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Carlos María Galmarini, Marilyn L. Clarke, Lars Jordheim, Cheryl L. Santos, Emeline Cros, et al.. Resistance to gemcitabine in a human follicular lymphoma cell line is due to partial deletion of the deoxycytidine kinase gene.. BMC Pharmacology, 2004, 4, pp.8. 10.1186/1471-2210-4-8 . inserm-00112022

HAL Id: inserm-00112022

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Resistance to gemcitabine in a human follicular lymphoma cell line is due to partial deletion of the deoxycytidine kinase gene

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Published: 24 May 2004

BMC Pharmacology 2004, **4**:8

Received: 28 January 2004
Accepted: 24 May 2004

This article is available from: http://www.biomedcentral.com/1471-2210/4/8

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Abstract

Background: Gemcitabine is an analogue of deoxycytidine with activity against several solid tumors. In order to elucidate the mechanisms by which tumor cells become resistant to gemcitabine, we developed the resistant subline RL-G from the human follicular lymphoma cell line RL-7 by prolonged exposure of parental cells to increasing concentrations of gemcitabine.

Results: *In vitro*, the IC_{50} increased from 0.015 μM in parental RL-7 cells to 25 μM in the resistant variant, RL-G. Xenografts of both cell lines developed in nude mice were treated with repeated injections of gemcitabine. Under conditions of gemcitabine treatment which totally inhibited the development of RL-7 tumors, RL-G derived tumors grew similarly to those of untreated animals, demonstrating the *in vivo* resistance of RL-G cells to gemcitabine. HPLC experiments showed that RL-G cells accumulated and incorporated less gemcitabine metabolites into DNA and RNA than RL-7 cells. Gemcitabine induced an S-phase arrest in RL-7 cells but not in RL-G cells. Exposure to gemcitabine induced a higher degree of apoptosis in RL-7 than in RL-G cells, with poly-(ADP-ribose) polymerase cleavage in RL-7 cells. No modifications of Bcl-2 nor of Bax expression were observed in RL-7 or RL-G cells exposed to gemcitabine. These alterations were associated with the absence of the deoxycytidine kinase mRNA expression observed by quantitative RT-PCR in RL-G cells. PCR amplification of désoxycytidine kinase gene exons showed a partial deletion of the dCK gene in RL-G cells.

Conclusions: These results suggest that partial deletion of the dCK gene observed after selection in the presence of gemcitabine is involved with resistance to this agent both *in vitro* and *in vivo*.

Background

Gemcitabine is an analogue of deoxycytidine with activity against several solid tumors (reviewed in [1]). Gemcitabine enters the cell via a facilitated nucleoside transport mechanism [2,3] and is phosphorylated into gemcitabine 5'-monophosphate (dFd-CMP) by deoxycytidine kinase (dCK) [4]. It is then subsequently phosphorylated by other pyrimidine kinases to the active 5'-diphosphate (dFd-CDP) and triphosphate (dFd-CTP) derivatives [5]. Inactivation of gemcitabine and dFd-CMP occurs by

deamination by cytidine deaminase (CDD) and dCMP deaminase, respectively [6,7]. Dephosphorylation of dFd-CMP by cytoplasmic 5'-nucleotidase activities can also prevent the production of the active forms [8].

Cytotoxic actions of gemcitabine are exerted by the phosphorylated metabolites [9]. The active diphosphate metabolite, dFd-CDP, inhibits DNA synthesis indirectly through inhibition of ribonucleotide reductase (RNR) [10]. This effect blocks the *de novo* DNA synthesis pathway and self-potentiates gemcitabine activity by decreasing intracellular concentrations of normal deoxynucleoside triphosphates, thereby favoring further gemcitabine phosphorylation and dFd-CTP incorporation into DNA [11,12]. The other active metabolite, dFd-CTP, is incorporated into the growing DNA strand by replication synthesis after which DNA polymerase is able to add one more nucleotide before DNA polymerisation is terminated [13]. Inhibition of DNA synthesis leads to growth inhibition or cell death. Gemcitabine can also be incorporated into RNA and inhibit RNA synthesis. While incorporation of gemcitabine metabolites into RNA are concentration-dependent, the extent of RNA synthesis inhibition seems to be cell-specific [9]. Finally gemcitabine inhibits dCMP deaminase and CTP synthetase activities [11,14].

Various general mechanisms of resistance to gemcitabine have been described (reviewed in [15,16]). A primary mechanism of resistance may arise from an insufficient intracellular concentration of dFdCTP because of an altered activation/degradation pathway. This may results from inefficient cellular uptake, reduced levels of gemcitabine activation or increased drug degradation. Deficiency of dCK is the most frequent described mechanism of resistance to gemcitabine (for review see [17]). Several authors have described a relation between dCK activity and sensitivity to gemcitabine in cells with an acquired resistance to gemcitabine in cell and animal models [18-20]. Deficiency of nucleoside transporters in tumour plasma membranes, as well as increased activity of CDD, dCMP deaminase or 5'-nucleotidase enzyme activities have also been suggested as mechanisms of cellular resistance to gemcitabine [2,6-8].

A second type of resistance mechanism may concern altered interactions with intracellular targets [21]. Various authors showed a correlation between gemcitabine resistance and a increase in ribonucleotide reductase (RNR) activity [19,21]. Finally, alterations of apoptosis-regulating genes (principally *p53*) also appear to be involved in the sensitivity of tumor cells to the cytotoxic effect of gemcitabine [22].

In order to elucidate the mechanisms by which tumor cells become resistant to gemcitabine, we developed the resistant subline RL-G from the human follicular lymphoma cell line RL-7 by prolonged exposure of parental cells to increasing concentrations of gemcitabine. Here, we describe the biochemical and molecular characterization of RL-G cells in comparison to the parental RL-7 cells, with particular reference to their differential responses to exposure to gemcitabine. Analysis of the mechanisms of resistance identified deficiency of dCK as the main cause of resistance in RL-G cells *in vitro* and in mouse xenografts.

Results

In vitro and in vivo chemosensitivity studies

We have previously shown that RL-G cells were 2,092-fold more resistant than RL-7 cells to gemcitabine (RL-G IC50: 25.1 ± 5.0 μM; RL-7 IC50: 0.015 ± 0.0006 μM) [23]. RL-G cells were also cross resistant to ara-C.

