

Increased sensitivity to gemcitabine of P-glycoprotein and multidrug resistance-associated protein-overexpressing human cancer cell lines

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Gemcitabine (2',2'-difluorodeoxycytidine) is a deoxycytidine analogue that is activated by deoxycytidine kinase (dCK) to its monophosphate and subsequently to its triphosphate dFdCTP, which is incorporated into both RNA and DNA, leading to DNA damage. Multidrug resistance (MDR) is characterised by an overexpression of the membrane efflux pumps P-glycoprotein (P-gP) or multidrug resistance-associated protein (MRP). Gemcitabine was tested against human melanoma, non-small-cell lung cancer, small-cell lung cancer, epidermoid carcinoma and ovarian cancer cells with an MDR phenotype as a result of selection by drug exposure or by transfection with the *mdr1* gene. These cell lines were nine- to 72-fold more sensitive to gemcitabine than their parental cell lines. The doxorubicin-resistant cells 2R120 (MRP1) and 2R160 (P-gP) were nine- and 28-fold more sensitive to gemcitabine than their parental SW1573 cells, respectively ($P < 0.01$), which was completely reverted by 25 μM verapamil. In 2R120 and 2R160 cells, dCK activities were seven- and four-fold higher than in SW1573, respectively, which was associated with an increased dCK mRNA and dCK protein. Inactivation by deoxycytidine deaminase was 2.9- and 2.2-fold decreased in 2R120 and 2R160, respectively. dFdCTP accumulation was similar in SW1573 and its MDR variants after 24 h exposure to 0.1 μM gemcitabine, but dFdCTP was retained longer in 2R120 ($P < 0.001$) and 2R160 ($P < 0.003$) cells. 2R120 and 2R160 cells also incorporated four- and six-fold more [³H]gemcitabine into DNA ($P < 0.05$), respectively. P-glycoprotein and MRP1 overexpression possibly caused a cellular stress resulting in increased gemcitabine metabolism and sensitivity, while reversal of collateral gemcitabine sensitivity by verapamil also suggests a direct relation between the presence of membrane efflux pumps and gemcitabine sensitivity.

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Gemcitabine (2',2'-difluorodeoxycytidine) is a deoxycytidine analogue with proven activity in ovarian, pancreatic and non-small cell lung cancer (NSCLC) *in vivo*, *in vitro* and in the clinic (Abratt *et al*, 1994; Lund *et al*, 1994; Braakhuis *et al*, 1995; Heinemann, 2001). Deoxycytidine kinase (dCK) phosphorylates both deoxycytidine (dCyd) and gemcitabine to their monophosphates, which are subsequently phosphorylated to deoxycytidine triphosphate (dCTP) and gemcitabine triphosphate (dFdCTP), respectively. Gemcitabine can be inactivated by deamination catalysed by deoxycytidine deaminase (dCDA) (Heinemann *et al*, 1988). dFdCTP can be incorporated into both DNA and RNA (Huang *et al*, 1991; Ruiz van Haperen *et al*, 1993b). The mitochondrial enzyme thymidine kinase 2 (TK2) phosphorylates the natural nucleosides thymidine and deoxycytidine, but also gemcitabine, although, to a lesser extent than dCK (Eriksson *et al*, 1991; Bergman *et al*, 1999). This is in contrast to the cytosolic enzyme thymidine kinase 1, which does not phosphorylate deoxycytidine (Eriksson *et al*, 1991). Since dCTP is the major natural feedback inhibitor of dCK and competes with dFdCTP for

DNA polymerase, an increase in dCTP pools will decrease gemcitabine sensitivity (Heinemann *et al*, 1990; Ruiz van Haperen and Peters, 1994).

Crossresistance to some structurally and functionally unrelated natural-derived drugs (e.g. daunomycin, etoposide, vincristine) is called multidrug resistance (MDR). Multidrug resistance is caused by overexpression of the plasma membrane drug efflux pumps P-glycoprotein (P-gP), the product of the *mdr1* gene, and multidrug resistance-associated protein (MRP) (Endicott and Ling, 1989; Grant *et al*, 1994). These pumps can be blocked by verapamil (Cornwell *et al*, 1987). The MRP family currently has seven members, all with different drug specificities (Borst *et al*, 2000). Although gemcitabine is a substrate for MRP5 efflux pump (Davidson *et al*, 2002), it is predominantly transported into the cell across cell membranes via facilitated diffusion (equilibrative nucleoside transporter, ENT) and sodium-dependent concentrative mechanisms (concentrative nucleoside transporter, CNT) (Mackey *et al*, 1998, 1999; Ritzel *et al*, 2001). Jensen *et al* (1997) observed that some small-cell lung cancer (SCLC) cells with P-gP overexpression were more sensitive to gemcitabine and the structurally related deoxycytidine analogue 1- β -D-arabinofuranosylcytidine (cytarabine, ara-C), although Grant *et al* (1995) observed crossresistance to ara-C in a P-gP-overexpressing leukaemia cell line.

