies, these relative minor changes were considered insufficient to explain the completely resistant phenotype. Therefore, an expression microarray was done with Colon 26-A versus Colon 26-G. Using independently grown nonresistant and resistant tumors, a striking increase in expression of the *RRM1* subunit gene was found in Colon 26-G. The expression of *RRM1* mRNA was 25-fold increased in the resistant tumor, as measured by real-time PCR, which was confirmed by Western blotting. In contrast, *RRM2* mRNA was 2-fold decreased. However, ribonucleotide reductase enzyme activity was only moderately increased in Colon 26-G. In conclusion, this is the first model with *in vivo* induced resistance to gemcitabine. In contrast to most *in vitro* studies, dCK activity was not the most important

determinant of gemcitabine resistance. Expression microarray identified *RRM1* as the gene with the highest increase in expression in the Colon 26-G, which might clarify its complete gemcitabine-resistant phenotype. This study is the first *in vivo* evidence for a key role for *RRM1* in acquired gemcitabine resistance. (Cancer Res 2005; 65(20): 9510-6)

Introduction

Gemcitabine (2',2'-difluorodeoxycytidine, dFdC, Gemzar) is a deoxycytidine (dCyd) analogue, with two fluorine atoms substituted at the 2'-position of the ribose ring, which was synthesized in the eighties by Eli Lilly, Inc., Indianapolis, IN (1). Unlike the structurally and functionally related dCyd analogue 1- β -D-arabinofuranosylcytosine (ara-C), which is used for the treatment of hematologic malignancies, gemcitabine had a remarkable activity in solid tumors. This potency might be related to a far more complicated metabolism than of ara-C with several self-potentiating mechanisms (2).

In the clinic, gemcitabine is used in combination with other drugs for the treatment of locally advanced or metastasized non-small cell lung cancer (NSCLC) and bladder cancer and as a single agent for the treatment of adenocarcinoma of the pancreas (3-5). Development of drug resistance is a major problem in the treatment of neoplasms. Resistance can be either inherent or acquired. Inherent resistance is a quality of several tumor types, which is reflected in low response rates in clinical trials (6). Acquired resistance can develop by selection of cells from a heterogeneous tumor cell population during repetitive treatment with a drug.

In the cell, gemcitabine is phosphorylated to a monophosphate, diphosphate, and triphosphate, before incorporation into DNA, which is required for its growth inhibiting activity (2). The first step in phosphorylation is catalyzed by dCyd kinase (dCK), which is the rate-limiting step for further phosphorylation to active metabolites and thus essential for the activation of gemcitabine (7). For this reason, dCK plays a pivotal role in gemcitabine activation. Gemcitabine may also be activated by the mitochondrial thymidine kinase 2 (TK2) but not by the cytoplasmic thymidine kinase 1 (TK1; ref. 8) and is inactivated by deamination, catalyzed by dCyd deaminase (dCDA) to 2',2'-difluorodeoxyuridine (dFdU; ref. 9).

Ribonucleotide reductase, which consists of two subunits M1 and M2, catalyzes *de novo* synthesis of deoxyribonucleoside diphosphates (dNDP), as building blocks of DNA. The enzyme reduces the hydroxyl at carbon 2 of the ribose sugar in ribonucleoside diphosphates (NDP) to a hydrogen, forming a deoxyribose sugar in the corresponding dNDP. In this reaction, a

Note: V.W.T. Ruiz van Haperen is currently at the Raad voor Gezondheidsonderzoek, The Hague, the Netherlands.

G. Veerman is currently at the New Drug Development Organization Oncology, Amsterdam, the Netherlands.

Requests for reprints: Godefridus J. Peters, Department of Medical Oncology, VU University Medical Center, P.O. Box 7057, 1007 MB Amsterdam, the Netherlands. Phone: 20-444-2633; Fax: 20-444-3844; E-mail: gj.peters@vumc.nl.

©2005 American Association for Cancer Research.

doi:10.1158/0008-5472.CAN-05-0989

free-radical mechanism is involved. The diphosphate of gemcitabine dFdCDP is an inhibitor of ribonucleoside reductase, resulting in a decrease in deoxynucleoside triphosphate (dNTP) pools, which are required for DNA repair and synthesis (10, 11). Moreover, a decrease in dCyd triphosphate (dCTP) pools will decrease feedback inhibition of dCK and thus increase gemcitabine phosphorylation (10). The mechanism of inhibition of ribonucleotide reductase by dFdCDP is not completely clarified yet, but several studies suggest that M1 is the targeted subunit of ribonucleotide reductase (11, 12). However, M2 holds the organic free radical that is essential for the enzyme activity (13).

In multiple *in vitro* studies, the main resistance mechanism against gemcitabine was a decrease in dCK activity (7). However, resistance to gemcitabine can include several other mechanisms besides dCK deficiency, including an increased activity of dCDA, increased ribonucleotide reductase activity, decreased accumulation of triphosphates, and an altered DNA polymerase (7).

All models for the development of gemcitabine resistance are *in vitro* models. Because of the wide use of gemcitabine, further insight into mechanisms of acquired resistance might be of great value. Because the translation of *in vitro* results to the clinic is usually hampered by the lack of suitable *in vivo* models, we developed an *in vivo* model of gemcitabine resistance. For that purpose, Colon 26-A, a murine tumor with a moderate *in vivo* sensitivity to gemcitabine, was used (14). Resistance was induced by repeated gemcitabine treatment. Initial studies focused on resistance mechanisms known from *in vitro* studies, dCK, dCDA, TK2, and DNA polymerase activity and accumulation of the triphosphate dFdCTP. Because this approach did not reveal a clear explanation for the resistance, parental and the gemcitabine-resistant tumors were analyzed by expression microarrays. Rather than dCK, dCDA, and DNA polymerase, this analysis identified RRM1 as a main player in gemcitabine resistance *in vivo*. Subsequent mechanistic studies concentrated on mRNA and protein expression of RRM1 and confirmed our findings that RRM1 is a major determinant of acquired gemcitabine resistance *in vivo*.

Materials and Methods

Materials. Gemcitabine and dFdU were kindly supplied by Eli Lilly. Deoxy-[5-³H]-cytidine (21.9 Ci/mmol) and ¹⁴C-CDP [cytosine-¹⁴C(U)] (60 mCi/mmol) were purchased from Moravek (Brea, CA) and [2-¹⁴C]-thymidine (58.8 Ci/mmol) from NEND (Dreiech, Germany). All other chemicals were of analytic grade and commercially available.

