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Original Paper

Postconfluent Multilayered Cell Line Cultures for Selective Screening of Gemcitabine

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The *in vitro* cytotoxicity of gemcitabine (dFdC) was tested in ovarian and colon cancer cell lines grown as monolayers and three-dimensional multilayered cell cultures. In our model, dFdC showed slight selectivity in cytotoxicity against ovarian over colon cancer cells, when cell lines were grown as monolayers. However, when cell lines were grown as multilayers, this selectivity was accentuated: A2780 multilayers were 14 times less sensitive than monolayers, but the colon cancer cell lines were more than 1000 times more resistant than their corresponding monolayers. The accumulation of the active metabolite, dFdCTP, after 24 h exposure to 1 μM dFdC varied between 1100 and 1900 pmol/10^6 cells in monolayers. This was 5 times lower in multilayers compared with monolayers of all four cell lines, which can, in part, explain the lower sensitivity of the multilayers. In addition, it appears that the amount of the active metabolite retained is more important than the amount accumulated initially, since the differences between the ovarian and the colon cancer cell lines were more evident in retention experiments. Exposure to dFdC caused a 2–3-fold increase in the levels of several nucleotides, except for the CTP pools in the colon cancer lines, which were reduced by 3-fold at the highest dFdC concentration (10 μM). The findings with the multilayer model are in better agreement with *in vivo* activity in ovarian cancer and colon cancer than those with the monolayer system. This indicates the potential of the multilayer system to be a better predictive model than the conventionally used monolayer cultures.

Key words: gemcitabine, three-dimensional cultures, ovarian cancer, colon cancer
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INTRODUCTION

THE ANTIMETABOLITE gemcitabine (2′,2′-difluoro-2′-deoxycytidine, Gemzar or dFdC) is a deoxycytidine analogue with reasonable antitumour activity against solid tumours in preclinical models, both *in vitro* in cell lines and *in vivo* against murine and human xenografts [1–4]. In clinical phase II studies, dFdC has shown remarkable activity against ovarian and lung cancer (partial response rate (PR) 20–30%), but not to colon cancer (PR 4%) [5–7]. Gemcitabine is now an established agent in the treatment of non-small cell lung cancer and pancreatic cancer [8].

dFdC inhibits the proliferation of cells mainly by incorporation of its triphosphate (dFdCTP) into DNA [9]. Although dFdC can also be incorporated into RNA, it is not clear how much this contributes to the cytotoxicity of the compound [10]. dFdC has to be phosphorylated by deoxycytidine kinase (dCK) [11, 12]. dCK is the rate limiting enzyme for active metabolite formation. Growth inhibition or cell kill can also be the result of multiple mechanisms of dFdC, such as incorporation into DNA, RNA and inhibition of nucleotide-synthesising enzymes. This can cause severe changes in ribo- or deoxyribonucleotide pools and can potentiate the activity of dFdC itself [13–15]. For example, the lowering of dCTP levels reduces the feedback inhibition of dCK, which increases the activation of dFdC [16].

Most initial preclinical studies on dFdC were performed in leukaemic cell lines and Chinese Hamster Ovary (CHO) cells [1, 17]. We reported that, in monolayered cell lines, accumulation of dFdCTP is time and concentration dependent, but qualitatively and quantitatively different from most leukaemic cells. The most sensitive cell line in the panel tested was the

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ovarian cancer line A2780, which accumulated the highest absolute dFdCTP concentrations when compared with two colon cancer cell lines [12]. Since solid tumours are organised in a three-dimensional structure, we developed an alternative *in vitro* system in which these cell lines grow in a three-dimensional way, resembling more the *in vivo* situation [18]. For this purpose, human tumour cell lines were cultured as postconfluent multilayers in 'V'-bottomed microtitre plates as opposed to exponentially growing subconfluent monolayers [19].