We determined the effect of gemcitabine on the growth of the RL-7 and RL-G tumors when grown subcutaneously as xenografts in athymic nude mice. Median tumor weights in mg of RL-7 and RL-G xenografts in Swiss nude female mice treated or not with gemcitabine are shown in Figure 1. At day 60 after tumor injection, the RL-7 xenografts treated with gemcitabine were significantly smaller than the control tumors (Student *t* test, $p = 0.04$) (Fig. 1). The T/C value for RL-7 xenografts was 0.24. Conversely, no significant differences in tumor size were observed between the treated and control groups of mice xenotransplanted with the RL-G cells. The T/C value for RL-G xenografted mice was 0.98. These results indicate that RL-7 tumors were significantly more sensitive to gemcitabine treatment than the RL-G tumors.

[^{3}H]Gemcitabine uptake, metabolism and incorporation into nucleic acids

To assess whether a nucleoside transport defect was a contributing factor to the gemcitabine resistance of RL-G cells, the initial uptake rates (2–10 s) of 10 μM [^{3}H]gemcitabine by RL-7 and RL-G cells were compared, either in the absence or presence of excess (100-fold) non-radioactive gemcitabine. Both RL-7 and RL-G cells were able to transport [^{3}H]gemcitabine with comparable initial uptake rates of 0.81 ± 0.28 and 0.5 ± 0.22 pmol/10^6 cells, respectively (Fig. 2A). In the presence of excess non-radioactive gemcitabine, initial uptake rates were decreased by 72% (RL-7) and 65% (RL-G), indicating that the uptake of [^{3}H]gemcitabine was mediated by a transporter in both cell lines. The initial uptake rates of 10 μM [^{3}H]uridine, a physiologic nucleoside, by RL-7 and RL-G cells were also measured and found to be comparable to those measured with gemcitabine (data not shown). These data suggested that RL-G cells were capable of transporting nucleosides intracellularly and that a transport defect was not involved in the gemcitabine-resistance mechanism.

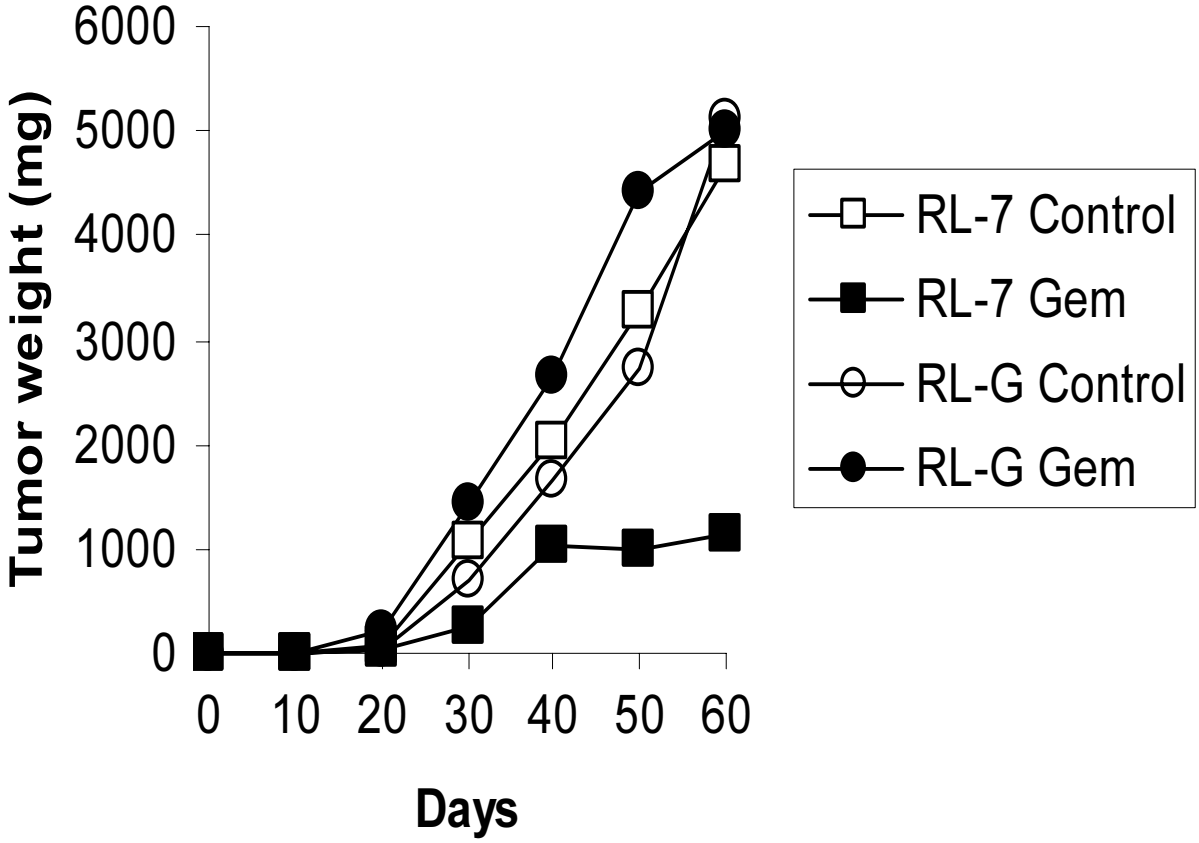

Figure 1
Median tumor weight in mg in nude Swiss female mice xenografted with RL-7 or RL-G cells and treated with gemcitabine (200 mg/Kg i.p. once a week; 6 doses) or untreated. RL-7 control : mice injected with RL-7 cells, untreated; RL-7 Gem : mice injected with RL-7 cells, treated with gemcitabine; RL-G control : mice injected with RL-G cells, untreated; RL-G Gem : mice injected with RL-G cells, treated with gemcitabine.

Although RL-G cells were capable of inward nucleoside transport, the cells were only able to metabolize [^{3}H]gemcitabine in trace amounts over a 4 h exposure period as shown by the lack of measurable phosphorylated metabolites detected by HPLC analysis. Trace amounts of [^{3}H]dFdCTP could be detected in some samples if the scale was greatly amplified. In contrast, RL-7 cells accumulated 16.4-fold more [^{3}H]gemcitabine-derived compounds than RL-G cells (Fig. 2B). Approximately 90% of the total radioactivity measured in acid extracts of RL-7 cells was recovered as the cytotoxic triphosphate (2.2 μmol/mg protein) and diphosphate (0.98 μmol/mg protein) derivatives with a [^{3}H]dFdCTP/[^{3}H]dFdCDP ratio of 2.3. ATP/ADP ratios for untreated vs. gemcitabine-treated cells, were 3.6 vs. 2.8 for RL-7, and 3.1 vs. 2.1 for RL-G, respectively. Since gemcitabine has the ability to be incorporated into both DNA and RNA, the amount of [^{3}H]gemcitabine present in the nucleic acid extractions was determined. No [^{3}H]gemcitabine was detected in the DNA or RNA of RL-G cells as expected (Fig. 2C). In RL-7 cells, the incorporation of [^{3}H]gemcitabine was higher in genomic DNA (11.9-fold) than in total RNA (Fig. 2C).