Murine tumors and establishment of resistance. Sources and characteristics of the Colon 26 were described previously (15). Colon 26 tumors were grown in female BALB/c mice (Harlan/Olac, Zeist, The Netherlands). The mice were kept in an area maintained on a standardized light/dark cycle and had access to food (RMH-B 10 mm code 2100, Hope Farms, Woerden, The Netherlands) and water *ad libitum*. Tumors were transplanted s.c. in both flanks in the thoracic region in small fragments of 1 to 5 mm².

Mice were treated by i.p. bolus injection. The maximum tolerable dose (MTD) was assessed in non-tumor-bearing mice and defined as the dose that caused a maximal weight loss of 15%. In BALB/c mice, the MTD was 120 mg/kg gemcitabine every 3 days for four times (q3dX4; ref. 14). Resistance to gemcitabine was induced by continued treatment of six Colon 26-A tumors in three mice at the MTD. Treatment started 10 days after each transplantation. One day after the last dose, the most resistant tumor was transplanted and treatment of six tumors in three mice was repeated. After six generations, mice were treated 17 times without transplantation of the tumor. When weight of the mice decreased to below 15% of the initial

weight, treatment was temporarily delayed. Finally, a tumor was created with a gemcitabine resistant phenotype and termed Colon 26-G (gemcitabine). Different generations of Colon 26-A and Colon 26-G were analyzed separately.

To determine the *in vivo* antitumor efficacy of gemcitabine treatment, tumor-bearing mice were treated at the MTD. Each group consisted of at least six mice. Experiments and their evaluation were done essentially as described previously (15). Tumor sizes were determined by caliper measurement and growth was evaluated by calculation of a T/C value defined as volume of tumors of treated mice divided by the volume of tumors of control mice and by a growth delay factor (GDF) defined as the number of tumor doubling times gained by the treatment.

Tissue preparation for enzyme assays. Frozen murine tumors were pulverized using a microdismembrator as previously described (16). Subsequently, the frozen powder was weighed and suspended in ice-cold assay buffer [0.3 mmol/L Tris-HCl (pH 8.0)] at a concentration of 1 g tissue per 3 to 4 mL buffer. The suspension was centrifuged twice (10 minutes at 4,000 × g at 4°C; the supernatant subsequently, 20 minutes at 10,000 × g at 4°C). One part of the undiluted tumor supernatant was taken for measurement of the protein content with the Bio-Rad Bradford protein assay (17), the other part was used for enzyme assays.

Deoxycytidine and thymidine kinase enzyme activities. For determination of dCK and TK activities in tumors, the abovementioned supernatant was used. For dCK activity, a substrate mixture was added to the supernatant resulting in final concentrations of 10 mmol/L ATP, 5 mmol/L MgCl₂, 0.18 mol/L Tris-HCl, 25 μmol/L β-mercaptoethanol, and 230 μmol/L dCyd (specific activity, 0.04 Ci/mmol), pH 7.4 and incubated for 30 minutes at 37°C, essentially as described (18). Thymidine was added at 1 mmol/L to inhibit TK2-mediated phosphorylation of dCyd. TK activities were measured similar to dCK by using thymidine as a substrate. The reaction mixture contained 21.9 μmol/L [2-¹⁴C]-thymidine (specific activity, 1.8 Ci/mmol) and enzyme suspension essentially as described previously (19). To discriminate between TK1 and TK2, we added dCTP (final concentration, 10 mmol/L) to inhibit TK2. TK2 activity can be estimated by subtracting TK1 activity from total TK activity. Substrates were separated from products by TLC as described previously (18). Enzyme activities were expressed in nmol product formed per hour per mg protein (nmol/h/mg protein).

Deoxycytidine deaminase activity. Activity of dCDA was determined as described earlier (18). Briefly, in the abovementioned supernatant enzyme activity was determined at 37°C with 500 μmol/L dCyd as a substrate for 15 or 25 minutes, after which proteins were precipitated by TCA and nucleosides were extracted by triethylamine/1,1,2-trichloro-trifluoroethane (v/v, 4:1). The substrate dCyd and its product deoxyuridine were analyzed using reversed phase high-performance liquid chromatography.

DNA polymerase assay. Total DNA polymerase activity was assayed by measurement of ¹⁴C-dTTP incorporation into DNA (20). Pulverized tissues were suspended in TEMG buffer [50 mmol/L Tris-HCl, 1 mmol/L EDTA, 20% glycerol (pH 7.4)] supplemented with 0.8 mol/L KCl, centrifuged at 10,000 × g for 10 minutes at 4°C, and the supernatant subsequently for 60 minutes at 100,000 × g and 4°C. The 100,000 × g supernatant was dialyzed against KCl-free TEMG buffer. The assay mixture (200 μL) contained the equivalent of 400-2,000 μg protein, 3.3 mmol/L DTT, 50 mmol/L Tris-HCl, 30 μmol/L dATP, 30 μmol/L dCTP, 30 μmol/L dGTP, 7.2 mmol/L MgCl₂, and 50 μg activated DNA (pH 7.2) and was carried out in 96-well filter plates as used previously (21). The reaction was started by addition of 0.125 μCi ¹⁴C-dTTP (final concentration, 0.1 μmol/L) and terminated after 5 to 40 minutes by removal of the solution through the filter. The filters were subsequently washed with 5% TCA containing 0.1% pyrophosphate followed by two washes with 5% TCA and twice with 96% ethanol. The filters were dried, taken off the plate, and put in liquid scintillation vials. DNA was solubilized with 100 μL of 2 mmol/L NaOH for 3 hours and subsequently counted. Activated DNA was prepared by incubation of 350 μg calf thymus DNA with 15 units DNase I in 350 μL of 50 mmol/L Tris-HCl (pH 7.2), 2 mmol/L MgCl₂, 1 mmol/L ZnCl₂ at 37°C for 15 minutes followed by heating for 5 minutes at 77°C and chilling on ice.

