Pergamon

Biochemical Pharmacology, Vol. 48, No. 7, pp. 1327–1339, 1994.

0006-2952/94 $7.00 + 0.00

0006-2952(94)00315-7

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

VERONIQUE W. T. RUIZ VAN HAPEREN, GIJSBERT VEERMAN, EPIE BOVEN, PAUL NOORDHUIS, JAN B. VERMORKEN and GODEFRIDUS J. PETERS*

Department of Oncology, Free University Hospital, P.O. Box 7057, 1007 MB Amsterdam, The Netherlands

(*Received* 10 *February* 1994; *accepted* 28 *June* 1994)

Abstract—2′,2′-Difluorodeoxycytidine (Gemcitabine, dFdC) is a relatively new deoxycytidine antimetabolite, with established activity against ovarian cancer and non-small-cell lung cancer. dFdC is assumed to exert its antitumour effect mainly by incorporation of the triphosphate dFdCTP into DNA. We determined the sensitivity to dFdC of six cell lines derived from solid tumours; two ovarian carcinoma (A2780 and OVCAR-3), two colon carcinoma (WiDr and C26-10) and two squamous cell carcinoma cell lines (UM-SCC-14C and UM-SCC-22B). *In vitro* sensitivity to dFdC was strongly time dependent. Under all conditions A2780 was the most sensitive cell line with an IC_{50} (the concentration of dFdC causing 50% growth inhibition) of 31 and 0.6 nM at 1 and 48 hr exposure, respectively. WiDr and C26-10 cells were relatively insensitive, with IC_{50}s of 468 and 1133 nM, respectively, at 1 hr exposure, but of 11 and 6 nM at 48 hr exposure. Accumulation of the triphosphate dFdCTP was also time dependent. After 4 hr exposure to 10 μM dFdC, A2780, WiDr and C26-10 cells accumulated 223, 136 and 267 pmol/10^6 cells, respectively; after 24 hr exposure they accumulated 1045, 619 and 617 pmol/10^6 cells, respectively. A2780 cells retained the high dFdCTP concentration longer than 24 hr. For comparison purposes we also studied dFdCTP kinetics in the corresponding solid tumours, showing the same sensitivity pattern as the cell lines. In general, sensitivity to dFdC *in vitro* related with dFdCTP accumulation and retention, but *in vivo* this relation was less clear. Unexpectedly, remarkable *in vitro* and *in vivo* changes were observed in the ribonucleotide pools. The most predominant *in vitro* cell line dependent changes were a decrease in CTP concentrations, accompanied by an increase in UTP and GTP concentrations. *In vivo* CTP, UTP and GTP pools increased in all tumours. In conclusion, in this study we demonstrate that dFdCTP is accumulated and retained in solid tumours and cell lines. dFdCTP is not only important as a DNA precursor, but also appears to interfere with normal ribonucleotide metabolism.

Key words: gemcitabine; deoxycytidine; CTP synthetase; deoxycytidine kinase; nucleotides; ovarian cancer

dFdC† is a relatively new deoxycytidine analogue which has shown remarkably good antitumour activity in murine experimental tumour models and human tumour xenografts [1–3]. In clinical phase II studies antitumour activity was observed against ovarian cancer and non-small-cell lung cancer [4]. The mechanism of action of this antimetabolite is quite complex. dFdC itself is not active; after transport into the cell, mediated by facilitated diffusion [5], the drug needs to be phosphorylated to its corresponding triphosphate dFdCTP. The first, rate-limiting step in this phosphorylation is catalysed by deoxycytidine kinase. The triphosphate is the main metabolite formed [5]. The main mechanism of action is assumed to be incorporation of the triphosphate dFdCTP into DNA, causing inhibition of DNA synthesis and cell death [6]. The drug also induces apoptosis [7]. Recently we showed that a metabolite of dFdC can also be incorporated into RNA [8]. Furthermore, several metabolites of dFdC can inhibit various enzymes, leading to self-potentiation of dFdC metabolism. dFdCDP is a potent inhibitor of ribonucleotide diphosphate reductase [9], whereas dFdCTP can inhibit CTP synthetase [10] and dCMP deaminase [11]. Inhibition of ribonucleotide diphosphate reductase will lead to depletion of dCTP, a potent feedback inhibitor of deoxycytidine kinase [12], leading to a more efficient phosphorylation of dFdC. Moreover, the competition between dCTP and dFdCTP for incorporation into DNA will be shifted favourably towards dFdCTP. Inhibition of dCMP deaminase will decrease the catabolism of dFdCMP, resulting in more dFdCTP formation. The significance of the postulated inhibition of CTP synthetase is not yet clear, since for CTP and UTP different effects on deoxycytidine kinase have been reported [13–15].

* Corresponding author.

† Abbreviations: dFdC, 2′,2′-difluorodeoxycytidine, Gemcitabine; dFdCTP, dFdC-triphosphate; TCA, trichloro-acetic acid.

Thus far, most studies on dFdC metabolism and consequently dFdCTP accumulation have been limited to leukaemic cell lines, CHO cells and 2008 ovarian carcinoma cells [5, 16–18] and (leukaemic) lymphocytes of patients treated with dFdC [19, 20]. Although the antitumour effect of dFdC has been shown to be highly dose and schedule dependent, until now dFdCTP accumulation has been studied in a limited range of exposure times and concentrations. No data have been reported on dFdCTP accumulation and retention in solid tumours. Since our initial studies showed that dFdC caused marked, unexpected changes in normal ribonucleotides, we also focused on the concentration and time dependence of these alterations.

This study was performed to gain better insight into the metabolism of dFdC and its effect on normal ribonucleotide metabolism in solid tumour cell lines and solid tumours. Sensitivity to dFdC in a panel of six solid tumour cell lines with different histological phenotypes was related to the accumulation and retention of dFdCTP and changes in normal ribonucleotides in three of these cell lines and their corresponding xenografts.

MATERIALS AND METHODS

Chemicals

dFdC and dFdCTP were kindly provided by Eli Lilly & Co. (Indianapolis, IN, U.S.A.). ATP, ADP, CTP, GTP and UTP were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). All other chemicals were of analytical grade and commercially available.

Cell culture

The sources of the human ovarian carcinomas A2780 and OVCAR-3, the human colon carcinoma WiDr, the murine colon carcinoma C26-10 cells [21–23], the human squamous cell carcinomas UM-SCC-14C and UM-SCC-22B [2] have been described previously. Cells were maintained in exponential growth in Dulbecco's Modification of Eagle's Medium (Gibco Laboratories, Grand Island, NY, U.S.A.) supplemented with 5% heat-inactivated foetal calf serum (Gibco), 1 mM L-glutamine (Sigma) and 250 ng/mL gentamicine at 37° and 5% CO_2.

Chemosensitivity

Chemosensitivity was assessed using the sulforhodamine-B (SRB)-assay, essentially as described previously [24]. In essence, 2000 to 15,000 cells per well, depending on the cell line, were seeded in 96-well flat-bottom plates (in triplicate) at day 0. Twenty-four hours after seeding, when cells were in exponential growth, dFdC was added in a concentration range from 10^{-4} to 10^{-11} M. After 30 min, 1, 2, 4, 24, 48 and 72 hr, the drug-containing medium was removed, and cells were washed once and subsequently cultured in drug-free medium. Growth of the cells was enumerated with the SRB-assay 72 hr after initial drug addition. Chemosensitivity was expressed in IC_{50}, which was defined as the concentration of dFdC causing 50% growth inhibition. Relative growth was calculated as described previously [25]: $[(OD_{treated}/OD_{zero}) - 1]/[(OD_{control}/OD_{zero}) - 1] \times 100\%$, OD being the optical density read at 540 nm. OD_{zero} reflects the cell number at the moment of drug addition, $OD_{control}$ reflects the cell number of untreated wells and $OD_{treated}$ the cell number in treated wells at the day of the assay.