Detection of gemcitabine-induced cell cycle perturbations and cell cycle-related events

Flow cytometry of RL-7 and RL-G cells demonstrated a diploid cell population. After 24 h of continuous exposure to gemcitabine, a substantial accumulation of RL-7

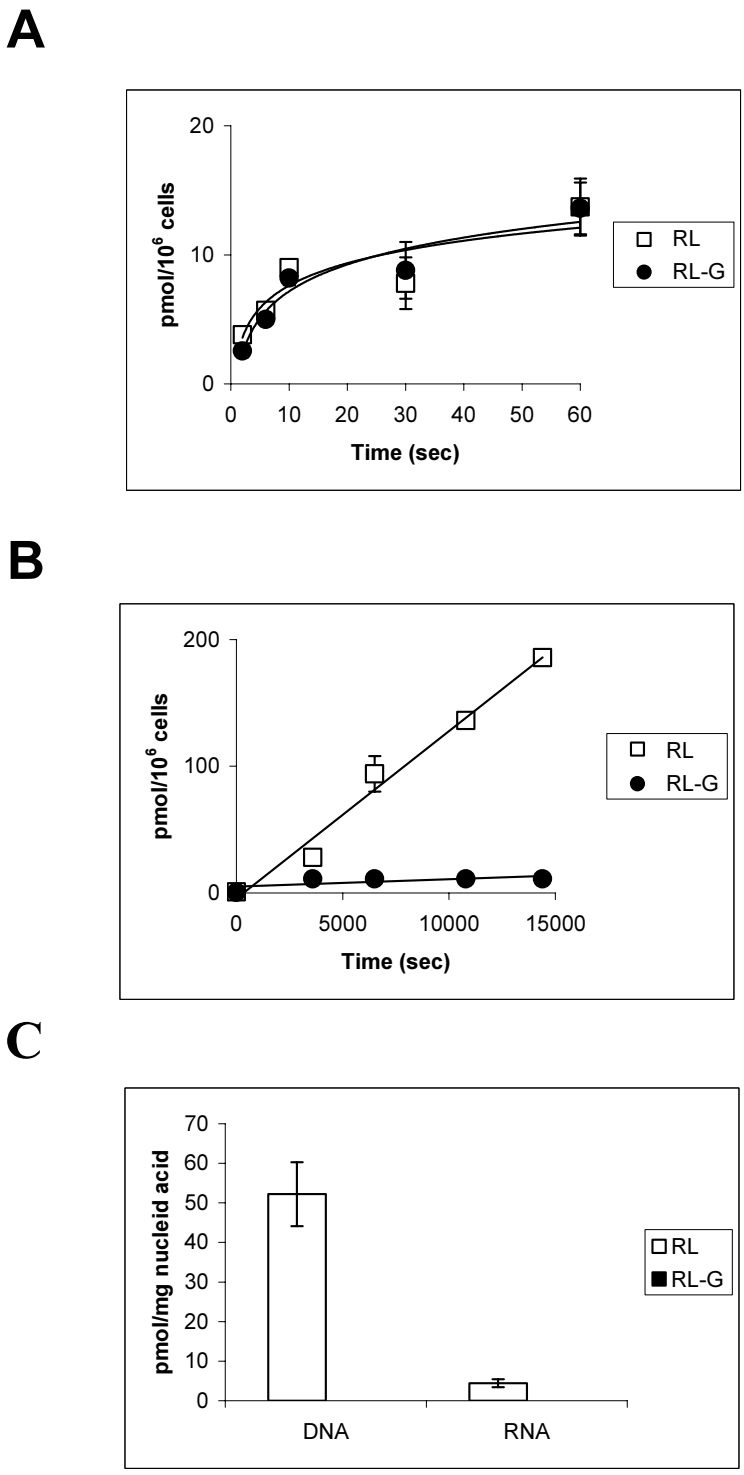

Figure 2

Comparative uptake of [^{3}H]gemcitabine and incorporation into nucleic acids of RL-7 and RL-G cells. (A) Cells were exposed to [^{3}H]gemcitabine for 2–60 s. **(B)** Accumulation of [^{3}H]gemcitabine in RL-7 and RL-G cells over a 4 h exposure period. Each time point represents the average (± S.D.) of three determinations **(C)** Incorporation of [^{3}H]gemcitabine (pmol/mg) into nucleic acids after a 4-h exposure in two separate experiments.

Table 1: Primer sequences used for semi-quantitative PCR assessment of the deoxycytidine kinase gene.

Exon	Forward Primer 5'-3'	Reverse Primer 5'-3'	Product size
5'-UTR	AGTCACCGTTTTCCCCTTCT	CTTTGTTCAACACGCCAAAA	206
2	GGCAGGGAGCCTTTTCATTT	CTCCTCTAAACCAAGAGGCT	339
3	CCAGACCTCAGACAATCTGA	CCCAGCTCAGCCATTCATTA	490
4	GTGCCACTGGATTTAGGAGA	GTTGGGAGGCTGGAGTTAAA	358
5	AGCGTGAGTAGAGAGACACA	CGCTATCAATACCACCAAGG	439
6	CTGCCAGATGAAGTACTGCA	TGTCAGATTCTGAGATTCCG	458
7	AGTTACTTATACAGCTGGGG	TATCTGGAACCATTTGGCTGC	180

cells in G0/G1 phase was observed with a decrease in the G2/M fraction (Table 2). At 72 h, a maximal arrest of cells in the S phase fraction (84%) was detected. The G0/G1 fraction dropped dramatically as the cells moved into S phase. Conversely, in RL-G cells gemcitabine treatment slightly arrested the cell cycle in S-phase at the expense of the G2/M fraction (Table 2). No G1 phase arrest was observed in this cell line (Table 2).

Comparison of apoptosis and apoptotic pathways between RL-7 and RL-G cells

Exposure of cells for 24 h and 72 h with gemcitabine caused a proportional increase of annexin-V staining typical of apoptosis in RL-7 cells. However, the amount of apoptotic cells did not increase in the RL-G cell line (Fig. 3A). Poly-(ADP-ribose) polymerase (PARP) cleavage was detected after 72 h of exposure to gemcitabine of RL-7 cells (Fig. 3B). As shown in Figure 3C, no significant modifications of the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax levels were detected after gemcitabine exposure.