Ribonucleotide reductase activity. The assay for ribonucleotide reductase activity is based on the conversion of ^{14}C -CDP to ^{14}C -dCDP in extracts from tumors as described earlier by Fukushima et al. (22). After the assay, both substrate and product are degraded by snake venom diesterase to cytidine and dCyd. Briefly, tumors are pulverized in a microdismembrator as described above. The powder was suspended (1 part powder and 3 parts assay buffer) in assay buffer [5 mmol/L MgCl_2 /10 mmol/L NaF/1 mmol/L FeCl_3 /5 mmol/L DL-DTT/50 mmol/L HEPES and protease inhibitor cocktail (pH 7.4); Roche Laboratories, Woerden, The Netherlands], centrifuged for 10 minutes at high speed and 4°C . The supernatant was used for the ribonucleotide reductase assay, which consisted of 65 μL (diluted) supernatant, whereas the reaction was started by addition of 10 μL ^{14}C -CDP (specific activity, 60 mCi/mmol; final concentration in assay mixture, 50 $\mu\text{mol/L}$) and 10 μL of 42.5 mmol/L ATP (neutralized to pH 7.4), bringing the total reaction volume to 85 μL . The reaction was linear up to 15 minutes (depending on the source of the enzyme) and stopped by boiling for 3 minutes at 95°C to denature all proteins followed by chilling on ice and a short centrifugation step. To degrade the substrate ^{14}C -CDP and the product ^{14}C -dCDP to ^{14}C -cytidine and ^{14}C -dCyd, we added 10 μL of snake venom diesterase (200 mg/mL; *Crotalus adamanteus*, Eastern Diamondback Rattlesnake, venom, Sigma, St. Louis, MO) in 15 mmol/L MgCl_2 to the assay mixture and incubated this for 2 hours at 37°C ; this reaction was also stopped by heating at 95°C for 3 minutes followed by addition of 5 μL of unlabeled 100 mmol/L cytidine/100 mmol/L dCyd to facilitate detection on TLC sheets. At least 10 μL of this mixture was spotted onto a TLC Al sheet silica gel 60 F₂₅₄ (Merck, Amsterdam, The Netherlands), which were developed with 0.87 mol/L H_3BO_3 , 0.2 mol/L LiCl in 50% ethanol. R_f values for cytidine and dCyd (as detected under UV light) were 0.4 and 0.75, respectively. Spots were cut out and radioactivity was estimated by liquid scintillation counting.

dFdCTP accumulation in vivo. Colon 26-A- and Colon 26-G-bearing BALB/c mice were treated with a single dose of 120 mg/kg gemcitabine. After 2, 6, 8, and 24 hours, tumors were removed under anesthesia, immediately frozen and pulverized, after which, nucleotides were extracted. Briefly, proteins in frozen tissue powder were precipitated by TCA, spun down, after which, the supernatant was neutralized with tri-octylamine/1,1,2-tri-chloro-trifluoroethane. Finally, dFdCTP was analyzed on high-performance liquid chromatography. Nucleotides were detected at 254 and 280 nm (23).

Total RNA isolation. Total RNA isolation from separate generations of Colon 26-A and Colon 26-G tumors grown in different animals were done using the TriZol (Invitrogen, Leek, The Netherlands) method according to the manufacturer's protocol. Total RNA concentration was measured by A260 and RNA integrity judged on a 1.2% agarose gel. Samples were dissolved in 100% DMPC-treated H_2O and stored at -80°C before use in either the microarray hybridizations or real-time PCR confirmation of the microarray results.

Microarray procedures. The mouse oligoLibrary (compugen/Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands) containing 7,524 oligonucleotides (65 bp) representing 7,230 separate genes⁴ was resuspended to a concentration of 10 $\mu\text{mol/L}$ in 150 mmol/L sodium phosphate buffer, pH 8.5 and spotted in duplicate using the SpotArray 72 with Telechem SMP pins, partially described (24). Single-stranded cDNA was synthesized from 30 μg of total RNA by reverse transcription essentially according to DeRisi et al. (25) using aminoallyl-labeled dUTP (Ambion Ltd., Huntingdon, United Kingdom). Labeling was done according to the aminoallyl-labeling protocol developed by DeRisi.⁵ Briefly, cDNA was incubated at room temperature for 1 hour with fluorolink monofunctional Cy5 or Cy3 dye (Amersham, Roosendaal, The Netherlands) followed by 15 minutes of 4 mol/L hydroxylamine treatment. Uncoupled dyes were removed using Qiaquick PCR purification columns (Qiagen, Westburg B.V., Leusden, The Netherlands) and mixed with 12 μg poly(dA) (Amersham), 60 μg yeast tRNA (Sigma-Aldrich Chemie), and 24 μg Cot-1 DNA

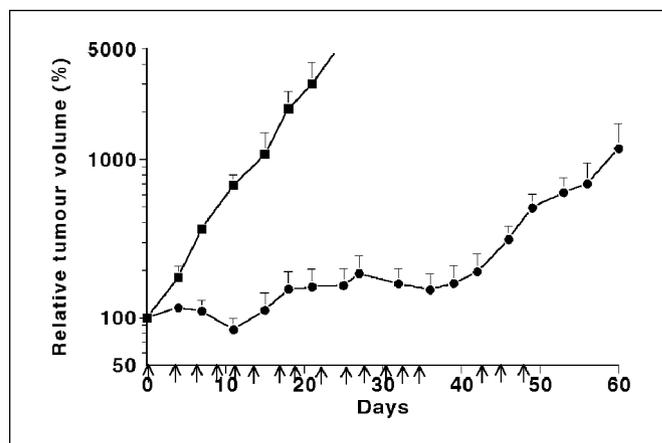


Figure 1. Development of the Colon 26-G tumor by repetitive treatment at the MTD dose. ■, control and ●, gemcitabine (120 mg/kg) at each day (arrows). Points, means of at least six tumors; bars, \pm SE. Tumors were treated 17 times until they showed the same growth rate as untreated tumors. Due to toxicity, treatment was delayed several times.

(Invitrogen). The labeled target was dissolved in 127- μL hybridization mixture containing 46% formamide (Invitrogen), 9.5% dextran sulfate (U.S. Biochemical Corp., Cleveland, OH), $2\times$ SSC, and 0.2% SDS. The labeled target was heated to 70°C for 10 minutes and annealed at 37°C for 1 hour. Slides were prehybridized in hybridization mix with 30 μg salmon sperm DNA (Invitrogen) for 1 hour at 37°C followed by 14 hours at 37°C overnight (HybArray 12, Perkin-Elmer, Zaventem, Belgium). After hybridization, the slides were washed in the HybArray 12, with 50% formamide (FLUKA, Sigma-Aldrich Chemie), $2\times$ SSC (pH 7) at 35°C for 15 minutes followed by PI buffer [0.1 mol/L sodium phosphate, 0.1% Igepal Ca630 (pH 8)] at room temperature and three washes of $0.2\times$ SSC, $0.1\times$ SSC, and $0.01\times$ SSC at room temperature followed by centrifugation. Arrays were scanned using a laser scanner (ScanArray Express, Perkin-Elmer) and analyzed using Imagen version 5.6 (Westburg). Cy3/Cy5 ratios are calculated by taking the \log_2 of the "signal mean" of each spot. This is followed by a standard normalization for spot intensity and calculation of the ratios. Three separate experiments and a self-self experiment were done to find differences between three different generations of Colon 26-G tumors and three different generations of Colon 26-A tumors.