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We tested the sensitivities to gemcitabine of various pairs of cell lines and their MDR variants derived from different human tumours. The human NSCLC cell line SW1573 and its P-gP- and MRP1-overexpressing variants were used for mechanistic studies on gemcitabine sensitivity of MDR cells. Moreover, a possible role of the membrane efflux pumps in gemcitabine sensitivity was investigated.

MATERIALS AND METHODS

Chemicals and reagents

Dulbecco's modified eagle's medium (DMEM) and Rosswell Park Memorial Institute (RPMI) medium were purchased from Flow Laboratories (Irvine, UK); foetal calf serum from Life Technologies (New York, NY, USA); trichloroacetic acid (TCA), glutamine and gentamicin from Merck (Darmstadt, Germany); trypsin and sulphorhodamine B (SRB) from Sigma Chemical Co. (St Louis, USA); and VP-16 (etoposide) from Bristol-Myers Squibb (Weesp, the Netherlands). Eli Lilly (Indianapolis, IN, USA) kindly supplied gemcitabine and [^3H]gemcitabine (16.7 Ci mmol^{-1}). [^3H]Deoxycytidine was purchased from Moravex (Brea, CA, USA) [^3H]thymidine (58.8 Ci mmol^{-1}) was from Dupont de Nemours NEND (Dreieich, Germany) and [^3H]uridine (27.8 Ci mmol^{-1}) from Amersham International (Buckinghamshire, England). All other chemicals were of analytical grade and commercially available.

Cell culture

The *in vitro* experiments were performed with five parental cell lines and eight MDR variants (Table 1). All cell lines were grown in monolayers in DMEM, except for GLC4S and GLC4/ADR, which grew as semimultilayers in RPMI, at 37°C and 5% CO_2 . These cells grow in several layers, but are only loosely attached to the flask. Both media were supplemented with 7.5% heat-inactivated foetal calf serum, and 250 ng ml^{-1} gentamicin. Cells were regularly screened for *Mycoplasma* contamination by using a rapid detection system with a ^3H -labelled DNA probe (Gen-Probe, San Diego, CA, USA) and were found to be negative.

Chemosensitivity testing

The determination of the IC_{50} (the drug concentration causing 50% growth inhibition) in monolayer cell lines was performed using the SRB assay. The assay was performed using the NCI protocol with some small modifications (Skehan *et al*, 1990; Keepers *et al*, 1991). For GLC4S and GLC4/ADR, both growing as semimultilayers, the tetrazolium (MTT) assay was performed as previously described (Keepers *et al*, 1991). Culture conditions were optimised for all cell lines. For both assays, the cells were plated in 96-well plates at day 1, in different densities, depending on their doubling times (4000–15 000 cells per well). The optimal plating number was the highest number of cells, which enabled log-linear growth for 4 days. Log-linear growth or exponential growth is the phase of growth in which each descendant of the parental cell will divide as well.

On day 2, cells were exposed to gemcitabine (final concentrations ranged from 5×10^{-16} to $5 \times 10^{-5}\text{ M}$), with or without $25\text{ }\mu\text{M}$ verapamil for 72 h and optical density (OD) was estimated either by the SRB or MTT assay and set at 100%; the OD of cells at the day of drug administration was set at 0%. The IC_{50} was the drug concentration resulting in a relative OD of 50%, total growth inhibition when OD was similar to the initial value, 0%, and an OD lower than the initial value 0% represents cell kill (Peters *et al*, 1993).

dCK enzyme activity

For determination of dCK activities, 10 000 g supernatants were prepared with cold dCK buffer, containing 0.3 M Tris-HCl (pH 8.0), essentially as described (Ruiz van Haperen *et al*, 1993a). Protein content was estimated with the Biorad protein assay. To $25\text{ }\mu\text{l}$ of supernatant containing $0.2\text{--}4.0 \times 10^5$ cells, $25\text{ }\mu\text{l}$ of a substrate mixture was added (final concentrations: 10 mM ATP, 5 mM MgCl_2 , 30 mM Tris-HCl and $230\text{ }\mu\text{M}$ ^3H -dCyd (final specific activity 0.04 Ci mmol^{-1})), with or without 1 mM thymidine to inhibit TK2-mediated phosphorylation of dCyd (Eriksson *et al*, 1991), and incubated at 37°C for 15 min. The radio-labelled product dCMP was quantitated in a liquid scintillation counter, after thin layer chromatography on polyethyleneimine cellulose layers.