Analysis of factors involved in gemcitabine metabolism and drug targets by quantitative reverse-transcriptase polymerase chain reaction (RT-PCR)

To test whether resistance of RL-G cells to gemcitabine was due to multiple mechanisms, the mRNA levels of several biological parameters (and potential drug targets) involved in gemcitabine metabolism, were analyzed by real time-PCR. According to quantitative RT-PCR results shown in Figure 4A, RL-G cells had no detectable dCK expression compared to RL-7 cells. In contrast, the 5'-nucleotidases, cN-II and dNT-2, were slightly increased in RL-G cells (2-fold). Similarly, expression levels of hENT1 and RNR subunits 1 and 2 were increased in 2.7-, 3.4- and 1.8-fold in RL-G cells, respectively (Figure 4B). hENT2, 5'-nucleotidase dNT1 and DNA POL mRNA were expressed at similar levels in both cell lines. Neither RL-7 cells nor RL-G cells expressed detectable CDD (data not shown).

PCR analysis of dCK gene

To determine the cellular modification responsible for dCK mRNA down-regulation, we performed a qualitative study of the dCK gene. For this purpose, exons of the dCK gene as well as 5'-untranslated regions were amplified by PCR as described in the methods section. Figure 5 shows PCR products obtained using genomic DNA from RL-7 and RL-G cells. In resistant RL-G cells, no amplicons were obtained for regions located before the fourth exon, suggesting a partial deletion of the dCK gene in this cell line. Exons 4, 5, 6 and 7 of the human dCK gene were amplified in both cell lines. These results suggest a genetic modification of the dCK gene in RL-G cells.

Discussion

Our results show that dCK-deficient RL-G lymphoma cells are resistant to gemcitabine both *in vitro* and *in vivo*. RL-G cells accumulated only trace amounts of phosphorylated derivatives of [^{3}H]gemcitabine compared to their parental counterpart. This lack of accumulation was not due to decreased inward transport of gemcitabine by nucleoside transporters because mRNA expression of nucleoside transporters and the initial uptake rates measured were similar for both cell lines. The lack of [^{3}H]gemcitabine nucleotide accumulation and incorporation into nucleic acids in RL-G cells was explained by the lack of dCK expression in these cells.

A relationship between lack of dCK expression and decreased dFd-CTP accumulation has already been shown by other investigators, both in selected and non-selected cell lines which had various levels of dCK activity, including dCK-deficient lines (reviewed in [15]). The observed cross-resistance of the RL-G cells to other pyrimidine and purine analogues may also be due to dCK deficiency. Other authors have also shown cross-resistance of gemcitabine-resistant cells to other nucleoside analogues due to dCK deficiency [7,24,25].

In keeping with the *in vitro* results, the RL-G mouse xenografts were resistant to gemcitabine treatment in contrast to the parental RL-7 tumors. These data are are

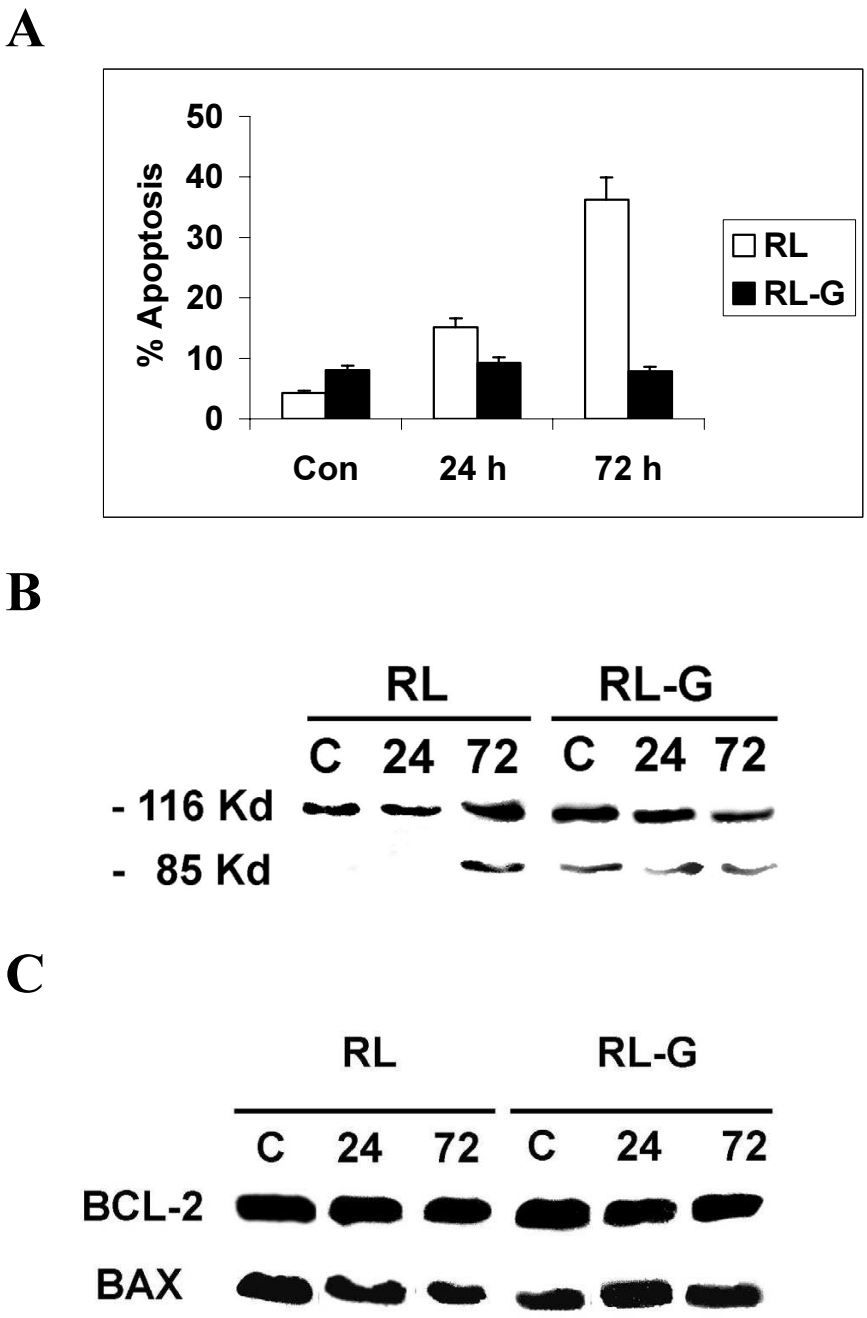

Figure 3

Effect of gemcitabine (0.3 μM) on apoptosis and protein expression of PARP and pro- and antiapoptotic members of the Bcl-2/ Bax family. (A) Flow cytometric detection of the Annexin-V staining in RL-7 and RL-G cells measured at different time intervals after incubation with gemcitabine 0.3 μM (B) PARP cleavage as detected by western blot after gemcitabine treatment at different time intervals (C) Bcl-2 and Bax expression by western blot. RL-7 and RL-G cells were treated with gemcitabine 0.3 μM for 24 and 72 h and subsequently lysed, subjected to SDS-PAGE, and immunoblotted with the corresponding monoclonal antibody as described in Material and Methods. The experiments shown are representative of two performed. C: control.