Real-time light cycler-PCR. The assays for RRM1 and RRM2 mRNA expression were done by real-time PCR with a LightCycler 1.0 (Roche Diagnostics, Almere, The Netherlands). Primers for murine RRM1 and RRM2 were based on the sequence of the gene (Entrez-PubMed) and designed by the program Primer3;⁶ forward primer, 5'-CCAATGAGTGCTCTGGTCT and 5'-CCTACTAACCCACGCGTTGA; reverse primer, 5'-GTTCTGCTGGTGTCTCTCC and 5'-GTTTCAGAGCTTCCCAGTGC, respectively. The primers for murine β -actin are forward, 5'-TGTTACCAACTGGGACGACA and reverse, 5'-GGGGTGTGAAGGTCTCAA. Synthesis of double-stranded DNA during the various PCR cycles was monitored using SYBR Green I (Roche Laboratories). A Master SYBR Green I working solution was prepared by mixing 60 μL of LC-FastStart Reaction Mix SYBR Green I with 4 μL of LC-FastStart Enzyme (Roche Laboratories). Two volumes of this working solution were mixed with 16 volumes of a solution containing varying concentrations of MgCl_2 , RRM1, RRM2, or β -actin primers and H_2O . Thereafter, 18 μL of this solution were pipetted into a light cycler capillary. The reaction was started after the addition of 2- μL cDNA of varying dilutions of tumor cells. For RRM1 and RRM2, the final optimal concentration of MgCl_2 was 3 mmol/L and that of the primer 0.7 $\mu\text{mol/L}$. For β -actin, these concentrations were 5 mmol/L and 0.9 $\mu\text{mol/L}$.

⁴ <http://www.labonweb.com>.

⁵ <http://cmgm.stanford.edu/pbrown/protocols/index.html>.

⁶ <http://frodo.wi.mit.edu>.

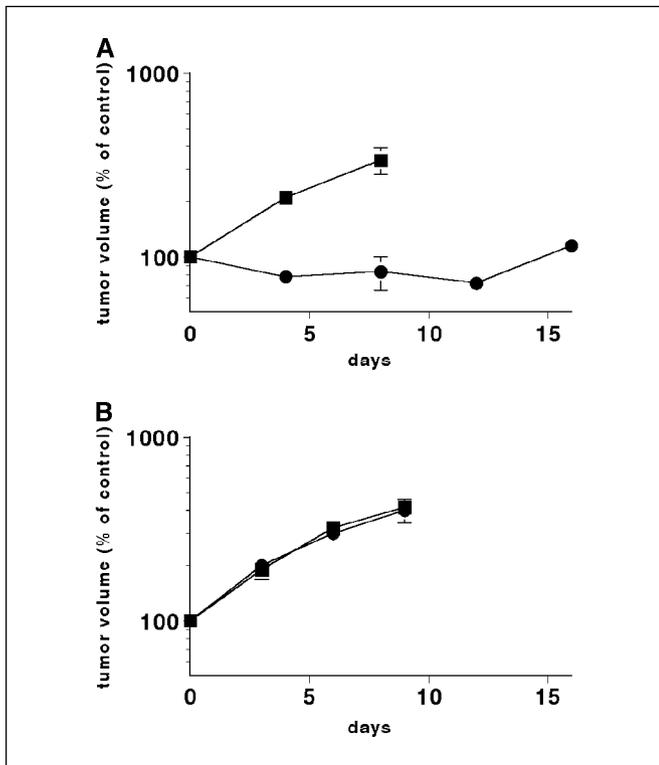


Figure 2. Sensitivity of the gemcitabine sensitive and resistant Colon 26 variants. Tumor volumes of (A) parental Colon 26-A tumors and (B) gemcitabine-resistant Colon 26-G tumors. ■, control and ●, gemcitabine (120 mg/kg) q3dX 4.

The RRM1 and RRM2 PCR program consisted of an initial denaturation step at 95°C for 10 minutes followed by 45 cycles of 10 seconds at 95°C, 5 seconds at 60°C, and 17 seconds at 72°C using AmpliTaq Gold DNA Polymerase. For β -actin, the PCR program was similar to that of RRM1 and RRM2. To verify the purity of the products, a melting curve was produced after each run as described previously (26).

The expression of both RRM1 and RRM2 were quantified relative to β -actin. Construction of calibration lines and calculations were done as described previously for dCK (26).

Western blot for ribonucleotide reductase subunits M1 and M2. Western blotting by goat anti-human, anti-mouse monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) against ribonucleotide reductase subunits M1 and M2 was done essentially as previously for dCK (27). In short, proteins were separated on 12.5% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and probed with the M1 and M2 antibody at a dilution of 1:500 or the β -actin mouse anti-actin monoclonal (1:3,000; Chemicon International, Temecula, CA), which was followed by incubation with the second antibody rabbit-anti goat (DAKO, Glostrup, Denmark) conjugated to horseradish peroxidase (1:2,500). Immune complexes were visualized by the enhanced chemiluminescence reaction (Amersham Pharmacia Biotech, Roosendaal, The Netherlands) and quantified by scanning on a CS-690 Bio-Rad scanner (Bio-Rad, Hercules, CA). Levels of expression were reported relative to the parental tumor Colon 26-A (which was set at 1).

Statistics. To evaluate possible significant differences in mRNA expression, enzyme activities and dFdCTP accumulation between the parental tumor and the gemcitabine resistant variant, a *t* test was used, one tailed, unpaired two-sample unequal variance. The computer program SPSS (version 7.5, SPSS, Inc., Chicago, IL) was used for statistical analysis. No statistical analysis was done on the microarray gene expression profiling. Genes of interest were selected visually. Statistical analysis of these arrays will be published separately.