Table 1 MDR phenotype, sensitivity to gemcitabine and sensitivity factors of human cancer cell lines

Cell line	Origin	MDR phenotype	Doubling time (h)	IC_{50} (nM) gemcitabine	Sensitivity factor
SW1573	NSCLC	Parental	35 ± 5	17.0 ± 2	1
2R120 ^a		DOX resistant, MRP1	50 ± 6	1.9 ± 1.2	8.9***
2R160 ^a		DOX resistant, P-gP	50 ± 6	0.6 ± 0.4	27.9***
SW1573/S1(1.1) ^{b, c}		P-gP transfected	30 ± 2	5.7 ± 2.4	3.0**
SW1573/S1(MRP) ^{b, c}		MRP1 transfected	35 ± 8	0.05 ± 0.02	340***
GLC4	SCLC	Parental	22 ± 5	4.1 ± 0.6	1
GLC4/ADR ^b		DOX resistant, MRP1	31 ± 9	0.4 ± 0.2	11*
A2780	Ovarian	Parental	22 ± 9	1.6 ± 0.3	1
2780AD ^d		DOX resistant, P-gP	26 ± 8	0.03 ± 0.03	55**
KB3-1	Epidermoid	Parental	16 ± 3	59 ± 24	1
KB8-5 ^e		Colchicine resistant, P-gP	19 ± 5	33.0 ± 20	1.8 NS
BRO	Melanoma	Parental	21 ± 3	300 ± 87	1
BROmdr ^f		P-gP transfected	29 ± 4	33 ± 12	9.0**

MDR = multidrug resistance; NSCLC = non-small-cell lung cancer; SCLC = small-cell lung cancer; DOX = doxorubicin; P-gP = P-glycoprotein; MRP = multidrug resistance-associated protein. Cells were exposed to gemcitabine for 72 h. Values represent the mean $\text{IC}_{50} \pm \text{s.d.}$ of at least three experiments. IC_{50} = 50% growth-inhibiting concentration, sensitivity factor; IC_{50} parental/ IC_{50} MDR variant. Statistical analysis by *t*-test (independent samples); NS = not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. References: ^aKuiper *et al* (1990), ^bZaman *et al* (1993), ^cZaman *et al* (1994), ^dLouie *et al* (1986), ^eShen *et al* (1986), ^fLincke *et al* (1990).

dCDA enzyme activity

Activity of dCDA was determined as described earlier (Ruiz van Haperen *et al*, 1993a). Briefly, 10 000 g supernatants were prepared and enzyme assays were performed at 37°C in $1-5 \times 10^6$ cells with $500 \mu\text{M}$ dCyd as a substrate for 15 or 25 min. The deaminated product deoxyuridine was analysed using reversed phase high-performance liquid chromatography (HPLC) (Ruiz van Haperen *et al*, 1993a).

Quantitative reverse transcriptase-polymerase chain reaction assay for dCK-mRNA with competitive templates

RNA was extracted from confluent growing cells using the RNazole method and cDNA samples were prepared as previously described (Rots *et al*, 2000). Competitive template reverse transcriptase-polymerase chain reaction (RT-PCR) assays for dCK mRNA and the housekeeping gene β -actin were performed as described previously (Kroep *et al*, 2002). Levels of expression were reported as units of dCK-mRNA/ 10^6 β -actin mRNA molecules.

Western blot for dCK

Western blotting by affinity-purified rabbit antibody against human dCK was performed as previously described (Hatzis *et al*, 1998).

dFdCTP accumulation and retention

For dFdCTP accumulation and retention, cells were plated in six-well plates at $0.5-1.0 \times 10^6$ cells per well in 2 ml medium, and exposed to 0.1 or $1.0 \mu\text{M}$ gemcitabine for 24 h. For the retention, cells were cultured in drug-free medium for 4 or 24 h after incubation. After harvesting, nucleotides were extracted and quantitated with HPLC as previously described (Ruiz van Haperen *et al*, 1994).

[³H]Gemcitabine incorporation into DNA and RNA

Incorporation studies were performed using 96-well filter plates essentially as described previously (Van der Wilt *et al*, 1993; Van Moorsel *et al*, 1999a). A total of 1.0×10^5 cells per well were plated in a volume of $100 \mu\text{l}$, whereafter $100 \mu\text{l}$ of [³H]gemcitabine containing medium (4 Ci mmol^{-1}) was added resulting in final concentrations of 0.1 or $1.0 \mu\text{M}$. After 24 h incubation, the medium was removed by suction through the filters. Filters were washed with TCA and phosphate-buffered saline (PBS), followed by incubation with RNase A or without RNase A at 37°C. After termination of the reaction, the filters were washed again with TCA, H₂O and 70% ethanol, removed and radioactivity was counted as described previously (Van Moorsel *et al*, 1999a). Counts in wells incubated with RNase A were considered to represent [³H]gemcitabine incorporated into DNA, the difference in counts between incubation with or without RNase A was considered to represent [³H]gemcitabine incorporation into RNA. To correct for DNA and RNA synthesis, experiments were performed simultaneously with cells exposed to 0.1 or $1.0 \mu\text{M}$ cold gemcitabine and $5.6 \mu\text{M}$ [¹⁴C]thymidine ($62.8 \text{ mCi mmol}^{-1}$) or $0.165 \mu\text{M}$ [³H]uridine (25 Ci mmol^{-1}). Cell numbers were estimated by performing this experiment with cells exposed to unlabelled gemcitabine in a concentration of 0.1 or $1.0 \mu\text{M}$ (Van Moorsel *et al*, 1999a).