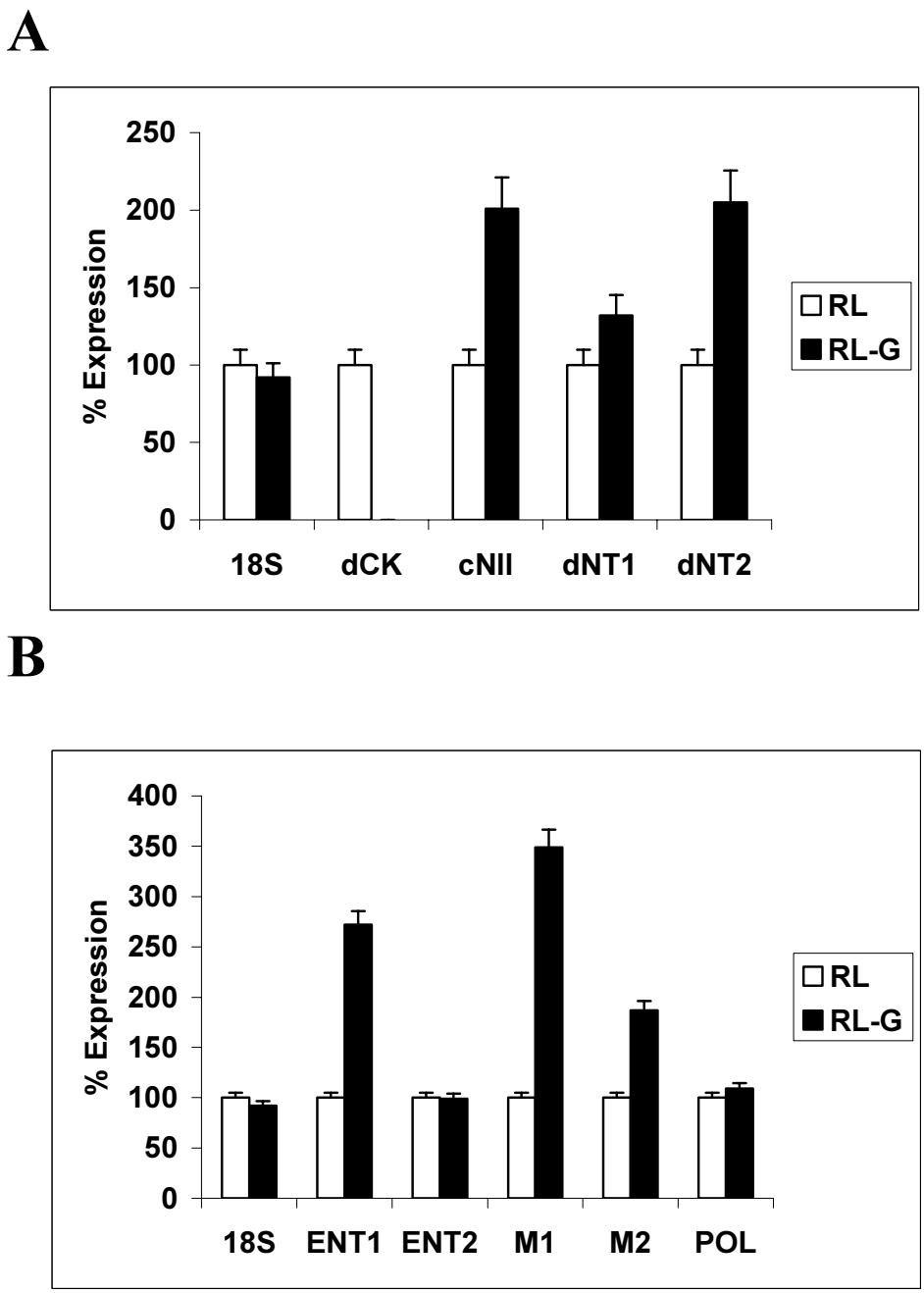

Figure 4

Analysis of other known mechanisms of resistance to gemcitabine. (A) Quantitative real time RT-PCR analysis of factors implicated in the activation or degradation of gemcitabine in RL-7 and RL-G cells; 18S ribosomal RNA (18S), deoxycytidine kinase (dCK), high Km 5'-nucleotidase (cNII), 5'-3'-nucleotidase (dNT1) and mitochondrial 5'-nucleotidase (dNT2) **(B)** Quantitative real time PCR analysis of factors implicated in the transport and intracellular targeting of gemcitabine; human equilibrative nucleoside transporter 1 and 2 (ENT1; ENT2), ribonucleotide reductase M1 and M2 subunits (M1, M2) and DNA polymerase α (Pol). RT-PCR of these genes was performed as described in Material and Methods. Results are expressed as % of PCR arbitrary unit expression in RL-G cells related to RL-7 PCR arbitrary unit expression.