Table 1. Activities of various enzymes in the murine colon carcinoma Colon 26-A and its gemcitabine-resistant variant Colon 26-G

Enzyme	Substrate	Colon 26-A	Colon 26-G	P
dCK	dCyd + TdR	4.9 \pm 0.7	2.9 \pm 0.4	<0.01
dCDA	dCyd	2.0 \pm 0.2	2.3 \pm 0.7	NS
TK1	TdR + dCTP	0.8 \pm 0.1	0.4 \pm 0.1	<0.01
TK1/2	TdR	7.9 \pm 0.7	1.0 \pm 0.2	<0.001
DNA polymerase	dTTP	0.320 \pm 0.090	0.188 \pm 0.040	NS

NOTE: For measurement of dCyd phosphorylation by dCK, TK2 was inhibited by thymidine. The TK assay contained dCTP to inhibit TK2. TK1 is the activity present in the assay containing dCTP. TK2 was calculated by the difference in total TK activity and TK1 activity. Average enzyme activities \pm SE of at least three experiments. dCK and dCDA activities in nmol/h/mg protein and DNA polymerase activity in fmol/h/mg protein. Statistical analysis by *t* test (independent samples).

Abbreviations: TdR, thymidine; NS, not significant.

Results

Establishment of a gemcitabine resistant tumor. Resistance to gemcitabine was achieved by continuously treating Colon 26-A tumors with gemcitabine at 120 mg/kg (q3dX4). The tumors least affected in each treatment course were chosen for transplantation, after which, the mice were treated again with gemcitabine at 120 mg/kg. After six transplantations, the mice were treated for a longer period (2 months; Fig. 1), after which, a stable gemcitabine-resistant phenotype was achieved. Unfortunately, this approach prevented storage of intermittent tumors for later analysis, because that particular tumor was used for transplantation. These tumors were transplanted and treatment with gemcitabine was continued. Initially, the resultant tumor, termed Colon26-G (Fig. 2), was continued to be treated with gemcitabine to be sure that a stable phenotype was maintained and that resistance did not reverse. In various subsequent experiments, sensitivity of both tumors was assessed over a period of several years and tumors remained resistant without the necessity of having to treat mice at each generation. In Colon 26-A and Colon

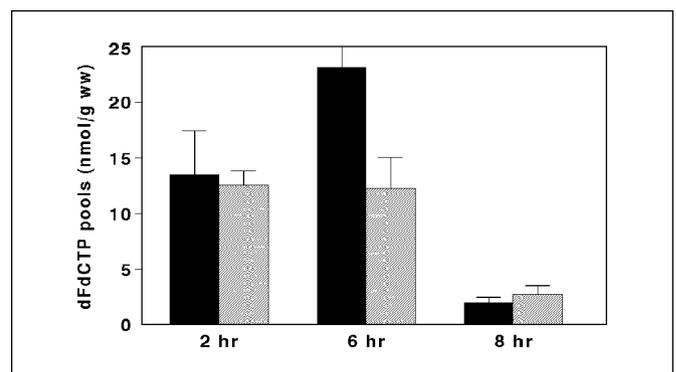


Figure 3. Accumulation of dFdCTP in the Colon 26-A (solid columns) and Colon 26-G (striped columns) tumors, 2, 6, and 8 hours after one i.p. dose of 120 mg/kg gemcitabine. Columns, means of at least three tumors; bars, \pm SE. Significant difference in accumulation after 6 hours ($P < 0.05$).

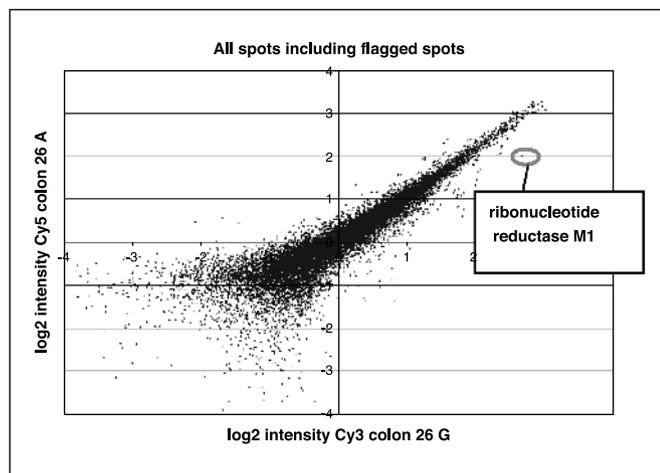


Figure 4. A representative microarray analysis of Colon 26-A and Colon 26-G tumors. This array was repeated thrice with different tumors with similar results. A dye swap (Cy3 and Cy5 switched) gives similar data, as well as a self-self array. All flagged spots on the array are included and no corrections were done.

26-G tumors treated with gemcitabine at 120 mg/kg, the T/C were 0.25 and 0.96, respectively, and the GDF 12.2 and 0.03, respectively (Fig. 2A-B).

Some initial attempts to elucidate the mechanism of resistance of Colon 26-G tumors focused on the sensitivity to other pyrimidine analogs. Because Colon 26-A is intrinsically resistant to cytarabine (arabinosyl-cytosine), we did not test Colon 26-G for cytarabine. However, Colon 26-A is moderately sensitive to 5-fluorouracil in combination with leucovorin (GDF = 2.6), but Colon 26-G is completely resistant to this combination (GDF = -0.1).

Enzyme activities. Because dCK, dCDA, TK, and DNA polymerase activities are determinants of gemcitabine activity, we measured their activity in resistant tumors (Table 1). The activity of dCK in Colon 26-G tumors was 1.7-fold lower than in the parental Colon 26-A tumor ($P < 0.01$), but no difference was found in dCDA activity between Colon 26-A and Colon 26-G tumors. Surprisingly, the activities of both TK1 and TK2 were decreased in Colon 26-G. The decrease was most pronounced for TK2 (12-fold, $P < 0.001$).

Because decreased DNA polymerase activity is associated with sensitivity to various drugs that act by causing DNA damage, we also determined the activity of this enzyme. However, DNA polymerase activity was not altered in Colon 26-G tumors (Table 1).

Accumulation of dFdCTP. To determine whether resistance would be due to a decrease of dFdCTP accumulation, we treated mice with a single 120 mg/kg dose of gemcitabine and removed the tumors at 2, 4, 8, and 24 hours after injection. Accumulation of dFdCTP in the parent tumor and the gemcitabine-resistant variant was similar at 2 hours after injection but higher in Colon 26-A at 6 hours ($P < 0.05$; Fig. 3). dFdCTP was retained similarly in both tumors. After 24 hours, no dFdCTP was detectable in both tumors.