Tumours

In vivo experiments were performed with three of the cell lines selected for their *in vitro* sensitivity pattern. The two human cell lines A2780 and WiDr were established as xenografts grown s.c. in female nude mice (HSd; athymic nude mice) as described previously [26]. Colon 26 is a murine colon adenocarcinoma line, maintained in female Balb/c mice as described [23, 27]. The C26-10 cell line was derived from this tumour [23]. Sensitivity to dFdC was determined in six mice bearing a tumour in each flank, with six tumour-bearing mice as controls as previously described [2, 3, 23, 27]. Treatment was started after transplantation, when the tumours had reached a volume of 50–150 mm^3, with 120 mg dFdC/kg body weight i.p. q3d × 4. Tumour volumes were measured twice weekly with a calliper. Tumour volume was calculated as follows: length × width × height × 0.5 [28, 29]. Antitumour activity was evaluated using T/C (tumour volume treated/tumour volume control) and the specific growth delay factor (GDF) ([doubling time treated tumour doubling time control tumour]/doubling time control tumour) [2, 3, 23, 29].

Accumulation and retention of dFdCTP

In vitro. For determination of dFdCTP accumulation $2–4.10^5$ cells were seeded in triplicate in 6-well plates. After 2 days, when cells were growing in log-phase, dFdC was added to a final concentration of 1, 10 and 100 μM for 4 and 24 hr. As a control non-exposed cells were cultured for the same period. For retention experiments, cells were exposed to 1 and 10 μM dFdC for 24 hr in duplicate. Thereafter, the drug-containing medium was removed and cells were washed once with drug-free medium and cultured for an additional 1, 4 and 24 hr in drug-free medium. At the end of the incubation cells were washed with ice-cold PBS, harvested by rapid trypsinization (2 min at room temperature), suspended in ice-cold culture medium with serum and immediately chilled on ice. They were then counted with a haemocytometer, washed once with ice-cold PBS and extracted for the nucleotides, as described previously [30]. Briefly, the cell pellet was suspended in 150 μL ice-cold PBS, immediately followed by the addition of 50 μL of 40% w/v TCA (10% final concentration), mixed and chilled on ice for 20 min. Then the mixture was centrifuged for 5 min at 10,000 *g* and 4°. The supernatant was neutralized by adding a 2-fold excess (400 μL) of freshly prepared trioctylamine: 1,1,2-trichlorotrifluoroethane (1:4). After centrifugation of 1 min at 10,000 *g* and 4°, the upper aqueous layer (nucleotide extract) was carefully taken off and stored at −20° until HPLC analysis.

In vivo. For *in vivo* experiments tumour pieces of 5 mm^3 were implanted s.c. in both flanks. When the tumours reached 300–500 mm^3, the animals were

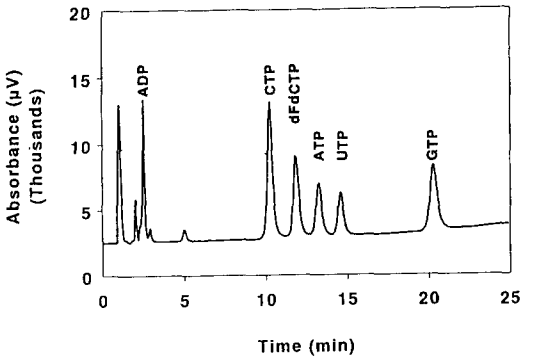

Fig. 1. Typical chromatogram of the separation of a standard mixture of normal ribonucleotides and dFdCTP, using 1000 pmol per nucleotide. For details on the methodology see Materials and Methods.

treated with a single i.p. bolus injection of 120 mg/kg dFdC, which is the maximum tolerated dose in the schedule of q3d × 4 [2, 3]. At 2, 4, 6, 8 and 24 hr after dFdC administration the mice were anaesthesized with ether, after which the tumours were removed as quickly as possible and immediately frozen in liquid nitrogen to avoid nucleotide breakdown. The mice were then sacrificed by cervical dislocation. Tumours from untreated animals were removed similarly. In order to extract nucleotides, the frozen tumours were pulverized using a microdismembrator (B. Braun, Melsungen, Germany) [31]. The frozen powder was reconstituted in ice-cold PBS at 1 g tumour per 3 mL buffer and centrifuged for 10 min at 2800 *g* and 4°. The supernatant was subsequently centrifuged for 20 min at 10,000 *g* and 4°. This second supernatant was used for the nucleotide extraction, as described above.

HPLC analysis. The HPLC methodology was based on that reported previously [5, 22]. The applied method enabled the measurement not only of dFdCTP, but also of ADP, ATP, UTP, CTP and GTP in one run and thus the assurance of the different nucleotides not interfering with each other. Calculation of the ATP/ADP ratio gives an indication of the quality of the extraction procedure and the effect of treatment on the energy status of the cell or tumour. Separation and quantitation of the normal ribonucleotides and dFdCTP was achieved with a gradient system consisting of two Gyncotek pumps (Model 300, Separations Analytical Instruments B.V., Hendrik Ido Ambacht, The Netherlands) connected to an automatic injector (Separations, Promis II, Separations Analytical Instruments B.V.). The system was connected to a photo-diode array detector (Separations, Model 1000S, Separations Analytical Instruments B.V.), regularly set at 254 and 280 nm. Peaks were quantitated using peak heights and calculated using the data acquisition program AXXIOM (Model 737, Axxiom Chromatography Inc. Calabasas, CA, U.S.A.). Separation of the nucleotides was achieved by injection of 175 μL of the extracts onto a Partisphere SAX anion exchange column (length 110 mm, internal diameter 4.7 mm, particle size 5 μm). As standards 1000 pmol of each nucleotide were injected. A linear gradient was started with 65% of buffer A (5 mM $NH_4H_2PO_4$, pH 2.80) and 35% of buffer B (0.5 M $NH_4H_2PO_4$ and 0.25 M KCl, pH 3.00), completing in 30 min to 100% B. Re-equilibration to 35% B was achieved in 15 min. The gradient system was controlled by the AXXIOM program. Retention times of ADP, CTP, dFdCTP, ATP, UTP and GTP were 2.9, 10.2, 12.0, 13.2, 14.7 and 20.5 min, respectively. A typical chromatogram obtained after injection of a standard mixture of 1000 pmol per nucleotide is shown in Fig. 1. The detection limit for dFdCTP was 50 pmol per injection, the quantification limit was 75 pmol.

RESULTS

Chemosensitivity

Chemosensitivity testing was first performed in a few selected cell lines, and focused on the time dependence of growth inhibition. It was evident that continuous exposure was more effective than short-term exposure. However, no significant difference was observed between 30 min and 1 hr exposure, 2 and 4 hr exposure and 48 and 72 hr exposure (data not shown). Subsequent experiments with additional cell lines were limited to four different exposure periods: 1, 4, 24 and 48 hr. The results are

Table 1. Sensitivity of six solid tumour cell lines to dFdC

Cell line	DT*	Exposure time (hr)				Ratio		
		1	4	24	48	1/48 hr	4/48 hr	24/48 hr
C26-10	23	1133 ± 133	238 ± 48	22 ± 4	6 ± 1	200	40	3.7
WiDr	32	468 ± 66	205 ± 61	51 ± 15	11 ± 2	42	19	4.6
A2780	26	31 ± 5	7 ± 1	2 ± 0.6	0.6 ± 0.1	53	12	3.3
OVCAR3	37	130 ± 17	36 ± 8	2 ± 0.8	0.6 ± 0.1	200	60	3.3
14C	32	192 ± 47	34 ± 6	6 ± 0.5	4 ± 0.9	48	8.5	1.5
22B	50	39 ± 3	5 ± 0.5	1 ± 0.3	0.6 ± 0.2	67	8.3	1.7

IC_{50} values (in nM) are expressed as means ± SEM of 4–10 separate experiments. Total culture time after addition of dFdC was 72 hr.