Fluorometric analysis of DNA unwinding assay

To measure the gemcitabine-induced DNA damage, the fluorometric analysis of DNA unwinding (FADU) assay was used (Birnboim and Jevcak, 1981; Bergman *et al*, 1996). Essentially,

3×10^6 cells were incubated for 24 h at 37°C with gemcitabine in a concentration of 0.1 or $1.0 \mu\text{M}$. Cells exposed to $50 \mu\text{M}$ VP-16 for 24 h were used as a positive control, since this drug is known to introduce double-strand (ds) DNA breaks very effectively, and cells not exposed to drugs were used as a negative control. Cells were lysed and exposed to an alkaline environment, allowing the DNA to unwind. The extent of strand breaks in the DNA determines the extent of DNA unwinding at the end of incubation. The dsDNA was stained by ethidium bromide and the ratio of fluorescence between treated and control samples was used to determine the percentage dsDNA (Bergman *et al*, 1996).

Statistical analysis

In case of significant differences between parental and MDR cells, a *t*-test was used to compare unpaired data of the two cell lines. The computer program SPSS (version 7.5, SPSS, Inc., Chicago, IL, USA) was used for statistical analysis.

RESULTS

Growth inhibition tests

The parental cell lines showed large differences in sensitivity to gemcitabine, which was not related to their doubling time, this indicates that differences in metabolic activation and mechanisms of action are predominantly responsible for sensitivity to gemcitabine. As a control, clonogenic assays were performed for some cell lines as described previously (Bergman *et al*, 2000), which also showed a similar difference in sensitivity (data not shown). SW1573 cells were intermediately sensitive to gemcitabine compared to the other parental cells; BRO, GLC4, KB3-1 and A2780 cells. All SW1573 variants were significantly ($P < 0.01$) more sensitive to gemcitabine than the parent (Figure 1, Table 1). 2R120 and 2R160 cells, the MRP1- and P-gP-overexpressing variants of SW1573, respectively, were nine- and 28-fold more sensitive to gemcitabine, respectively. The P-gP-transfected variant S1(1.1) was three-fold more sensitive, but the largest difference was found for the MRP1-transfected S1(MRP) cells, which were 340-fold more sensitive to gemcitabine than SW1573 cells ($P < 0.001$). The doxorubicin-selected, MRP1-overexpressing variant of GLC4, GLC4/ADR, was 11.1-fold more sensitive to gemcitabine than its parental cells ($P < 0.05$). The human ovarian cancer A2780 cells were the most sensitive parental cells, but the doxorubicin-

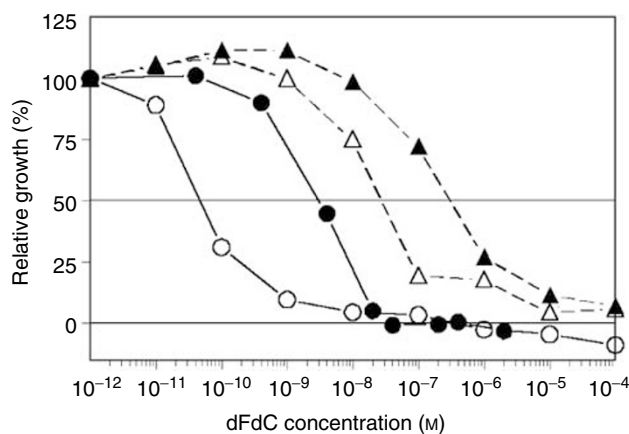


Figure 1 Representative growth inhibition curves of the human melanoma cell line BRO (▲) and its transfected variant BROmdr (△) (broken line) and the human ovarian carcinoma cell line A2780 (●) and its doxorubicin-resistant, P-gP-overexpressing variant A2780AD (○) (solid line). Cells were exposed to gemcitabine for 72 h.

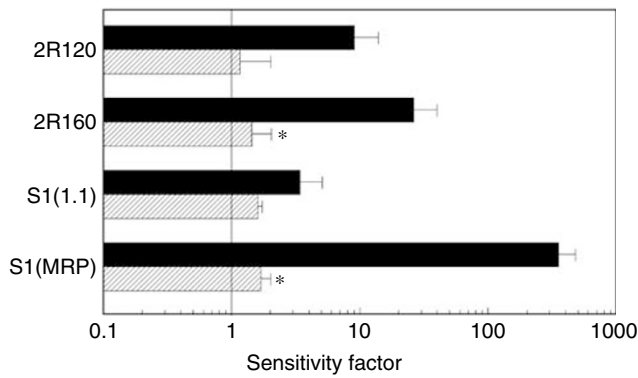


Figure 2 Sensitivity factors to gemcitabine, without 25 μM verapamil (■) or with 25 μM verapamil (▨) (IC_{50} of the MDR variants relative to IC_{50} of SW1573 was set at 1), of the MRP1-overexpressing 2R120, the P-gP-overexpressing 2R160, the *mdr1*-transfected S1(1.1) and the MRP1-transfected S1(MRP). Verapamil itself did not affect cellular growth. Values are means \pm s.d. of at least three experiments. *Sensitivity with 25 μM verapamil statistically significantly different from gemcitabine alone (*t*-test, independent samples, $P < 0.05$).