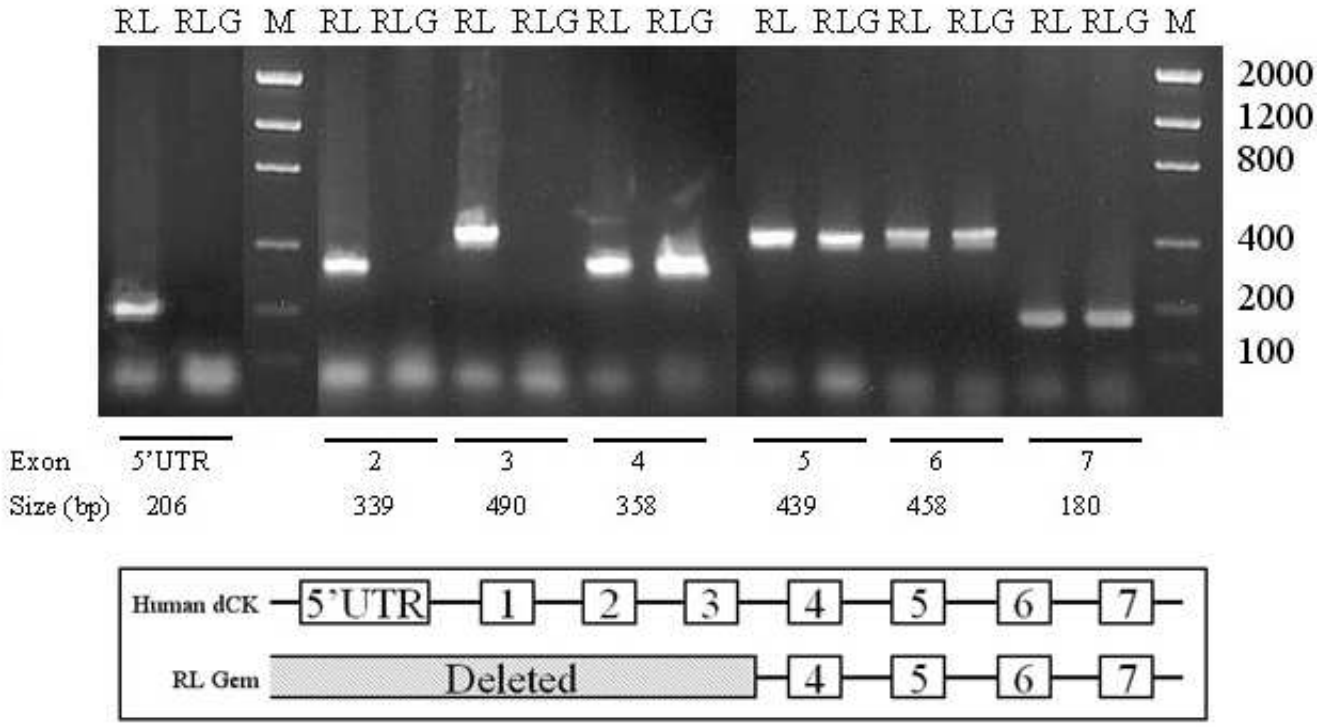

Figure 5
Semi-quantitative PCR assesment of exons 2 to 7 of the dCK gene and a 5'-untranscribed region. Genomic DNA and PCR were performed as described in Methods section.

consistent with the observations reported by other authors. Ruiz van Haperen [18] demonstrated that human ovarian carcinoma A2780 xenografts were highly resistant to gemcitabine, mainly because of a decreased dCK activity. More recently, Blackstock [26] demonstrated that transfection of the *dCK* gene into HT-29 human colon carcinoma xenografts resulted in increased expression of dCK, enhanced dFd-CTP accumulation and a potentiated *in vivo* tumor response to gemcitabine. Our results confirm a major role for dCK in *in vitro* and *in vivo* resistance to gemcitabine.

To elucidate why dCK mRNA expression was absent in the resistant cells, we analyzed the dCK gene by PCR. Our data show that the deficiency in dCK mRNA expression as detected by quantitative PCR is due to a partial deletion in the 5'-region of the dCK gene developed during the stepwise selection process with increasing concentrations of gemcitabine. It was previously reported that deletions of exon 3 conferred resistance to pyrimidine nucleoside analogues in a human melanoma cell line [27]. Deletions of exon 4 and 5 were also related with resistance to ara-C and gemcitabine in leukemic, fibrosarcoma and ovarian cell lines and leukemic blasts from acute myeloid leukemia patients [18,27-29]. The nature of the deletion mutant seems to be dependent on the nucleoside analogue which was used for establishing resistant cells, although the chemical structures and the molecular targets of these nucleosides are similar.

It is common to observe the presence of multiple resistance mechanisms when drug resistance is induced artificially *in vitro* by prolonged exposure [19,30]. Gemcitabine resistance demonstrated by RL-G cells may involve minor contributions from other resistance mechanisms in addition to dCK deficiency. Previous detailed investigations using the purified ribonucleotide reductase have elucidated the mechanism-based inhibition of this protein by dFd-CDP [12]. *In vitro* data suggests irreversible binding of the R1 subunit as the reasonable target for enzyme inactivation [31]. Such inhibition of the R1 subunit depends on the availability of dFd-CDP and on the synthesis of new proteins to restore enzyme activity. Thus, an increase in RNR-M1 expression may also have contributed to the

resistance phenotype of RL-G cells. However, as indicated earlier, RL-G cells accumulated only trace amounts of phosphorylated derivatives of [^{3}H]gemcitabine indicating that the major mechanism of resistance to gemcitabine in these cells involved drug activation.

RL-G cells also expressed a 2-fold increase of the 5'-nucleotidases cN-II and dNT-2. The role of 5'-nucleotidases in gemcitabine resistance is controversial. We previously observed that total 5'-nucleotidase activity was 14-fold increased in K562 human leukemia cells selected for resistance to gemcitabine [19]. In contrast, Schirmer [8] demonstrated that cladribine-resistant HL60 cells expressing high levels of cN-II were not resistant to gemcitabine. In addition, recent *in vitro* studies with different recombinant 5'-nucleotidases have not shown a direct degradation of dFd-CMP [32]. Thus, whether increased levels of these two 5'-nucleotidases contribute to gemcitabine resistance observed in our model is not clear.

In our model, RL-7 cells were more susceptible to gemcitabine-induced apoptosis than RL-G cells, in keeping with previous evidence on the role of gemcitabine as a potent inducer of apoptosis [22]. The presence of PARP cleavage found in the RL-7 cells demonstrated that apoptosis may involve the caspase-3 pathway. We also analyzed the expression of Bcl-2 and Bax proteins. These two proteins have opposing effects, with Bcl-2 serving to prolong cell survival and Bax acting as an accelerator of apoptosis [33-35]. Our results show no correlation between Bcl-2 and Bax protein levels and the extent of apoptosis in lymphoma cells exposed to gemcitabine. These results are in agreement with the findings of Gazzit [36] who reported that the extent of gemcitabine-induced apoptosis is independent of the levels of Bcl-2 expression. However, we have previously found that in breast cancer cells the extent of apoptosis was correlated with Bcl-2 expression [22], further supporting the cell type dependence of the response of the apoptotic pathway.

Conclusions

In this human lymphoma model, the *in vitro* and *in vivo* resistance to gemcitabine can mainly be attributed to the partial deletion of dCK gene. Therapeutic strategies aiming to overcome dCK deficiency may be useful in enhancing the cytotoxic effects of gemcitabine. In this setting, stimulation of dCK activity can be induced by pre-treating cancer cells with etoposide, other nucleoside analogues or gamma radiation before gemcitabine administration [37-40]. The effect seems to be cell-specific and dependent on the administration scheme and the basal dCK levels. dCK gene-directed therapies may also be useful in enhancing the cytotoxic effects of gemcitabine by affecting the metabolism of the drug [26]. Another possibility would be to administer mononucleotide prodrugs of gemcitabine [41,42] bearing transient phosphate protectors that would deliver the corresponding 5'-mononucleotide inside the cell without requiring dCK activation. This strategy has already shown succesful activity in overcoming ara-C resistance due to dCK deficiency [23].