* Mean doubling time in hours, as determined in our laboratory.

summarized in Table 1. At both 1 and 48 hr exposure A2780 and UM-SCC-22B were the most sensitive cell lines, while the two colon cell lines were the least sensitive under all conditions tested. Sensitivity, however, depended strongly on exposure time; the three-fold difference between the C26-10 and WiDr cells and the 6-fold difference between C26-10 and UM-SCC-14C cells disappeared. At the long exposure of 48 hr WiDr was the most insensitive cell line, while C26-10 was intermediately sensitive. These changes in sensitivity pattern were already evident after 4 hr (Table 1) and are illustrated by the change in the ratios between IC_{50} values of 1, 4 and 24 hr compared to 48 hr. This change was most pronounced in the C26-10 and OVCAR-3 cell lines and least in WiDr cells.

dFdCTP accumulation and retention in vitro

Three cell lines were selected for evaluation of dFdCTP accumulation and retention: the ovarian carcinoma cell line A2780, which was the most sensitive under all conditions; the murine colon carcinoma cell line C26-10 and the human colon carcinoma cell line WiDr. The latter two cell lines were chosen because of differences in sensitivity patterns in relation to drug exposure time. Furthermore, the doubling times of the three cell lines chosen fall within a small range, thus excluding possible influences of doubling time of the effects observed.

dFdCTP accumulation *in vitro* was time, concentration and cell line dependent (Fig. 2). The time dependence was most pronounced in A2780, at all concentrations tested, with a markedly higher accumulation after 24 hr exposure as compared to 4 hr exposure. This difference was much less pronounced in the C26-10 cell line. In WiDr cells concentration dependence was not as evident as in the other two cell lines; a clear saturation at 10 μM was observed after both 4 and 24 hr. In A2780 cells this was only observed at the 4 hr exposure. In the murine cell line C26-10 there seemed to exist a threshold level, since substantial accumulation was only seen at 10 and 100 μM. No saturation was observed in this cell line. A2780 cells accumulated the highest absolute dFdCTP concentrations in the low 1–10 μM range of dFdC concentrations. At 100 μM dFdC, well above the IC_{50} values for all cell lines, C26-10 cells accumulated the highest dFdCTP concentrations.

Retention of dFdCTP measured from 0 to 24 hr after removal of dFdC was not concentration dependent in A2780 and WiDr cells (Fig. 3). Although absolute dFdCTP accumulation after exposure to 10 μM was higher than at 1 μM, the pattern of retention at 1 and 10 μM was comparable. In C26-10 cells dFdCTP retention was poor; after exposure to 1 μM dFdC, dFdCTP decreased to non-detectable levels after 24 hr culture in drug-free medium. In human cell lines A2780 and WiDr, dFdCTP was retained much better than in murine C26-10 cells and dFdCTP concentrations did not drop below 50% after culture in drug-free medium for 24 hr.

Effects on normal ribonucleotide pools

Unexpectedly, normal ribonucleotide pools were considerably affected by dFdC treatment. In Table 2 ATP, ADP, CTP, UTP and GTP pools of untreated cells are summarized. Substantial differences were observed between the cell lines, with markedly higher ATP pools in C26-10 cells. Moreover, CTP was higher in this cell line than in the other lines. GTP and UTP pools in C26-10 cells were in the same range as in A2780 and WiDr cells. The ATP/ADP ratio, an indicator for the energy status of the cells, varied from 3.9 to 8.3 in the cell lines.

As a result of exposure to dFdC some remarkable changes were observed (Fig. 4). The most pronounced immediate effect was a depletion of the CTP pool, although this was concentration, time and cell line dependent. In A2780 cells the most rapid, clearest time and concentration dependent effect was observed with a 50% decrease at 1 μM dFdC after 4 hr exposure, and more than an 80% decrease at 10 μM. After 24 hr, however, CTP pools increased two-fold, at 100 μM. In contrast, the effect on CTP was not time but was concentration dependent in the two colon carcinoma cell lines. In WiDr cells maximal depletion was only measured after exposure to 10 μM dFdC. In C26-10 cells CTP levels decreased both at 10 μM and 100 μM dFdC exposure. Parallel to CTP depletion, an increase in UTP pools was observed in the two colon carcinoma cell lines. The most predominant immediate effect appeared in C26-10 cells. Unexpectedly, dFdC also caused perturbations of the GTP pools, which increased in all cell lines and were concentration dependent. The highest increase was registered in the colon carcinoma cell lines.

Replenishment of the medium did not result in restoration of the normal levels of these three ribonucleotides (Fig. 5). Again, the observed changes were dependent on dFdC concentration. In general, the depleted CTP pools initially restored but showed an overshoot to levels 4-fold normal concentrations. UTP and GTP pools were both retained at the levels achieved during dFdC exposure. In ATP and ADP pools no major changes were measured (data not shown).

In vivo *effects of dFdC*

The three solid tumours showed the same sensitivity pattern as assessed *in vitro* for their corresponding cell lines: A2780 was the most sensitive and Colon 26 and WiDr were the least sensitive tumours (Table 3). *In vivo* highest dFdCTP concentrations were measured 2–6 hr after i.p. injection of 120 mg/kg dFdC, depending on the tumour. Shortly after dFdC administration, highest dFdCTP concentrations were detected in the WiDr tumour (Fig. 6). However, after 6 hr the highest accumulation was observed in A2780 tumours. The murine colon tumour Colon 26 accumulated 4–8-fold less dFdCTP than either WiDr or A2780. The latter tumours retained dFdCTP longer than 24 hr, in contrast to Colon 26.

Marked differences in normal nucleotide pools and changes caused by dFdC administration were also observed *in vivo* (Table 4, Fig. 7). The

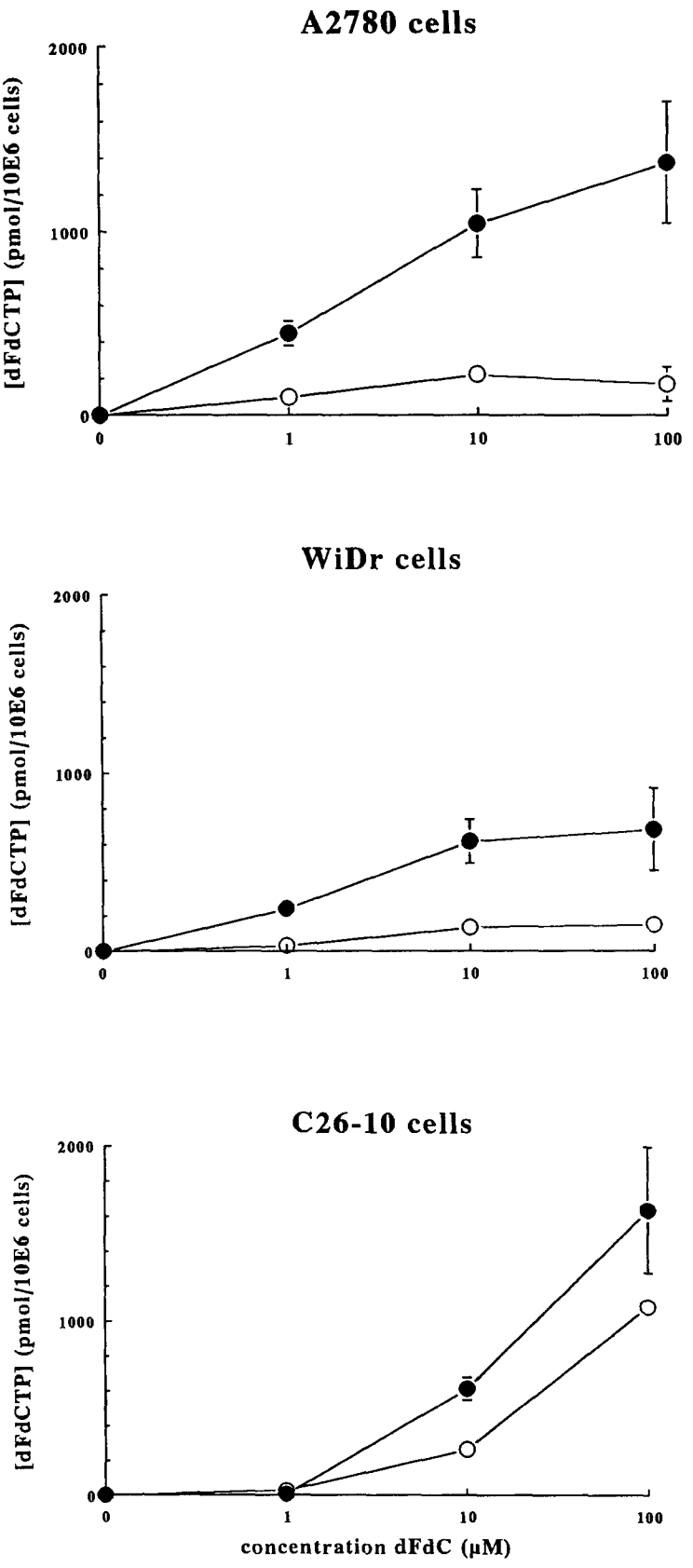

Fig. 2. Accumulation of dFdCTP in three solid tumour cell lines: A2780, WiDr and C26-10. The open circles represent accumulation at 4 hr exposure, the closed circles at 24 hr. Values shown represent the means ± SEM of 3–5 separate experiments.