resistant P-gP-overexpressing variant 2780AD was even more sensitive to gemcitabine ($P < 0.01$). KB8-5 cells were included since P-gP was induced by exposure to colchicine; also this phenotype was associated with increased sensitivity to gemcitabine. BRO cells were the least sensitive of the parental cells tested, but its *mdr1*-transfected variant was significantly more sensitive ($P < 0.01$).

The MDR phenotype of doxorubicin resistance can be reversed by the calcium-channel blocker verapamil. This phenomenon has been used as a characteristic of the efflux pump. In order to determine whether the activity of the pump was associated with the collateral sensitivity to gemcitabine, we exposed SW1573 and its variants to gemcitabine and verapamil. Verapamil almost completely reversed the gemcitabine sensitivity to the level of the parental SW1573 cells (Figure 2). This reversal was significant in 2R160 and S1(MRP) cells ($P < 0.05$).

Deoxycytidine kinase, thymidine kinase 2 and deoxycytidine deaminase assays

Since gemcitabine cytotoxicity is dependent on its phosphorylation, we determined the activities of the enzymes involved in this process. SW1573 cells had the lowest dCK activity, this activity was 6.6- and 4.0-fold higher in 2R120 ($P < 0.001$) and 2R160 cells (not significant), respectively (Table 2). In 2R120 cells, TK2 activity was unchanged compared to its parental SW1573 cells. However, TK2 activity was two-fold lower in 2R160 than in SW1573 cells (not significant).

The amount of dCK protein present in the cell as determined with Western blots, revealed a 7.2- and 2.2-fold higher expression in 2R120 and 2R160 cells, respectively, than in the parental SW1573 cells (Table 2). In order to determine whether the increase in dCK protein was the result of increased expression, we determined the expression of dCK-mRNA (Table 2). The dCK/ β -actin ratio was 6.1- and 5.5-fold higher in 2R120 and 2R160 cells than in the parental SW1573 cells, respectively ($P < 0.001$).

dCDA activity was decreased 2.9- and 2.2-fold in 2R120 and 2R160 cells, respectively (Table 2).

dFdCTP accumulation and retention

The active metabolite of gemcitabine is dFdCTP. Besides its accumulation, retention is also important for the cytotoxicity of gemcitabine. No significant difference in dFdCTP accumulation was found between the cell lines, both at 0.1 and 1.0 μM

Table 2 dCK, TK2 and dCDA activities and dCK protein and mRNA expression in the human NSCLC cell line SW1573 and its doxorubicin MDR variants; 2R120 (MRP1) and 2R160 (P-gP)

Enzyme		SW1573	2R120	2R160
dCK	Activity	0.3 \pm 0.08	2.1 \pm 0.3***	1.3 \pm 0.7 NS
	Protein	0.3	2.0	0.6
	mRNA	0.5	3.1	2.8
TK2		0.2 \pm 0.2	0.2 \pm 0.3	0.1 \pm 0.2 NS
dCDA		37 \pm 3.0	13 \pm 3***	17 \pm 1***

Enzyme activities are means \pm s.d. of at least three experiments in nmol h^{-1} per 10^6 cells. For dCK and TK2 activity, dCyd was used as a substrate with or without thymidine to block TK2 activity. The difference in dCMP formation between dCyd as a substrate and dCyd and thymidine as a substrate was considered as TK2 activity. The protein content of SW1573, 2R120 and 2R160 cells was about 273 \pm 77, 442 \pm 152 and 210 \pm 8 μg per 10^6 cells, respectively (means \pm s.d.). Protein and mRNA expression are means of two and three separate experiments in (OD mm^{-2}) and (dCK/ β -actin $\times 10^{-3}$), respectively. Statistically different from SW1573 by *t*-test (independent samples); NS = not significant, *** $P < 0.001$.

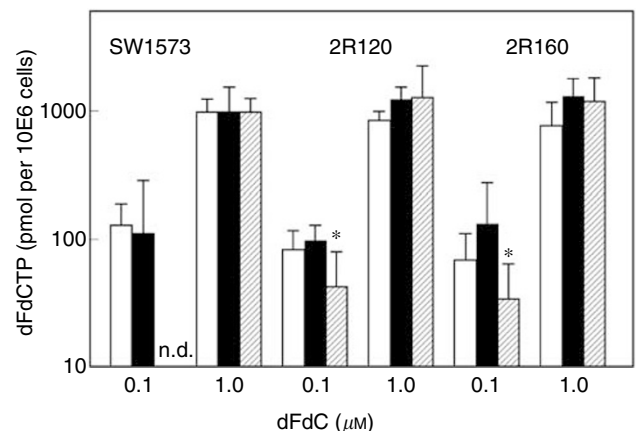


Figure 3 Accumulation (□), of dFdCTP after 24h exposure to 0.1 or 1.0 μM gemcitabine and its retention after suspension in drug-free medium for 4h (■) or 24h (▨) in the human NSCLC cell line SW1573 and its doxorubicin-resistant MDR cell lines; MRP1-overexpressing 2R120 and P-gP-overexpressing 2R160. Values are means \pm s.d. of at least three experiments. *Statistically significant different from SW1573 cells (*t*-test, independent samples, $P < 0.005$). n.d. = not detectable.