Methods

Reagents

Gemcitabine (Gemzar®) was a kind gift from Eli Lilly (Indianapolis, IN, USA). Stock solutions were prepared in distilled water and fresh dilutions were prepared before each experiment. Dilazep, tri-*N*-octylamine, 1,1,2-trichlorotrifluoroethane, propidium iodide and MTT were purchased from Sigma-Aldrich (St. Louis, MI, USA). Bradford protein assay solution was purchased from Bio-Rad Laboratories (Ontario, Canada). [^{3}H]Gemcitabine (14 Ci/mmol) and [^{3}H]uridine (17.7 Ci/mmol) were purchased from Moravek Biochemicals Inc. (Brea, CA, USA). Ecolite™ was purchased from ICN (Costa Mesa, CA, USA). Antibodies against Bax were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA); antibodies against Bcl-2 were purchased from DAKO (Glostrup, Denmark). Peroxidase-conjugated secondary antibodies were purchased from Covalab (Oullins, France). Enhanced chemiluminescence western blot detection reagents (ECL system) were purchased from Amersham (Amersham Corp, Buckinghamshire, UK). Other reagents used were analytical or HPLC grade and commercially available.

Cell lines

The RL-7 cells are derived from a human follicular lymphoma [43]. The resistant variant RL-G was developed using step-wise increases of concentration of gemcitabine over a 12 month period. The maximum concentration used during selection was 2 μM. All cells were grown on 25 cm^2 flasks at 37°C in RPMI containing 10% fetal calf serum, 1% L-glutamine and 2% penicillin-streptomycin in a humidified atmosphere containing 5% CO_2.

[^{3}H]Gemcitabine transport and accumulation

Cellular uptake assays using suspensed cells have been described previously [2,44]. Assays (10^6 cells/tube) were conducted at room temperature in transport buffer (20 mM Tris/HCl, 3 mM K_2HPO_4, 1 mM $MgCl_2.6H_2O$, 2 mM $CaCl_2$, 5 mM glucose and 130 mM NaCl, pH 7.4, 300 ± 15 mOsm) at various time intervals. Cell pellets were lysed with 5% Triton X-100 and mixed with Ecolite™ scintillation fluid to measure the cell-associated radioactivity (Beckman LS 6500 scintillation counter; Beckman-Coulter Canada, Mississauga, ON). Initial uptake rates were measured over 2–10 s and drug accumulation was determined after exposure of cells to 10 μM [^{3}H]gemcitabine for 4 h.

Extraction of nucleic acids for [^{3}H]Gemcitabine scintillation assay

Exponentially growing RL-7 and RL-G cells were exposed to 10 μM [^{3}H]gemcitabine for 4 h at 37°C. Cells were harvested by centrifugation and washed with phosphate-buffered saline. Aliquots (5 × 10^6 cells) were snap-frozen in liquid nitrogen and stored at -70°C before HPLC analysis and nucleic acid extraction. DNA was isolated using a Wizard genomic DNA kit (Promega Corp., Madison, WI, USA). Total RNA was extracted using an RNA easy kit (Qiagen, Valencia, CA, USA) and treated with RNase-free DNase 1 (Pharmacia Biotech) to remove any DNA contamination. Radioactivity incorporation was determined by scintillation counting. Experiments were performed in duplicate.

HPLC analysis

Cell pellets (prepared as described above) were extracted with 10% trichloroacetic acid on ice for 15 min and centrifuged to remove cell the protein precipitate. The acidic extract was neutralized with 2 volumes of tri-*N*-octylamine/1,1,2-trichlorotrifluoroethane (1:4) and the aqueous layer passed through a 0.22 μm filter before injection onto a Partisil SAX 10 column (250 × 4.6 mm internal diameter). The resolution of the nucleotides was achieved using a gradient (0%A-80%B) and based on a method described by Gandhi [45]. Buffer A, 10 mM, pH 2.9; buffer B, 750 mM ammonium phosphate, pH 4.5. The flow rate of the eluent was 1.5 mL/min and was monitored by UV absorbance (260 nm). Fractions were also collected for radioactivity measurements on a scintillation counter.

RNA extraction, RT-PCR and quantitative PCR

The level of mRNA expression of metabolic factors involved in gemcitabine resistance was assessed by quantitative real time RT-PCR, performed in a LightCycler detection system (Roche, Mannheim, Germany) as previously described [22]. Briefly, cDNA (5 μl) was mixed with primers (300 nM each), LightCycler-FastStart DNA Master SYBR Green I (Roche) (hENT1, hENT2, CDD, RNR-M1, RNR-M2 and DNA POL) or LightCycler-FastStart DNA master hybridization probes (Roche) (18S, dCK, cNII, dNT-1 and dNT-2), and probes (130 nM; if necessary) in a total volume of 20 μl. These reactions were prepared in duplicate. Primers and probes sequences for hENT1, dCK, CDD, cNII, dNT-1, RNR-M2 and DNA POL are published elsewhere [22]. Primers for hENT2 were as follows: for: atgagaacgggattcccagtag ; rev: gctctgattccggctcctt. Primers for RNR-M1 were as follows: for: gcagctgagagaggtgcttt; rev: aatggttgtagaattaagaatagc. Primers and probes for dNT-2 were as follows: for: catcagcatttgggagtcaa; rev: cgacacaatctgctccagaa; probe: 5'-fam-cgtcttcatctgcacaagcccca-tamra. All samples were analyzed in three separate experiments.

For each sample (RL-7 or RL-G cell lines) and the calibrator (K562 cell line), the relative amount of a target gene and a reference gene (18S) were determined. Crossing point values (C_t), which are the PCR cycle numbers at which the accumulated fluorescent signal in each reaction crosses a threshold above background, were obtained with the LightCycler software 3.5 (Roche) using the second derivative maximum method. (C_t) values are a function of the amplification efficiency of the respective PCR. These data were then exported into the RelQuant software (Roche) as *.txt files. This software provides efficiency-corrected, calibrator-normalized quantification results. Results are expressed as the target/reference ratio of the sample divided by the target/reference ratio of the calibrator and therefore are corrected for sample inhomogenities and detection-caused variances. The efficiency-corrected quantification performed by Relquant is based on relative standard curves describing the PCR efficiencies of each target and the reference gene. The relative standard curves are determined and are used for each analysis.