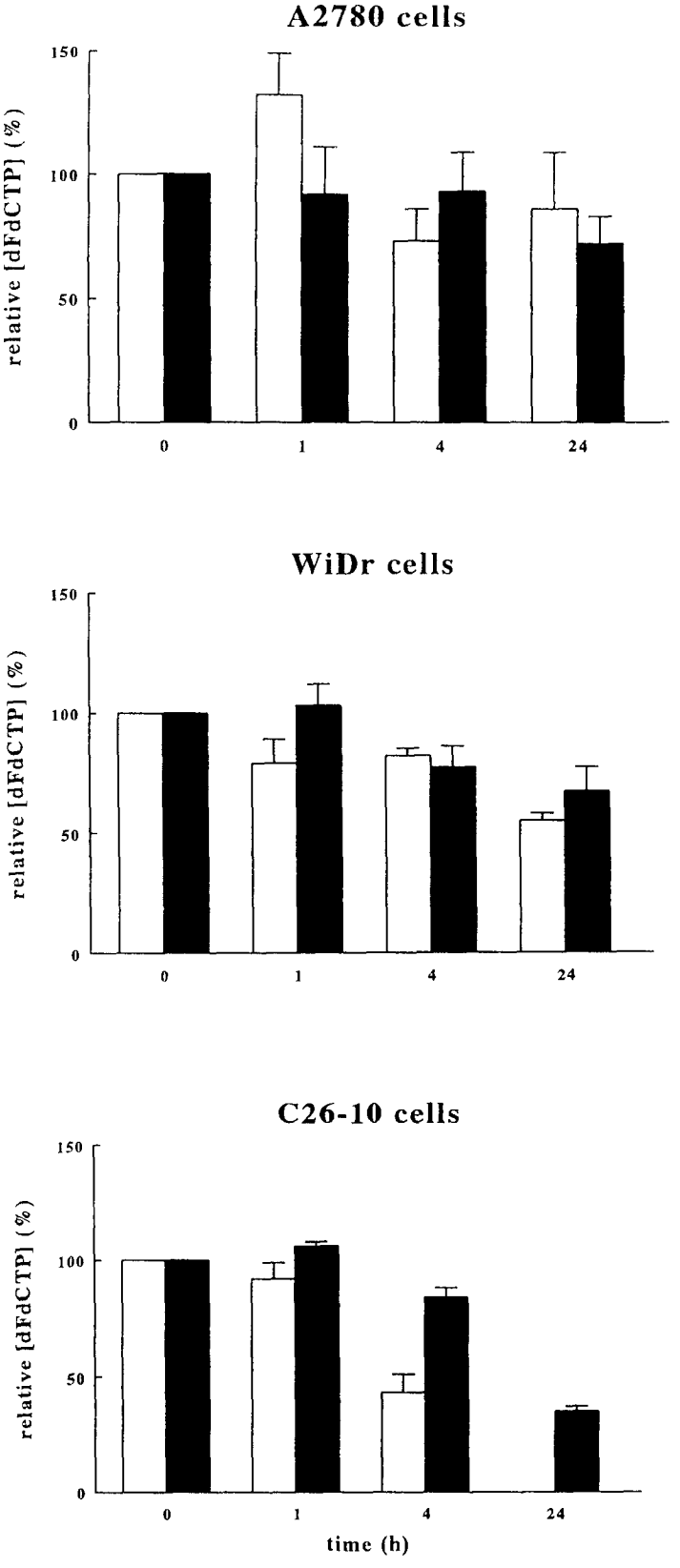

Fig. 3. Retention of dFdCTP in three solid tumour cell lines: A2780, WiDr and C26-10. The open bars represent the retention after exposure to 1 μM dFdC, the solid bars to 10 μM dFdC. The dFdCTP concentration accumulated after 24 hr exposure to 1 μM and 10 μM dFdC, respectively, was set at 100%. Values shown represent the means ± SEM of 3–5 separate experiments.

Table 2. Ribonucleotide concentrations in non-treated solid tumour cell lines

NTP	Cell line		
	A2780	WiDr	C26-10
UTP	630 ± 210	560 ± 41	789 ± 105
CTP	219 ± 65	168 ± 17	458 ± 38
GTP	343 ± 124	351 ± 36	443 ± 4
ATP	1796 ± 464	1602 ± 232	3173 ± 178
ADP	300 ± 41	546 ± 70	686 ± 52
ATP/ADP	8.3 ± 1.7	3.9 ± 0.7	5.1 ± 1.0

Concentrations are expressed in pmol/10^6 cells and are means ± SEM of 3–5 separately harvested cell pellets.

ATP/ADP ratios were calculated from the separate experiments and are expressed as means ± SEM.

ribonucleotide pools differ considerably with the tumours (Table 4), although the procedure used to remove the tumours and extract the nucleotides was similar. WiDr tumours showed approximately 10-fold higher ATP, GTP and UTP pools than A2780 and Colon 26 tumours. The CTP pool of the WiDr tumours was about 14-fold higher than that of the other tumours. The ADP pool was highest in the A2780 tumours, 2-fold higher than that in WiDr tumours and 5-fold higher than that in Colon 26 tumours. The normal nucleotides CTP, UTP and GTP in tumours changed considerably after dFdC treatment (Fig. 7). In contrast to the *in vitro* changes, CTP, UTP and GTP all increased in these tumours. CTP pools increased in all three tumours until 4–6 hr after dFdC administration, although this change was small in WiDr tumours compared to A2780 tumours. The pools then returned to normal levels in A2780 and WiDr tumours. In Colon 26, however, CTP pools again increased after an initial decrease at 6 hr after treatment. The same pattern was observed for UTP as well as GTP pools, although in Colon 26 tumours GTP pools decreased after 8 hr to approximately 50% of their level before dFdC treatment. It is noteworthy, that in the A2780 tumour all nucleotides initially increased about 10 to 15-fold before returning to pretreatment levels. ATP/ADP ratios also changed in time (Table 5). In A2780 and WiDr tumours the ratios were highest (2-fold increase) when dFdCTP concentrations also were highest. In Colon 26 tumours no significant changes were observed.

The dFdCTP/CTP ratio was calculated for all three tumours (Table 6) to determine the position of dFdCTP for competition with CTP in RNA synthesis and possible interference with the regulation of enzymes such as CTP-synthetase. A change in CTP pools (either an increase or decrease) is usually reflected by a similar change in dCTP. Thus, an altered dFdCTP/CTP ratio is considered to reflect a similar change in the dFdCTP/dCTP ratio and DNA synthesis. During the first 6 hr after dFdC administration the ratios were remarkably similar in all three tumours, although at 4 hr Colon 26 showed the highest ratio (0.90). However, 8 and 24 hr after i.p. injection of dFdC, the most sensitive tumour A2780 showed the highest ratios. The ratios in the two colon tumours WiDr and Colon 26 were 2–10-fold lower than in the ovarian tumour A2780.

DISCUSSION

In this study we demonstrated that the sensitivity to dFdC of solid tumour cell lines corresponded to the sensitivity of the corresponding tumours *in vivo*. Furthermore, we showed that the active metabolite dFdCTP was accumulated and retained in these cell lines and tumours, and that the dFdCTP concentrations accumulated in the 1–10 μM range generally related to sensitivity to dFdC *in vitro*. *In vivo* this relation was less evident. Previously, studies on dFdCTP accumulation and retention were reported for leukaemic cells and normal mononuclear cells. In accordance with our results, dFdCTP accumulation was concentration and time dependent in leukaemic cell lines [5, 32] as well as in leukaemic or normal mononuclear cells from patients [19, 20]. In studies performed on leukaemic cells, dFdCTP concentrations were expressed in μM. Since our cell lines grow as monolayers and are usually stretched and not spherical, we expressed our results in pmol/10^6 cells. In order to compare our data with those reported earlier, we determined the cell volumes of our three cell lines when suspended in medium in comparison with that of the leukaemic cell line CCRF-CEM. The cellular volume of A2780 cells was 12.3×10^{-13} L/cell, of WiDr cells 14.4×10^{-13} L/cell, of C26-10 cells 15.2×10^{-13} L/cell and that of CCRF-CEM cells 10.5×10^{-13} L/cell. Thus dFdCTP accumulation in our solid tumour cell lines exposed to 1 μM dFdC for 4 hr approximated 35–120 μM and about 95–245 μM, after exposure to 10 μM for 4 hr. dFdCTP accumulation in CCRF-CEM leukaemic cells appeared to be more rapid and three- to 10-fold higher than in cell lines derived from solid tumours. For these cells Heinemann *et al.* [17] reported dFdCTP concentrations of 346 and 525 μM after a 2 hr exposure to 1 and 10 μM dFdC, respectively. For the human leukaemia K562 cells Gandhi and Plunkett [32] reported 110, 215 and 125 μM of dFdCTP after 3 hr exposure to 1, 10 and 100 μM, respectively, more or less in the same range as our results. Bhalla *et al.* [18] reported comparable dFdCTP concentrations in the human ovarian carcinoma cell line 2008 (309 pmol/10^6 cells) and its cisplatin-resistant variant 2008/C13 (149 pmol/10^6 cells), after exposure of the cells to 1 mM dFdC for 4 hr. Our A2780 cells accumulated 258 pmol/10^6 cells at exposure to 100 μM for 4 hr.