gemcitabine (Figure 3). In all cells, accumulation was concentration dependent, six- to eight-fold more dFdCTP accumulated at 1.0 μM than at 0.1 μM gemcitabine. At 0.1 μM gemcitabine, dFdCTP was retained longer in 2R120 ($P = 0.001$) and 2R160 ($P = 0.003$) cells than in the parental cell line SW1573. At 1.0 μM gemcitabine, no difference in retention of dFdCTP was found between the cell lines. Another active metabolite of gemcitabine, gemcitabine-diphosphate was not detectable.

Gemcitabine incorporation into DNA and RNA, and DNA damage

Following its accumulation, dFdCTP may be incorporated into DNA and RNA. The extent and retention are dependent on DNA and RNA polymerases, but also on subsequent excision. We not only determined the total extent of incorporation but also its relation to synthesis of DNA and RNA, which are inhibited by gemcitabine. SW1573, 2R120 and 2R160 cells not exposed to gemcitabine incorporated 53 \pm 29, 8.7 \pm 5.0 and 41 \pm 9 pmol

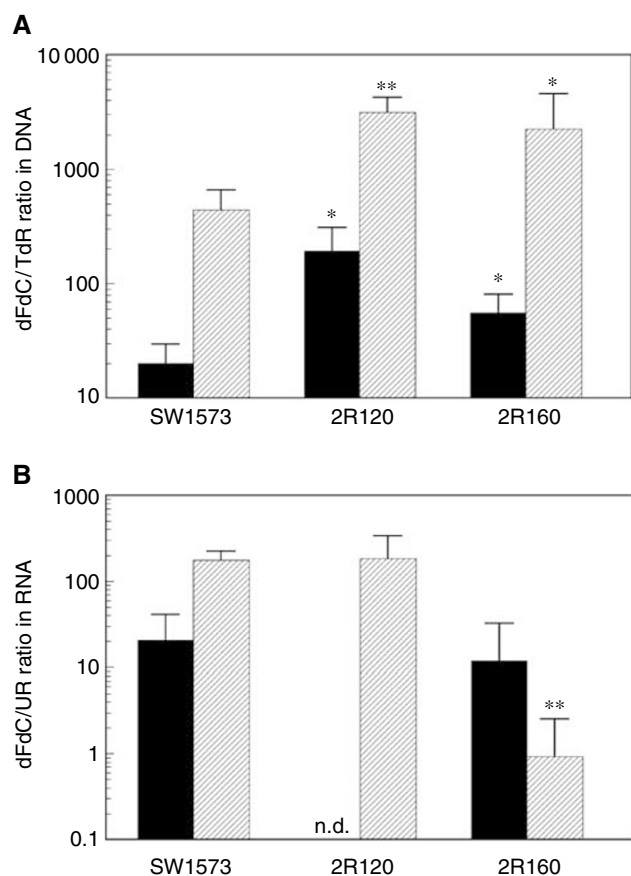


Figure 4 Incorporation of [^3H]gemcitabine into DNA relative to incorporation of [^{14}C]thymidine (TdR) into DNA (**A**) and incorporation of [^3H]gemcitabine into RNA relative to incorporation of [^3H]uridine (UR) into RNA (**B**) after 24 h exposure to 0.1 μM (■) or 1.0 μM (▨) gemcitabine in the human NSCLC cell line SW1573 and its doxorubicin-resistant MDR cell lines; MRP1-overexpressing 2R120 and P-gP-overexpressing 2R160. Values are means \pm s.d. of at least three experiments. *Statistically significant different from SW1573 cells (*t*-test, independent samples), $P < 0.05$, ** $P < 0.02$. n.d. = not detectable.

thymidine h^{-1} per 10^6 cells into DNA and 2.5 ± 0.7 , 2.7 ± 2.9 and 3.1 ± 0.5 pmol uridine h^{-1} per 10^6 cells into RNA, respectively. At 0.1 μM gemcitabine, inhibition of DNA synthesis varied between 79 and 91%, and at 1 μM gemcitabine between 94 and 97%. For inhibition of RNA synthesis, these values were less pronounced and varied between 0 and 4.7%, and 4.6 and 14.5%, respectively, for the three cell lines.