Microarray gene expression profiling. Because analysis of several known resistance variables for gemcitabine did not reveal a clear target, we did microarray expression analysis assay to identify changes in gene expression, which might explain the difference in sensitivity. Of the 7,230 spotted genes, the expression of the *RRM1* gene was clearly increased in Colon 26-G, as was judged by the eye (Fig. 4). This increased expression was found in three different sets of tumors (Table 2). However, no alteration in *RRM2* expression was found. Although dCK enzyme activity was slightly decreased in Colon 26-G, expression of the *dCK* gene was not consistently altered. In addition, *TK1* gene expression was not consistently altered. The 12-fold decreased *TK2* enzyme activity in Colon 26-G might be related to a decreased *TK2* gene expression. However, the \log_2 ratios for *TK2* are within the SD of the average of all the determined genes and are not as impressive as for *RRM1*.

Real-time light cycler-PCR, Western blot for ribonucleotide reductase subunits M1 and M2, and ribonucleotide reductase enzyme activity. Real-time light cycler-PCR was done to measure differences in expression of *RRM1* and *RRM2* mRNA. A 2-fold difference was found in the *RRM2*/ β -actin ratio between Colon 26-A and Colon 26-G, whereas the *RRM1*/ β -actin ratio was 25-fold increased in Colon 26-G (Fig. 5). The Western blots revealed a clearly increased *RRM1* protein expression in Colon 26-G; however, no difference in *RRM2* expression was found between Colon 26-A and Colon 26-G (Fig. 6).

To determine whether the increased expression of the *RRM1* unit was associated with an altered enzyme activity, we measured

Table 2. Microarray expression analysis of genes known to be involved in gemcitabine's mechanism of action in the murine colon carcinoma Colon 26-A versus its gemcitabine-resistant variant Colon 26-G

Enzyme	Array (\log_2 ratio)			
	1	2	3	4
Average	-0.0744 \pm 0.4454	-0.0909 \pm 0.2034	-0.0272 \pm 0.0116	0.0177 \pm 0.2271
<i>RRM1</i>	0.6606 \pm 0.0025	1.0750 \pm 0.0220	-0.9335 \pm 0.0278	0.1131 \pm 0.0212
<i>RRM2</i>	-0.3940 \pm 0.0304	-0.0792 \pm 0.1051	0.0115 \pm 0.0001	0.0510 \pm 0.0297
dCK	-0.4431 \pm 0.4259	-0.0395 \pm 0.4274	-0.2800 \pm 0.0681	0.0438 \pm 0.1118
TK1	-0.3040 \pm 0.0246	-0.1754 \pm 0.3931	0.1188 \pm 0.2095	0.0027 \pm 0.1436
TK2	-0.3547 \pm 0.0955	-0.2504 \pm 0.0132	0.2484 \pm 0.1090	0.1040 \pm 0.2251

NOTE: \log_2 ratio \pm SD of the determined genes. Average is the average \log_2 ratio \pm SD of all 7,230 spotted genes. Gene expression was measured in three different sets of tumors: two different Colon 26A (Cy5) versus Colon 26G (Cy3; arrays 1 and 2) and a dye swap Colon 26A (Cy3) versus Colon 26G (Cy5; array 3). The validity was confirmed by a self-self array of Colon 26G (array 4).

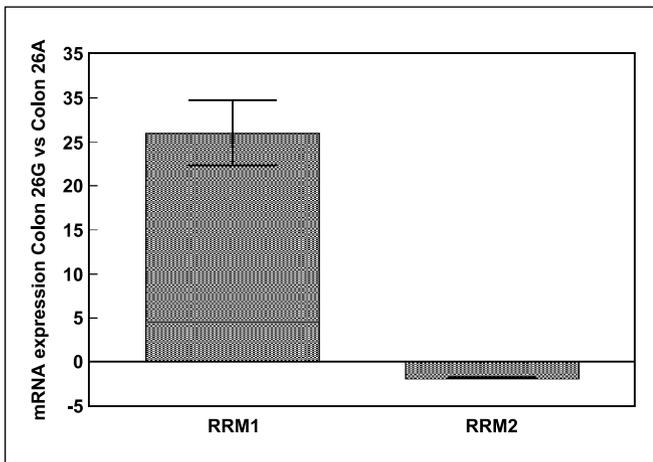


Figure 5. mRNA expression of RRM1 and RRM2 in the gemcitabine-resistant Colon 26G relative to its parental tumor Colon 26A (which was set at 1). Bars, \pm SE. Light cycler analysis of RRM1 and RRM2 mRNA expression was done in five different generations of Colon 26-G and Colon 26-A tumors. Expression relative to mRNA of the housekeeping gene β -actin in Colon 26A and Colon 26G: RRM1, 2.09 ± 0.48 and 52.63 ± 16.01 , respectively ($P = 0.001$) and RRM2, 1.12 ± 0.23 and 0.58 ± 0.11 , respectively ($P = 0.002$).

the activity of ribonucleotide reductase in extracts of the tumors. The activity of ribonucleotide reductase in Colon 26-A tumors was 353 ± 14 and that in Colon 26-G tumors 849 ± 21 pmol/h/mg protein.

Discussion

In this article, we describe the *in vivo* induction of resistance to gemcitabine, which was achieved by repetitive treatment of tumor-bearing mice. To our knowledge, this is the first animal tumor model with acquired resistance to gemcitabine. The parental tumor Colon 26-A was moderately sensitive to gemcitabine. Therefore, we can consider Colon 26-A as a murine model for human cancers with a moderate sensitivity to gemcitabine. Strikingly, establishment of resistance required a long period of treatment, especially considering the rapid growth rate of this tumor, indicating that resistance is not easily acquired. Although a decrease in dCK activity has frequently been associated with acquired resistance to gemcitabine (7), dCK activity was only moderately decreased in Colon 26-G and *dCK* gene expression was not consistently altered in all three expression microarray experiments. No difference in dCDA activity was found between Colon 26-A and Colon 26-G. Previously, no clear relation between intrinsic dCDA activity and sensitivity to gemcitabine has been described, although transfection of the *dCDA* gene can induce resistance (7, 28). DNA polymerase may play a role in recognition and repair of DNA damage and in the interaction between DNA-damaging agents and deoxynucleoside analogue incorporation into DNA (29). Moreover, when deoxynucleoside analogues are incorporated into DNA during resynthesis, new DNA is relatively resistant to repair excision and causes irreversible damage to the cell. However, no significant difference in DNA polymerase activity was found between the tumors. Because thymidine kinase may play a role in gemcitabine activation as well, we determined the activities of both TK forms, the cytoplasmic TK1 and the mitochondrial TK2. A striking 12-fold decrease in TK2 activity was found. The decreased TK1 activity was not accompanied by a decreased gene expression as analyzed by microarray and suggests a posttranslational

regulation. Both TK2 enzyme activity and gene expression were decreased in Colon-26G. However, gemcitabine is a poor substrate for TK2 and there is no direct evidence that the mitochondrial enzyme TK2 will phosphorylate gemcitabine in intact cells (8). Moreover, studies on the relation between TK2 activity and gemcitabine expression are not unequivocal (7).