Ratio results obtained with the RelQuant software were considered as final relative PCR arbitrary units. Results were then expressed as % of PCR arbitrary units in RL-G cells related to RL-7 cells PCR arbitrary unit expression.

DNA extraction and PCR assay for the dCK gene

Genomic DNA was prepared with a phenol/chloroform extraction method [46]. The coding sequence of the 7 last exons of the human dCK gene were amplified in a final reaction volume of 25 μl using PCR primers (Table 1), *Taq* DNA polymerase (Invitrogen) and 250 ng of genomic DNA. The PCR program profile was: 10 min at 94 °C followed by 50 cycles of 30 sec at 94 °C, 30 sec at 60 °C and 30 sec at 72 °C. The PCR products were separated on agarose gel 1,5% using low molecular DNA weight (Invitrogen) as marker of size.

Flow cytometric detection of cell cycle and apoptosis

For analysis of DNA content and cell cycle distribution, RL-7 and RL-G cells were treated with gemcitabine 0.3 μM for 24 h and 72 h. After drug-exposure, 10^6 cells/ml were resuspended in 2 ml of propidium iodide solution (50 μl/ml), incubated at 4°C overnight and then analyzed by flow cytometry. For apoptosis determination, we used the Annexin-V-Fluos Staining Kit (Boehringer Mannheim, Gmbh, Germany) as recommended by manufacturers. Flow cytometry was performed on a FACScalibur (Becton Dickinson, San Jose, CA, USA). For determination of the apoptotic fraction, analysis was performed using CellQuest™ software (Becton Dickinson, San Jose, CA, USA). Cell cycle distribution and DNA ploidy status were calculated after exclusion of cell doublets and aggregates on a FL2-area/FL2-width dot plot using Modfit LT 2.0™ software (Verity Software Inc, Topsham, ME, USA).

Table 2: Analysis of flow cytometric cell cycle distribution before and after exposure to gemcitabine (0.3 μM) [a].

	RL-7			RL-G		
	Control	24 h	72 h	Control	24 h	72 h
G1	53.2 ± 9.0	63.3 ± 1.5	15.0 ± 1.4	39.7 ± 7.5	38.2 ± 6.4	40.9 ± 2.6
S	39.2 ± 3.0	36.5 ± 1.3	84.7 ± 1.4	46.3 ± 10.6	50.3 ± 8.5	51.6 ± 1.4
G2/M	7.4 ± 6.1	0.3 ± 0.1	0.2 ± 0.3	13.8 ± 3.2	11.2 ± 2.2	7.2 ± 1.4

[a] The reported values (mean ± SD, n = 3) are expressed as percentage of total cells.

Western Blots

Protein expression was determined by western blot analysis in untreated RL-7 and RL-G cells and after 24 h incubation with gemcitabine 0.3 μM. Briefly, protein was extracted from cells with cold lysis buffer (20 mM Tris-HCL pH 6.8, 1 mM MgCl2, 2 mM EGTA, 0.5% NP40, STI 1 mg/ml, leupeptin 100 μg/ml, aprotinin 100 μg/ml, benzamidine 30 mg/ml, TPCK 1 mg/ml and PMSK 5 mg/ml). Cell lysates were resolved by 12% SDS-PAGE, and transferred onto a nitro-cellulose membrane (Hybond-ECL, Amersham Corp, Buckinghamshire, UK). The blots were incubated with the appropriate dilution of primary antibody, followed by incubation with peroxidase-conjugated secondary antibody. Protein signals were detected by chemiluminiscence and exposure to Kodak film (Eastman Kodak Company, Rochester, NY, USA). Horizontal scanning densitometry was performed on western blots by utilizing acquisition into Adobe PhotoShop (Apple, Cupertino, CA, USA).

Animal studies

Experiments were performed in 4 week-old female athymic nude mice (IFFA-CREDO, L'Arbresle, France). The animals were kept in conventional housing. Access to food and water was not restricted. RL-7 and RL-G xenografts were developed in groups of five mice. Each animal (average body weight 25 g before injection) received a subcutaneous injection in the right flank area containing 10^6 RL-7 or RL-G cells in serum-free RPMI 1640 (day 0). After 24 h, gemcitabine was given intraperitoneally at a dose of 200 mg/kg once a week (6 doses; 3 consecutive weeks followed by 1 resting week) in the treated group. Initial drug toxicity studies were performed in non-tumor bearing mice at these doses and no major toxicities were observed (weight loss <10%).

The mice were inspected daily for s.c. tumor development and evaluation of clinical condition, and once tumor development occurred, tumor size was determined by caliper measurements while monitoring changes in animal weight twice a week. Animals were euthanized when their total tumor burden reached approximately 5,000 mg (15% of body weigth) to avoid animal discomfort or if clinical conditions may anticipate the potential for animal suffering.

The endpoints for assessing tumor activity were as follows: (a) tumor weight (mg) = $(A \times B^2)/2$, where A and B are the tumor length and width (in mm), respectively; (b) tumor growth inhibition = T/C, where T is the weight of treated tumor and C is the weight of control group, both evaluated at 60 days after injection. All studies involving mice were performed under Institutional Review Board-approved protocol.

List of abbreviations used

dFdC, gemcitabine; hENT1, human equilibrative nucleoside transporter 1; dCK, deoxycytidine kinase; CDD, cytidine deaminase; 5 NT, 5'-nucleotidase; RNR-M1, ribonucleotide reductase subunit M1; RNR-M2, ribonucleotide reductase subunit M2; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; IC_{50}, inhibitory concentration 50; DNA POL, DNA polymerase α.

Authors' contributions

CMG participated in the design and coordination of the study and carried out flow cytometric detection of cell cycle and apoptosis, western blots and animal studies. MLC participated in the design and coordination of the study and carried out [^{3}H]Gemcitabine transport and accumulation assay, extraction of nucleic acids for [^{3}H]Gemcitabine scintillation assay, HPLC analysis. LJ performed the DNA extraction and PCR assay for the dCK gene. CLS performed the [^{3}H]Gemcitabine transport and accumulation assay, extraction of nucleic acids for [^{3}H]Gemcitabine scintillation assay and HPLC analysis. EC carried out RNA extraction, RT-PCR and quantitative PCR. JRM participated in the design of the study and coordinated the [^{3}H]Gemcitabine transport and accumulation assay, extraction of nucleic acids for [^{3}H]Gemcitabine scintillation assay and HPLC analysis. CD participated in the design of the study and developed the RL-G cell line. All authors read and approved the final manuscript.

Acknowledgements

This work was supported in part by the Ligue Contre le Cancer du Rhône, the Fondation de France and the National Cancer Institute of Canada.

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