The dFdCTP concentrations at 1 μM dFdC in A2780 and C26-10 cells correlate well with the incorporation of dFdC into nucleic acids; A2780 incorporated 5–10-fold more dFdC into DNA than C26-10 cells and 2- and 3-fold more dFdC into RNA at this dFdC concentration [8]. CCRF-CEM cells, however, incorporated about two-fold less dFdC into DNA and about 10-fold less into RNA than A2780 cells [8], although at short exposure CEM cells accumulated 9-fold more dFdCTP (2350 pmol/10^6 cells at 10 μM after 4 hr, unpublished results) than A2780 cells. Gandhi and Plunkett [32] observed saturation of dFdCTP accumulation in K562 cells at a 3 hr exposure to 10 μM of dFdC. This

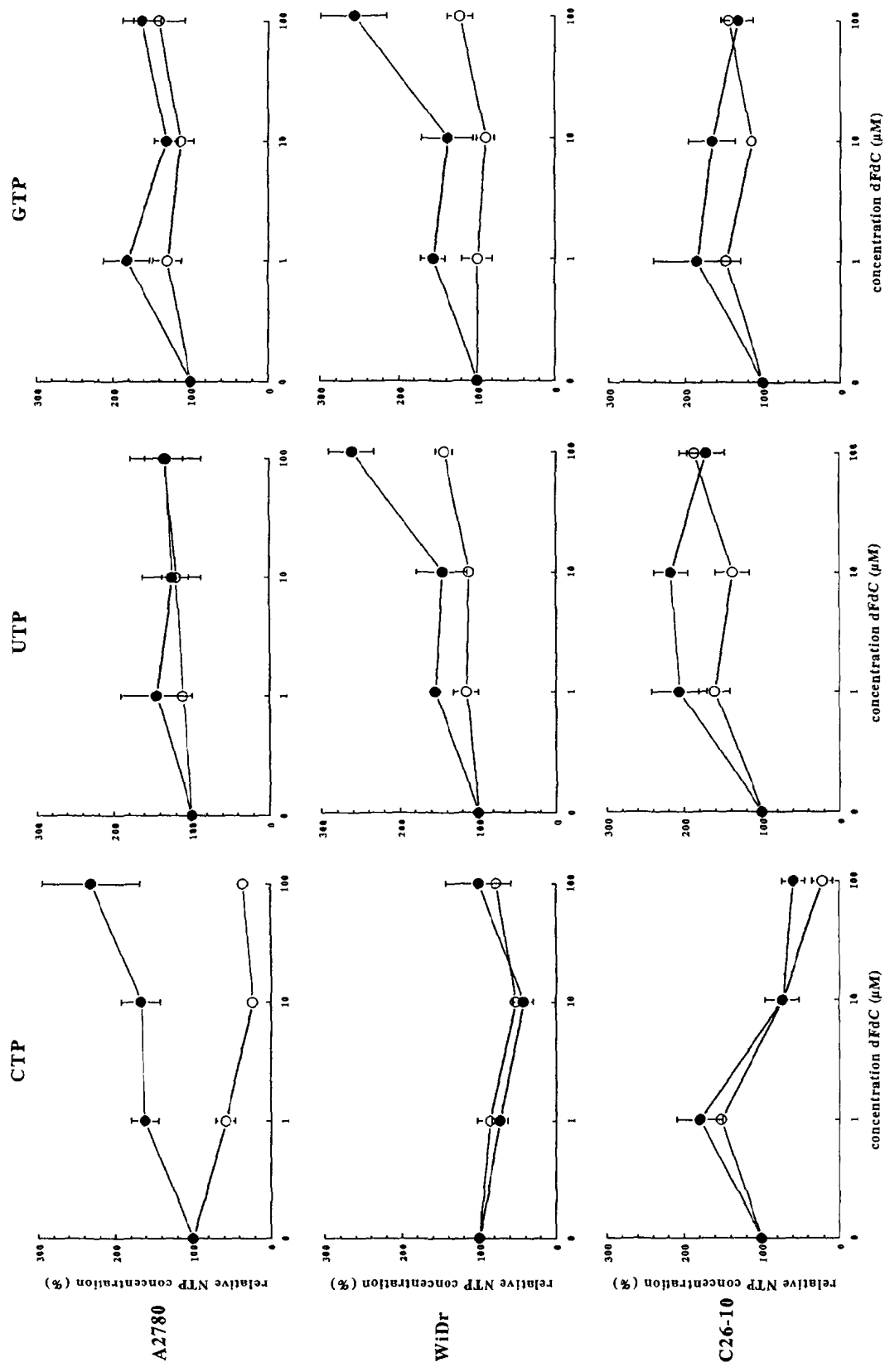

Fig. 4. Changes in ribonucleotide (NTP) pools during dFdC exposure in three solid tumour cell lines: A2780, WiDr and C26-10. The open circles represent the nucleotide pools at 4 hr dFdC exposure, the closed circles at 24 hr exposure. The concentrations of non-treated cells were set at 100%. Values represent means ± SEM of 3–5 separate experiments.