When corrected for the inhibition of DNA synthesis, [^3H]gemcitabine incorporation into DNA was greater at the higher concentrations in all three cell lines, suggesting that incorporation was concentration dependent (Figure 4). At 0.1 μM gemcitabine 2R120 and 2R160 cells incorporated 9.6- ($P = 0.04$) and 2.8-fold and at 1.0 μM gemcitabine 7.1- ($P = 0.01$) and 5.1-fold more [^3H]gemcitabine into DNA than SW1573 cells, respectively. When corrected for inhibition of RNA synthesis 1 μM [^3H]gemcitabine incorporation into RNA was about 100-fold lower in 2R160 compared to SW1573 and 2R120 cells (data not shown).

Incorporation of gemcitabine may cause DNA damage, which is the result of the extent of its incorporation and efficacy of the DNA repair mechanisms. In SW1573 and 2R120 cells, the amount of dsDNA breaks was concentration dependent (7 and 9% dsDNA breaks at 0.1 μM , 16 and 39% ds breaks at 1 μM gemcitabine, respectively). However, in 2R160 comparable DNA damage was found at 0.1 and 1.0 μM gemcitabine (19 and 25% dsDNA breaks, respectively).

DISCUSSION

In this paper, we describe an increased collateral sensitivity to gemcitabine in five pairs of cell lines with an MDR phenotype. Collateral sensitivity is defined as an increased sensitivity to one class of drugs, while made resistant to another class of drugs. This increased sensitivity was associated with an increase in the activity of dCK in the variant cell lines. Apparently, the increased dCK activity was related to an upregulation of the gene, reflected by an increased mRNA and protein content of the cells. Moreover, the increased dCK activity was stable for many passages, indicating the structural nature of the upregulation of the gene. Deoxycytidine kinase plays a pivotal role in gemcitabine activation since it catalyses the first step in phosphorylation, which is rate limiting for further phosphorylation to active metabolites. This is based on the observation that dCK deficiency is associated with gemcitabine resistance (Bergman *et al*, 1999, 2000) which can be reverted by transfection with human dCK (Van der Wilt *et al*, 2000). The increased dCK activity is the most logical explanation for the collateral sensitivity in MDR cells, while the decreased dCDA activity may add to enhanced intracellular gemcitabine levels available for phosphorylation. However, the role of dCDA activity in gemcitabine sensitivity is still not clear (Bergman *et al*, 2002). The levels of both dCK mRNA and protein were also higher in 2R120 and 2R160 cells. Comparable differences in dCK levels have been associated previously with differences in gemcitabine sensitivity (Kroep *et al*, 2002). The increase in the activating enzyme was not only associated with increased retention of dFdCTP pools, but also with more gemcitabine incorporation into DNA.

Jensen *et al* (1997) previously described that daunorubicin- and VM-26-resistant MDR variants of human SCLC cell lines were also more sensitive to gemcitabine and to the structurally and functionally related deoxycytidine analogue ara-C. However, the extent of collateral sensitivity to gemcitabine and ara-C in the SCLC cell lines was less than that for the present cell lines, although in the SCLC line also an increased dCK activity was present (Bergman *et al*, 2001a). In addition to these cell lines, an increase in ara-C sensitivity was found in refractory AML cells displaying a P-gP or MRP overexpression after treatment with MDR drugs (Schoorhuis *et al*, 1995). It was suggested that exposure of cells to an MDR drug might select for a subpopulation of P-gP- or MRP-overexpressing cells with the more favourable enzymatic profile for cell survival. Our *in vitro* data suggest that the selection process may involve an increase in dCK.

In addition to the structural increase in dCK, Sasvári-Székely *et al* (1998) reported that in human lymphocytes, inhibition of DNA synthesis by 2-chloro-2'-deoxyadenosine (Cl-Ado) resulted in a rapid, transient rise of dCK activity, which was however, not associated with an increase in the amount of dCK, while dCK mRNA levels even decreased. Apparently, dCK itself was activated, probably through a post-translation modification. Moreover, since dCK is a major enzyme in the supply of essential deoxynucleotides via the salvage pathway for DNA repair, its activation might be part of the cellular restoration process occurring after drug treatment. Unlike the transient increase in dCK activity in human lymphocytes during Cl-Ado exposure, the increase in dCK activity found in our NSCLC cell lines seemed to be stable. In 2R120 and SW1573 cells, 40% of the dCyd phosphorylation was the result of TK2 activity, while in 2R160 the TK2 activity was two-fold lower. Since TK2 only phosphorylates small 2'-substituted dCyd analogues and ara-C and gemcitabine are poor substrates (Wang *et al*, 1999), its role in the phosphorylation of gemcitabine might be limited, although its real contribution has not yet been established. A reduced TK2 activity might result in a higher dFdCTP/dCTP ratio in 2R160 cells than in SW1573 cells, which might contribute to an increased sensitivity to gemcitabine.