The single most striking increase in expression revealed by microarray expression profiling was of the *RRM1* gene, whose increase was confirmed by both reverse transcription-PCR as well as by Western blotting. In contrast, RRM2 expression, which was reported to be related to gemcitabine resistance previously (30), was not altered in Colon 26-G. Ribonucleotide reductase was already known as one of the targets for gemcitabine (10); however, reports from *in vitro* studies on the importance of ribonucleotide reductase inhibition in the cytotoxic properties of gemcitabine were not consistent (7). Goan et al. and Dumontet et al. were the first to suggest that ribonucleotide reductase was a primary target of gemcitabine as a regulator of dNTP pools, leaving a secondary role for dCK (30, 31). In human oropharyngeal epidermoid carcinoma KB cells made 10-fold resistant to gemcitabine, a 2-fold increase in ribonucleotide reductase activity was found, resulting in increased dATP and dCTP pools (30) due to an overexpression of RRM2. No difference was found in dCK expression, but dCK enzyme activity was decreased 2-fold. After removal of the endogenous dNTP pools from the extract by passing it over a G-25 column, no difference in dCK activity in extracts from parental and variant cells was found. Dumontet et al. observed a 4-fold increase in ribonucleotide reductase activity as well as a 4-fold decrease in dCK activity but did not specify the subunit (31). In contrast, Jordheim et al. (32) found a 2-fold reduction in the RRM2 unit similar to our data.

Davidson et al. identified an increased expression of RRM1 as the major determinant of gemcitabine resistance in two pairs of parental and gemcitabine resistant human NSCLC cell lines (33). In a microarray profiling assay, the *RRM1* gene was up-regulated at least 5-fold. Further studies revealed that there was no difference in the sequence of the cDNA encoding RRM1 between the parental and gemcitabine-resistant cells and that activity of ribonucleotide reductase was not altered. We only observed a moderately increased ribonucleotide reductase activity in the Colon 26-G tumors. This is not surprising because ribonucleotide reductase activity is predominantly associated with the RRM2 subunit, whereas the RRM1 subunit is involved in substrate regulation of the enzyme. Because the RRM2 subunit is responsible for the catalytic activity of the enzyme, Davidson et al. suggested that RRM1 might be acting as a "molecular sink"

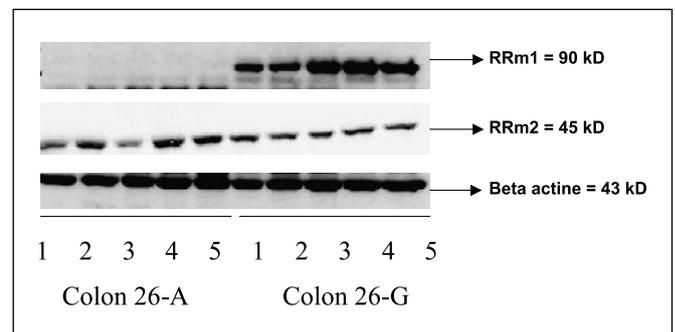


Figure 6. Western blots for RRM1 and M2 in five extracts of five different Colon 26-A and Colon 26-G tumors. Protein loading was checked by β -actin staining.

for gemcitabine, whereby the drug binds irreversibly to subunit RRM1 and inactivates it (33). To maintain sufficient ribonucleotide reductase activity for the cells to survive, the cells increase RRM1 expression.

Up to now, all studies on ribonucleotide reductase as a factor in gemcitabine resistance are based on *in vitro* acquired resistance. No data are available in animal model systems showing a relationship between ribonucleotide reductase expression and acquired or intrinsic gemcitabine resistance similar to that between gemcitabine and dCK (27, 34). However, patients with metastatic NSCLC treated with gemcitabine containing chemotherapy and low pretreatment expression of RRM1 mRNA had a significantly longer median survival than those with a high expression (35), indicating a role for RRM1 in intrinsic resistance to gemcitabine.

Our data do not rule out that other mechanisms of resistance determine sensitivity to gemcitabine *in vivo* and in patients. The used microarray and real-time PCR assays do not give insight in mutations, genomic polymorphisms, and posttranslational modifications such as protein phosphorylation, although other genes are coamplified or deleted (36). In addition, it is also known

that P53 can regulate ribonucleotide reductase expression and activity (37). Transporters for gemcitabine such as CNT can translocate from the membrane to intracellular vesicles. Future studies should therefore not only focus on RRM1 expression. Immunohistochemistry can give insight in the intracellular localization of the transporters and other target enzymes, such as dCK (38).

In conclusion, we developed the first *in vivo* model of resistance to gemcitabine as a result of repetitive treatment using a clinically relevant schedule. Microarray profiling revealed an marked increase in RRM1 expression, which is in line with *in vitro* studies. Ribonucleotide reductase was already known as a target for gemcitabine, but our data identify ribonucleotide reductase as a key target for acquired *in vivo* gemcitabine resistance.

Acknowledgments

Received 3/24/2005; revised 7/15/2005; accepted 8/1/2005.

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.