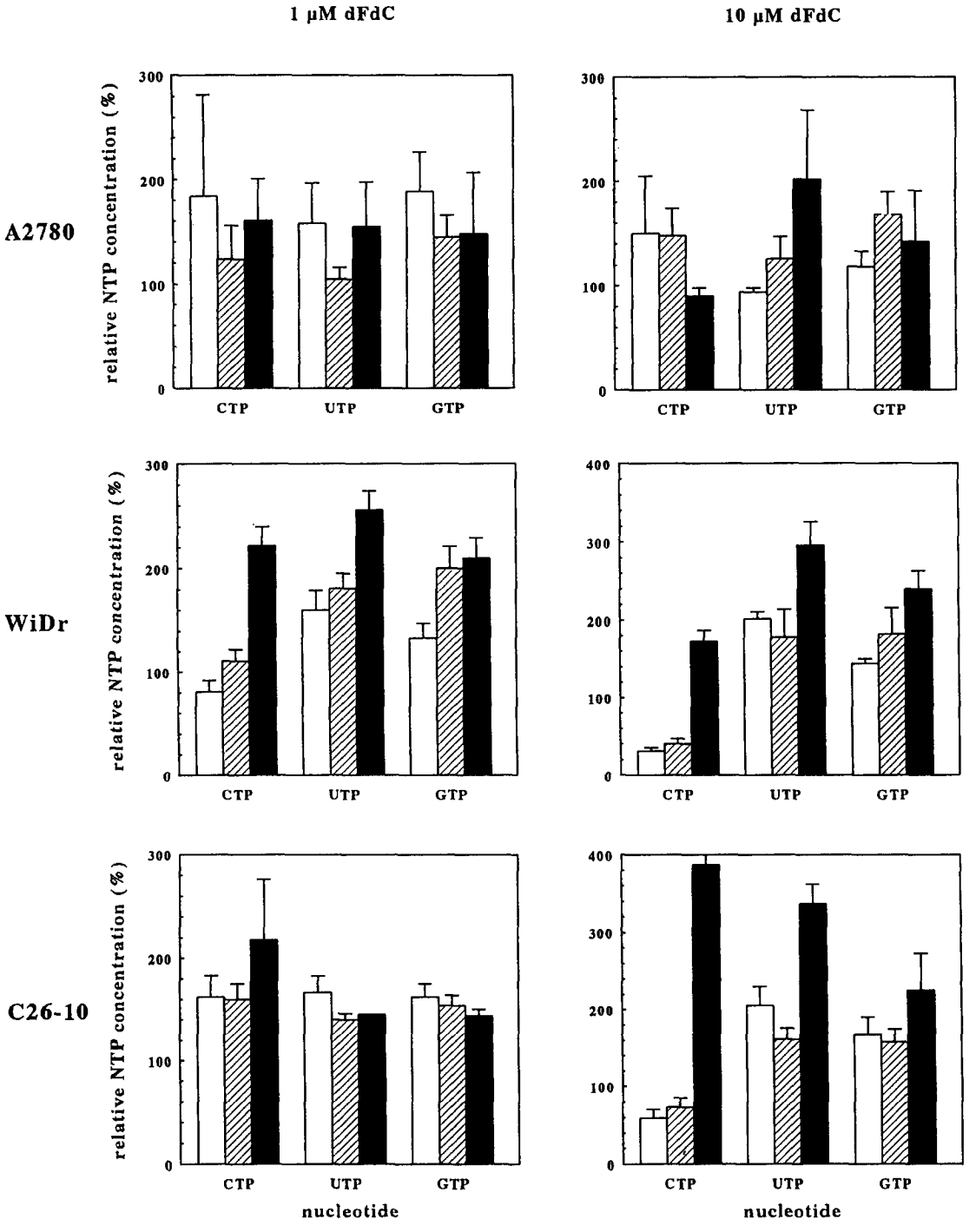

Fig. 5. Changes in ribonucleotide (NTP) pools in three solid tumour cell lines, after dFdC exposure. The open bars represent the nucleotide concentrations after 1 hr culture in drug-free medium, the hatched bars after 4 hr in drug-free medium and the solid bars after 24 hr in drug-free medium. The nucleotide concentrations in non-treated cells were set at 100% (see Fig. 4). The values shown represent means ± SEM of 3–5 separate experiments.

agrees with the accumulation which we measured in A2780 and WiDr cells after a 4 hr exposure to 10 μM dFdC. At longer exposure (24 hr), however, the initial saturation in A2780 and WiDr cells disappeared and dFdCTP levels 3–10-fold higher than the concentrations at 4 hr exposure were reached. In C26-10 cells no saturation whatsoever was observed. Heinemann *et al.* [17] observed that CCRF-CEM

Table 3. Sensitivity of solid tumours to dFdC

Tumour	DT*	GDF†	T/C‡
A2780	6.6	>6§	0.02 (day 24)
WiDr	8.3	2.4	0.28 (day 25)
Colon 26	2.4	3.6	0.25 (day 8)

* Tumour doubling time in days of non-treated tumours, as determined in our laboratory.

† Growth delay factor, calculated for the schedule 120 mg dFdC/kg, q3d × 4 reflects the number of doubling times gained by treatment. The GDF was calculated as follows: (doubling time treated tumours − doubling time control tumours)/(doubling time control tumours).

‡ Value of tumour volume treated/tumour volume control, measured at the indicated day.

§ Complete remissions: 2 out of 11 tumours, DT treated of A2780 could not be determined and was longer than 46 days.

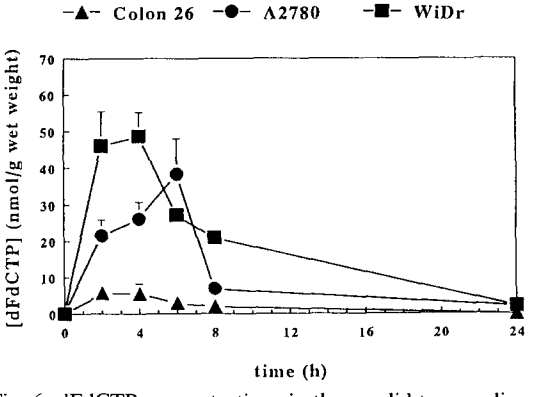

Fig. 6. dFdCTP concentrations in three solid tumour lines after administration of an i.p. injection of 120 mg dFdC/kg at $t = 0$. The circles represent dFdCTP in the A2780 tumour, the squares in the WiDr tumour and the triangles in the Colon 26 tumour. The values shown are means ± SEM of 3–5 separate tumours.

Table 4. Ribonucleotide concentrations in non-treated solid tumours

NTP	Tumour		
	A2780	WiDr	Colon 26
UTP	24.7 ± 5.8	235 ± 67	10.1 ± 1.3
CTP	5.9 ± 1.0	71.4 ± 21.0	4.9 ± 0.6
GTP	27.9 ± 5.2	205 ± 58	22.5 ± 0.2
ATP	75.1 ± 13.3	618 ± 196	56.1 ± 7.3
ADP	290 ± 57	143 ± 31	46.7 ± 4.1
ATP/ADP	0.3 ± 0.05	4.4 ± 2.4	1.3 ± 0.6

Concentrations are expressed as nmol/g wet weight and are means ± SEM of 4–6 separate tumours.

cells retained dFdCTP at least 10 hr. We showed that A2780 and WiDr cells retained dFdCTP longer than 24 hr.

In contrast to the observations of Gandhi and Plunkett [32] in K562 cells we observed substantial changes in ribonucleotide pools, even at a 4 hr exposure to 10 μM dFdC. The decrease in CTP observed in all three cell lines at 4 hr exposure and at 24 hr in the two colon cancer cell lines could be explained by an inhibition of CTP-synthetase, since this would lead to an increase in UTP pools, the substrate for CTP synthetase. Heinemann and Plunkett [10] reported inhibition of CTP-synthetase in CCRF-CEM cells. The inhibition of this important enzyme, rate limiting in the synthesis of cytidine nucleotides, may have consequences for the activity of deoxycytidine kinase, the activator of dFdC. A decrease in CTP concentrations as a result of inhibition of pyrimidine *de novo* synthesis has been related to a decrease in dCTP levels, as reported by Momparler *et al.* [33], for the combination of ara-C and the CTP-synthetase inhibitor 3-deazauridine in HL-60 cells, and for other inhibitors of the pyrimidine *de novo* synthesis reported by Peters *et al.* [22] and Schwartsmann *et al.* [34]. A decrease in dCTP pools can be favourable to the conversion of dFdC into its monophosphate dFdCMP, since dCTP is the most important and most potent feedback inhibitor of deoxycytidine kinase. Furthermore, White and Cappizzi [14] and Shewach *et al.* [15] showed that, at least in leukaemic cells, UTP is a more efficient phosphate donor of deoxycytidine kinase than is ATP. An increase in UTP levels would in this case also be favourable to dCK activity. Preliminary data in our solid tumour cell lines also revealed effects of UTP and CTP on dCK activity, although these were not always potentiating [13].

The increase in GTP pools might be indirectly related to the inhibition of CTP-synthetase. When this enzyme is inhibited, the L-glutamine normally used for amination of UTP accumulates. This may enhance activity of GMP-synthetase, which catalyses the conversion of xanthosine-5'-phosphate into GMP [35]. Another factor in the increase in UTP and GTP pools in all cell lines, and CTP pools at 24 hr exposure to dFdC in A2780, may be the inhibition of RNA synthesis, as we reported earlier [8]. In both C26-10 and A2780 cells RNA synthesis was inhibited 10–30% at a 4 hr exposure to 1 μM dFdC. The inhibition was concentration dependent, so at 10 or 100 μM dFdC inhibition could be even higher. This impaired RNA synthesis could also explain the further increase in ribonucleotide concentrations after removal of dFdC from the cells. More research is warranted to clarify this phenomenon.