The diphosphate of gemcitabine (dFdCDP) inhibits ribonucleotide reductase (RNR), resulting in a decreased conversion of CDP

to dCTP and eventually to a depletion of dCTP pools, which can favour dFdCTP incorporation into DNA (Baker *et al*, 1991; Plunkett *et al*, 1995). The dFdCTP accumulation and retention in several human tumour cell lines showed a relation with gemcitabine sensitivity *in vitro* (Ruiz van Haperen *et al*, 1994; Van Moorsel *et al*, 2000). dFdCTP is not only important as a DNA precursor, but also interferes with normal ribonucleotide metabolism, such as inhibition of CTP-synthetase and dCMP-deaminase leading to a depletion of CTP pools and indirectly a decrease of dCTP pools (Heinemann *et al*, 1990, 1995; Van Moorsel *et al*, 1999b, 2000). No difference was found in the retention of dFdCTP pools of SW1573 cells and its MDR variants after exposure to 1.0 μM gemcitabine, which might be related to differences in nucleoside transport or 5'-nucleotidase (5NT) activity, known to oppose the action of deoxynucleoside kinases (Bergman *et al*, 2002). However, MDR variants retained dFdCTP pools longer than SW1573 cells after exposure to 0.1 μM gemcitabine. After 24 h, no detectable dFdCTP pools were found in SW1573 cells, which might be related to the lower dCK activity or altered 5NT activity. The concentration-dependent [^3H]gemcitabine incorporation into DNA and correlation of DNA damage with sensitivity is in agreement with previous studies in ovarian, colon and leukaemia cell lines (Huang *et al*, 1991; Ruiz van Haperen *et al*, 1993b). However, no relation was found between [^3H]gemcitabine incorporation into RNA and sensitivity to gemcitabine, leaving a dubious role for RNA incorporation in gemcitabine toxicity (Bergman *et al*, 2002). Most likely, the increased dFdCTP retention, gemcitabine incorporation and increased sensitivity to gemcitabine are all downstream events of an increased dCK activity, underlining the pivotal role of dCK in gemcitabine sensitivity.

Verapamil is a strong inhibitor of P-gP, but only a partial inhibitor of MRP activity (Aszalos *et al*, 1999). A concentration of 10 μM verapamil increased sensitivity to doxorubicin of MRP1-transfected NIH/3T3 mouse fibroblasts, but reversal was incomplete (Breuninger *et al*, 1995). Since we used a 2.5-fold higher verapamil concentration, MRP1 inhibition might be more effective. Reversal of collateral sensitivity to gemcitabine was found both in P-gP- and MRP1-overexpressing cells. We did not find a direct correlation between the extent of P-gP and MRP1 activity and sensitivity to gemcitabine or dCK activity. Apparently, a certain threshold level of P-gP or MRP1 activity is required for an increase in gemcitabine sensitivity, in contrast to the resistance to, for example, doxorubicin. The observation that verapamil reverted the

collateral sensitivity of the MDR cells to gemcitabine suggests a possible relation between the presence of a certain membrane efflux pump activity, gemcitabine sensitivity and a transient regulation of dCK activity. One of these mechanisms may include regulation by protein kinase C (PKC) activity. Several studies report an increase in PKC activity in cells with an overexpression of P-gP or MRP (Beck *et al*, 1998; Ratnasinghe *et al*, 1998). P-glycoprotein and MRP are phosphorylated (Ma *et al*, 1995; Clavy *et al*, 1997); however, it is a matter of debate whether phosphorylation modulates the pump function (Ma *et al*, 1995; Smith and Zilfou, 1995; Clavy *et al*, 1997; Ratnasinghe *et al*, 1998). Since dCK may be phosphorylated by PKC, and exhibits a higher activity in the phosphorylated state (Wang and Kucera, 1994), PKC might play a role in the collateral sensitivity to gemcitabine of MDR cells.

Since P-gP and MRP act as an efflux pump for steroid hormones, such as cortisol, progesterone and aldosterone (Van Kalken *et al*, 1993; Mulder *et al*, 1996) and several studies reported a decrease in TK activity as a result of steroid hormone exposure in chicken embryo retina (Naray *et al*, 1977; Herzfield and Raper, 1980; Tesoriere *et al*, 1989), verapamil might inhibit the efflux of these compounds, leading to a transient downregulation of dCK. Clinically relevant concentrations of the steroid drug dexamethasone inhibited the effect of gemcitabine in cultured human glioma cells (Rieger *et al*, 1999). Moreover, dexamethasone decreased gemcitabine sensitivity of 2R120 and 2R160 cells, and decreased dCK activity in 2R160 cells, but only in the presence of verapamil (Bergman *et al*, 2001b).

In conclusion, MRP1- and P-gP-overexpressing cells were more sensitive to gemcitabine than their parental cells. This increased sensitivity was related to dCK and gemcitabine effects on DNA. Since relapsed tumours of patients after treatment with MDR drugs frequently display an MDR phenotype, screening for a P-gP or MRP1 overexpression, as a predictor of gemcitabine responsiveness, might be of clinical interest.

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