References

- Hertel LW, Kroin JS, Misner JW, Tustin JM. Synthesis of 2'-deoxy-D-ribose and 2'-deoxy-2',2'-difluoro-D-ribofuranosyl nucleosides. *J Org Chem* 1988;53:2406-9.
- Heinemann V, Hertel LW, Grindley GB, Plunkett W. Comparison of the cellular pharmacokinetics and toxicity of 2',2'-difluorodeoxycytidine and 1- β -D-arabinoarabofuranosylcytosine. *Cancer Res* 1988;48:4024-31.
- Ramalingam S, Belani CP. State-of-the-art chemotherapy for advanced non-small cell lung cancer. *Semin Oncol* 2004;31:68-74.
- Hussain SA, James ND. The systemic treatment of advanced and metastatic bladder cancer. *Lancet Oncol* 2003;8:489-97.
- Heinemann V. Gemcitabine in the treatment of advanced pancreatic cancer: a comparative analysis of randomized trials. *Semin Oncol* 2002;29:9-16.
- Ramalingam S, Belani CP. The role of gemcitabine in the treatment of other tumours. *Br J Cancer* 1998;78:21-5.
- Bergman AM, Pinedo HM, Peters GJ. Determinants of resistance to 2',2'-difluorodeoxycytidine (gemcitabine). *Drug Resist Updat* 2002;5:19-33.
- Wang L, Munch-Petersen B, Herstrom Sjoberg A, et al. Human thymidine kinase 2: molecular cloning and characterisation of the enzyme activity with antiviral and cytostatic nucleoside substrates. *FEBS Lett* 1999;443:170-4.
- 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.
- Heinemann V, Xu Y-Z, Chubb S, et al. Inhibition of ribonucleotide reduction in CCRF-CEM cells by 2',2'-difluorodeoxycytidine. *Mol Pharmacol* 1990;38:567-72.
- Pereira S, Fernandes PA, Ramos MJ. Theoretical study of ribonucleotide reductase mechanism-based inhibition by 2'-azido-2'-deoxyribonucleoside 5'-diphosphates. *J Comput Chem* 2004;30:1286-94.
- Van der Donk WA, Yu G, Perez L, Sanchez RJ, Stubbe J. Detection of a new substrate-derived radical during inactivation of ribonucleotide reductase from *Escherichia coli* by gemcitabine 5'-diphosphate. *Biochemistry* 1998;37:6419-26.
- Reichard P. From RNA to DNA, why so many ribonucleotide reductases? *Science* 1993;260:1773-7.
- Veerman G, Ruiz van Haperen VW, Vermorken JB, Pinedo HM, Peters GJ. Antitumor activity of prolonged as compared with bolus administration of 2',2'-difluorodeoxycytidine *in vivo* against murine colon tumors. *Cancer Chem Pharmacol* 1996;38:335-42.
- Peters GJ, Van Dijk J, Nadal JC, Van Groeningen CJ, Lankelma J, Pinedo HM. Diurnal variation in the therapeutic efficacy of 5-fluorouracil against murine colon cancer. *In vivo* 1987;1:113-8.
- Peters GJ, Laurensse E, Leyva A, Lankelma J, Pinedo HM. Tissue homogenization using a micro-dismembrator for the measurement of enzyme activities. *Clin Chim Acta* 1986;158:193-8.
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248-54.
- Ruiz van Haperen VW, Veerman G, Braakhuis BJ, et al. Deoxycytidine kinase and deoxycytidine deaminase activities in human tumour xenografts. *Eur J Cancer* 1993;29:2132-7.
- Munch-Petersen B, Cloos L, Tyrsted G, Eriksson E. Diverging substrate specificity of pure human thymidine kinases 1 and 2 against antiviral dideoxynucleosides. *J Biol Chem* 1991;266:9032-8.
- Tyrsted G, Munch-Petersen B. Early effects of phytohemagglutinin on induction of DNA polymerase, thymidine kinase, deoxyribonucleoside triphosphate pools and DNA synthesis in human lymphocytes. *Nucleic Acids Res* 1977;27:13-23.
- Smid K, Van Moorsel CJ, Noordhuis P, Voorn DA, Peters GJ. Interference of gemcitabine triphosphate with the measurements of deoxynucleotides using an optimized DNA polymerase elongation assay. *Int J Oncol* 2001;19:157-62.
- Fukushima M, Fujioka A, Uchida J, Nakagawa F, Takechi T. Thymidylate synthase (TS) and ribonucleotide reductase (RNR) may be involved in acquired resistance to 5-fluorouracil (5-FU) in human cancer xenografts *in vivo*. *Eur J Cancer* 2001;37:1681-7.
- Ruiz van Haperen VW, Veerman G, Boven E, Noordhuis P, Vermorken JB, Peters GJ. Schedule dependence of sensitivity to 2',2'-difluorodeoxycytidine (Gemcitabine) in relation to accumulation and retention of its triphosphate in solid tumour cell lines and solid tumours. *Biochem Pharmacol* 1994;48:1327-39.
- Buermans HP, Redout EM, Schiel AE, et al. Microarray analysis reveals pivotal divergent mRNA expression profiles early in the development of either compensated ventricular hypertrophy or heart failure. *Physiol Genomics* 2005;21:314-23.
- DeRisi JL, Iyer VR, Brown PO. Exploring the metabolic and genetic control of gene expression on a genomic scale. *Science* 1997;278:680-6.
- Sigmond J, Kroep JR, Loves WJ, Codacci-Pisanelli G, Peters GJ. Quantitative real time PCR of deoxycytidine kinase mRNA by light cycler PCR in relation to enzyme activity and gemcitabine sensitivity. *Cancer Lett* 2004; 213:173-9.
- Kroep JR, Loves WJ, Van der Wilt CL, et al. Pretreatment deoxycytidine kinase levels predict *in vivo* gemcitabine sensitivity. *Mol Cancer Ther* 2002;1:371-6.
- Eliopoulos N, Cournoyer D, Momparler RL. Drug resistance to 5-aza-2'-deoxycytidine, 2',2'-difluorodeoxycytidine, and cytosine arabinoside conferred by retroviral-mediated transfer of human cytidine deaminase cDNA into murine cells. *Cancer Chemother Pharmacol* 1998;42:373-8.
- Yang LY, Li L, Keating MJ, Plunkett W. Arabinosyl-2'-fluoro-adenine augments cisplatin cytotoxicity and inhibits cisplatin-DNA cross-link repair. *Mol Pharmacol* 1995;47:1072-9.
- Goan Y-G, 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.
- Dumontet C, Fabianowska-Majewska K, Mantincic D, et al. Common resistance mechanisms to deoxynucleoside analogues in variants of the human erythroleukemic line K562. *Br J Haematol* 1999;106:78-85.
- 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.
- 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.
- Blackstock AW, Lightfoot H, Case LD, et al. Tumor uptake and elimination of 2',2'-difluoro-2'-deoxycytidine (gemcitabine) after deoxycytidine kinase gene transfer: correlation with *in vivo* tumor response. *Clin Cancer Res* 2001;7:3263-8.
- 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.
- Van de Wiel MA, Costa JL, Smid K, et al. Expression microarray analysis and oligo array CGH of acquired gemcitabine resistance in mouse colon reveals selection for chromosomal aberrations. *Cancer Res*. In press 2005.
- 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.
- Hubeek I, Peters GJ, Broekhuizen AJ, et al. Immunocytochemical detection of deoxycytidine kinase in haematological malignancies and solid tumours. *J Clin Pathol* 2005;58:695-9.