The relation between *in vivo* dFdCTP accumulation and *in vivo* sensitivity was less clear. Although WiDr tumours were less sensitive than A2780 tumours, the accumulation of dFdCTP was higher at time points shortly after dFdC administration. The higher dFdCTP/CTP ratio in the A2780 tumour 8 to 24 hr after drug administration may imply that dFdCTP retention is very important and that in these A2780 tumours dFdCTP has a more favourable position in the competition for RNA and DNA incorporation and enzyme regulation than in WiDr

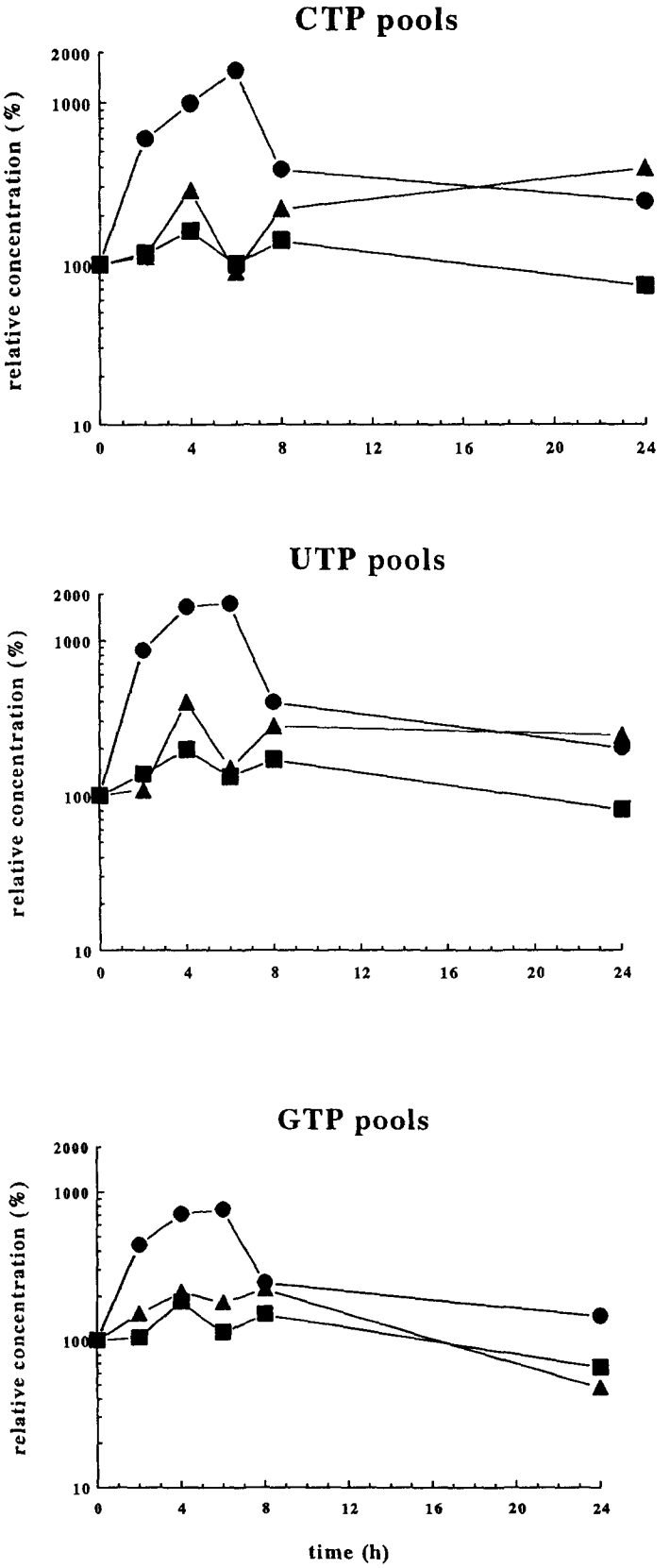

Fig. 7. Relative ribonucleotide concentrations in three solid tumours as a result of an i.p. injection of 120 mg dFdC/kg at $t = 0$. The circles represent nucleotide pools in the A2780 tumour, the squares in the WiDr tumour and the triangles in the Colon 26 tumour. Nucleotide concentrations of non-treated tumours were set at 100%. The variation in the absolute values was less than 30% (Table 4).

Table 5. Changes in ATP/ADP ratios in solid tumours after treatment with one i.p. injection of 120 mg dFdC/kg

Tumour		Time after injection (hr) 0	2	4	6	8	24
A2780	ratio	0.3	1.1	1.5	2.3	0.5	0.5
	ATP	75	471	892	969	243	147
WiDr	ratio	4.4	7.1	7.9	5.6	5.2	4.3
	ATP	618	938	1391	875	1063	500
Colon 26	ratio	1.3	1.1	0.7	1.2	2.1	0.8
	ATP	56	56	31	66	119	33

Ratios were calculated from ATP and ADP concentrations of 3–5 separate tumours.

ATP concentrations are expressed as nmol/g wet weight in the mean of 3–7 separate tumours. Variation was similar to that described in Table 4.

Table 6. Ratios of dFdCTP and CTP concentrations in solid tumours

Time after injection (hr)	A2780	WiDr	Colon 26
2	0.64 ± 0.06	0.57 ± 0.04	0.68*
4	0.44 ± 0.06	0.44 ± 0.05	0.90 ± 0.17
6	0.41 ± 0.02	0.38 ± 0.03	0.46 ± 0.04
8	0.39 ± 0.08	0.22 ± 0.03	0.21 ± 0.07
24	0.27 ± 0.07	0.04 ± 0.01	0†

Ratios were calculated from dFdCTP and CTP concentrations of 3–5 separate tumours and represent the means ± SEM.

* Value based on dFdCTP and CTP concentration of one tumour.

† In this tumour dFdCTP was not detectable 24 hr after dFdC administration.

and Colon 26 tumours, which could explain the higher sensitivity of A2780 tumours to treatment with dFdC. Although Colon 26 tumours showed the highest dFdCTP/CTP ratio 4 hr after treatment, the absolute dFdCTP levels were much lower than in A2780 tumours, which would explain the relatively low sensitivity of Colon 26 to dFdC.

As observed *in vitro*, the ribonucleotide pools changed, while *in vivo* the concentrations of all three nucleotides CTP, UTP and GTP increased initially and returned to more or less normal levels 24 hr after dFdC administration. This increase is possibly related to the increase in the pools of ATP, which is a phosphate donor for the synthesis of these nucleotides. This is in line with the differences between the tumours, since in A2780 the relative increase in all nucleotides was highest, but in Colon 26 this effect was negligible. The increase in NTP synthesis as a result of the increase in ATP levels possibly masks a differential effect on CTP and UTP pools as observed *in vitro*: an increased salvage synthesis of CTP and UTP (from cytidine and uridine) would mask an inhibition of CTP synthesis from UTP.

In contrast to the *in vitro* results, *in vivo* we observed significant effects on ATP/ADP ratios. Major changes were observed, especially in the A2780 tumour, where relatively low ATP concentrations as well as a low ATP/ADP ratio were found, which increased substantially after dFdC treatment due to an increase in ATP pools. Interestingly, the dFdCTP peak was observed when the higher ATP/ADP ratio was measured. This effect cannot be ascribed to the method of tumour excision, freezing and extraction, because all tumours were handled in the same way (see Materials and Methods). Thus, the observed changes are considered to be due to dFdC treatment. One explanation may be a decreased turnover of ATP as a result of the disturbing effects of dFdC(TP) on ribonucleotide homeostasis. When dFdCTP concentrations decrease again, the use of ATP in normal metabolic processes may be regained, resulting in the observed decrease of ATP/ADP ratios parallel to the decrease in dFdCTP.

In conclusion, we demonstrate in this study that dFdCTP accumulation and retention in solid tumour cell lines is clearly related to sensitivity. In solid tumours, the relation was less clear. The antitumour effect of dFdC may also be related to the marked changes in ribonucleotide pools, leading to and due to both inhibition and stimulation of several important enzymes involved in RNA and DNA synthesis. The changes, possibly leading to self-potentiation, suggest that the mechanism of action of dFdC cannot solely be attributed to DNA damage. The disturbance of normal pyrimidine and purine metabolism in solid tumour cells may be of equal importance.

REFERENCES

1. Hertel LW, Boder GB, Kroin JS, Rinzel SM, Poore GA, Todd GC and Grindey GB, Evaluation of the antitumour activity of gemcitabine (2′,2′-difluoro-2′-deoxycytidine). *Cancer Res* **50**: 4417–4422, 1990.
2. Braakhuis BJM, Van Dongen GAMS, Vermorken JB and Snow GB, Preclinical *in vivo* activity of 2′,2′-difluorodeoxycytidine (gemcitabine) against human head and neck cancer. *Cancer Res* **51**: 211–214, 1991.
3. Boven E, Schipper H, Erkelens CAM, Hatty SA and Pinedo HM, The influence of schedule and the dose of gemcitabine on the anti-tumour efficacy in experimental human cancer. *Br J Cancer* **68**: 52–56, 1993.
4. Lund B, Kristjanssen PEG and Hansen HH, Clinical and preclinical activity of 2′,2′-difluorodeoxycytidine (gemcitabine). *Cancer Treat Rev* **19**: 45–55, 1993.
5. Heinemann V, Hertel LW, Grindey GB and Plunkett W, Comparison of the cellular pharmacokinetics and toxicity of 2′,2′-difluorodeoxycytidine and 1-β-D-arabinofuranosylcytosine. *Cancer Res* **48**: 4024–4031, 1988.
6. Huang P, Chubb S, Hertel LW, Grindey GB and Plunkett W, Action of 2′,2′-difluorodeoxycytidine on DNA synthesis. *Cancer Res* **51**: 6110–6117, 1991.
7. Huang P and Plunkett W, A quantitative assay for fragmented DNA in apoptotic cells. *Anal Biochem* **207**: 163–167, 1992.
8. Ruiz van Haperen VWT, Veerman G, Vermorken JB and Peters GJ, 2′,2′-Difluorodeoxycytidine (gemcitabine) incorporation into RNA and DNA of tumour cell lines. *Biochem Pharmacol* **46**: 762–766, 1993.
9. Heinemann V, Xu Y-Z, Chubb S, Sen A, Hertel

LW, Grindey GB and Plunkett W, Inhibition of ribonucleotide reduction in CCRF-CEM cells by 2',2'-difluorodeoxycytidine. *Mol Pharmacol* **38**: 567–572, 1990.

10. Heinemann V and Plunkett W, Inhibitory action of 2',2'-difluorodeoxycytidine (dFdC) on cytidine 5'-triphosphate synthetase. *Ann Oncol* **3** (suppl. 1): 187 (abstr. 510), 1992.
11. Xu Y-Z and Plunkett W, Modulation of deoxycytidylate deaminase in intact human leukemia cells—Action of 2',2'-difluorodeoxycytidine. *Biochem Pharmacol* **44**: 1819–1827, 1992.
12. Durham JP and Ives DH, Deoxycytidine kinase II Purification and general properties of the calf thymus enzyme. *J Biol Chem* **245**: 2276–2284, 1970.
13. Ruiz van Haperen VWT and Peters GJ, New targets for pyrimidine antimetabolites for the treatment of solid tumours, II, Deoxycytidine kinase. *Pharmacy World Sci* **16**: 104–112, 1994.
14. White JC and Capizzi RL, A critical role for uridine nucleotides in the regulation of deoxycytidine kinase and the concentration dependence of 1-β-D-arabinofuranosylcytosine phosphorylation in human leukemia cells. *Cancer Res* **51**: 2559–2565, 1991.
15. Shewach DS, Reynolds KK and Hertel LW, Nucleotide specificity of human deoxycytidine kinase. *Mol Pharmacol* **42**: 518–524, 1992.
16. Plunkett W, Gandhi V, Chubb S, Nowak B, Heinemann V, Mineishi S, Sen A, Hertel LW and Grindey GB, 2',2'-Difluorodeoxycytidine metabolism and mechanism of action in human leukemia cells. *Nucleosides Nucleotides* **8**: 775–785, 1989.
17. Heinemann V, Xu Y-Z, Chubb S, Sen A, Hertel LW, Grindey GB and Plunkett W, Cellular elimination of 2',2'-difluorodeoxycytidine 5'-triphosphate: a mechanism of self-potentiation. *Cancer Res* **52**: 533–539, 1992.
18. Bhalla K, Holladay C, Lutzky J, Ibrado AM, Bullock G, Jasiok M and Singh S, Deoxycytidine protects normal bone marrow progenitors against ara-C and gemcitabine cytotoxicity without compromising their activity against cisplatin-resistant human ovarian cancer cells. *Gynecol Oncol* **45**: 32–39, 1992.
19. Grunewald R, Abbruzzese JL, Tarassoff P and Plunkett W, Saturation of 2',2'-difluorodeoxycytidine 5'-triphosphate accumulation by mononuclear cells during a phase I trial of gemcitabine. *Cancer Chemother Pharmacol* **27**: 258–262, 1991.
20. Grunewald R, Kantarjian H, Du M, Faucher K, Tarassoff P and Plunkett W, Gemcitabine in leukemia: a phase I clinical, plasma and cellular pharmacology study. *J Clin Oncol* **10**: 406–413, 1992.
21. Ruiz van Haperen VWT, Veerman G, Braakhuis BJM, Vermorken JB, Boven E, Leyva A and Peters GJ, Deoxycytidine kinase and deoxycytidine deaminase activities in human tumour xenografts. *Eur J Cancer* **29A**: 2132–2137, 1993.
22. Peters GJ, Laurensse E, Leyva A, Lankelma J and Pinedo HM, Sensitivity of human, murine and rat cells to 5-fluorouracil and 5'-deoxy-5-fluorouridine in relation to drug-metabolizing enzymes. *Cancer Res* **46**: 20–28, 1986.
23. Peters GJ, Kraal I and Pinedo HM, *In vitro* and *in vivo* studies on the combination of brequinar sodium (DUP 785, NSC 368390) with 5-fluorouracil; effects of uridine. *Br J Cancer* **65**: 229–233, 1992.
24. Keepers YPAM, Pizao PE, Peters GJ, Van Ark-Otte J, Winograd B and Pinedo HM, Comparison of the sulforhodamine B protein and tetrazolium (MTT) assays for *in vitro* chemosensitivity testing. *Eur J Cancer* **27**: 897–900, 1991.
25. Peters GJ, Wets M, Keepers YPAM, Oskam R, Van Ark-Otte J, Noordhuis P, Smid K and Pinedo HM, Transformation of mouse fibroblasts with the oncogenes H-ras or trk is associated with pronounced changes in drug sensitivity and metabolism. *Int J Cancer* **54**: 450–455, 1993.
26. Molthoff CFM, Calame JJ, Pinedo HM and Boven E, Human ovarian cancer xenografts in nude mice: characterization and analysis of antigen expression. *Int J Cancer* **47**: 72–79, 1991.
27. Van der Wilt CL, Van Laar JAM, Gyergyay F, Smid K and Peters GJ, Biochemical modification of the toxicity and antitumour effect of 5-fluorouracil and cisplatinum by WR2721 (ethiofos) in mice. *Eur J Cancer* **28A**: 4922–4928, 1992.
28. Tomayako MM and Reynolds CP, Determination of subcutaneous tumor size in athymic (nude) mice. *Cancer Chemother Pharmacol* **24**: 148–154, 1989.
29. Boven E, Winograd B, Berger DP, Dumont MP, Braakhuis BJM, Fodstad O, Langdon S and Fiebig HH, Phase II preclinical drug screening in human tumor xenografts: a first European multicenter collaborative study. *Cancer Res* **52**: 5940–5947, 1992.
30. Peters GJ, Van Groeningen CJ, Laurensse E, Lankelma J, Leyva A and Pinedo HM, Uridine-induced hypothermia in mice and rats in relation to plasma and tissue levels of uridine and its metabolites. *Cancer Chemother Pharmacol* **20**: 101–108, 1987.
31. Peters GJ, Laurensse E, Leyva A and Pinedo HM, Tissue homogenisation using a micro-dismembrator for the measurement of enzyme activities. *Clin Chim Acta* **158**: 193–198, 1986.
32. Gandhi V and Plunkett W, Modulatory activity of 2',2'-difluorodeoxycytidine on the phosphorylation and cytotoxicity of arabinosyl nucleosides. *Cancer Res* **50**: 3675–3680, 1990.
33. Momparler RL, Bouffard DY, Momparler LF, Marquet J, Zittoun J, Marie J-P and Zittoun R, Enhancement of antineoplastic activity of cytosine arabinoside against human HL-60 myeloid leukemic cells by 3-deazauridine. *Int J Cancer* **49**: 573–576, 1991.
34. Schwartsmann G, Peters GJ, Laurensse E, De Waal FC, Loonen AH, Leyva A and Pinedo HM, DUP 785 (NSC 368390): schedule-dependency of growth-inhibitory and antipyrimidine effects. *Biochem Pharmacol* **37**: 3257–3266, 1988.
35. Weber G, Biochemical strategy of cancer cells and the design of chemotherapy: GHA Clowes Memorial Lecture. *Cancer Res* **43**: 3466–3492, 1983.