In order to determine whether this *in vitro* system resembles *in vivo* tumours and has any predictive value, we studied sensitivity to dFdC, dFdCTP accumulation and retention and disturbances in the normal ribonucleotide pools in three colon cancer cell lines and one representative ovarian cancer cell line under different growth conditions.

MATERIALS AND METHODS

Chemicals

dFdC and dFdCTP were provided by Eli Lilly & Co, Indianapolis, Indiana, U.S.A. All other chemicals were of analytical grade and commercially available.

Cell culture

The human colon cancer lines HT29, WiDr and SW620 and the human ovarian cancer cell line A2780 are cultured routinely and have been described previously [12, 19].

Cells were grown, mycoplasma-free, in *N*-2-hydroxyethylpiperazine-*N*-2-ethane-sulphonic acid (HEPES) buffered Dulbecco's modified Eagles medium (DMEM) supplemented with 5% heat inactivated fetal calf serum (Gibco, BRL, Paisley, U.K.) and 1 mM L-glutamine in 80 cm^2 flasks in a 37°C, 5% CO_2, 95% humidified air incubator and were subcultured twice a week.

Chemosensitivity assays

Chemosensitivity assays were performed in 96 well microtitre plates with a V-shaped bottom (Greiner Labortechnik, Solingen, Germany) using a slightly modified sulphorhodamine B (SRB) assay [20, 21]. The cells were plated (15 000 cells/50 µl per well) on day 0 (d0). There was exponential growth initially followed by the formation of multiple cell layers when cultures became supraconfluent (after ± 5 days, depending on the cell line [18].

Twenty-four hours after plating (d1), monolayered cells were exposed for 24 h to serial dilutions of dFdC (10^{-6}–10^{-10} M for A2780 and 10^{-5}–10^{-10} for colon cancer cell lines) in a total volume of 150 µl medium per well, supplemented with gentamicin (50 µg/ml), in triplicate. On d2, the drug-containing medium was aspirated gently, the cells were washed once with medium and 150 µl fresh medium was added to each well for the next 24 h. On d3, d4 and d5, medium was replaced with 150 µl and at d6 (after 96 h culture in drug-free medium), the experiment was stopped by fixation by adding 50 µl ice-cold 50% trichloroacetic acid (TCA) to the wells.

The wells in which multilayers were cultured also received fresh medium on d2, d3 and d4, in order to prevent exhaustion of the medium. On d5, the multilayers received 150 µl drug-containing medium in triplicate (10^{-3}–10^{-10} M). At d6, cells were washed and medium was replaced with 150 µl drug-free medium. This was refreshed at d7, d8 and d9. At the end of the assay, on d10, cells were fixed using 50 µl TCA. On d1 and d5, a separate control plate was fixed in order to compare the amount of cells at the time of drug addition. After staining with SRB, absorbance was measured with a Titertek Multiscan MCC/340 (Flow Laboratories) at 450 (suboptimal) or 492 nm. Previously we demonstrated that, in this model, cell counting, the microtitre tetrazolium (MTT) assay and the SRB assay produced similar results [18]. Since the latter assay is more convenient and sensitive, we used the SRB assay for all experiments.

The IC_{50} was defined as the concentration of dFdC inducing 50% growth inhibition, corrected for initial absorbance of the control plates. To calculate the percentage growth inhibition (IC_{50}) or cell kill, the formulas of the National Cancer Institute were used [22, 23]. The cytotoxicity for the multilayers was expressed as the concentration of dFdC resulting in an optical density (OD) which was 50% of that of untreated wells (EC_{50}), usually implying some cell kill. The difference between EC_{50} and IC_{50} in monolayers is negligibly small, but is considerable in multilayers [19]. Experiments were repeated at least three times.

Accumulation and retention of dFdCTP

Cells were cultured as described in the previous section and were exposed on d1 (mono) or d5 (multi) to medium containing 0, 0.1 and 1 µM dFdC (A2780) or to 0, 1 and 10 µM dFdC (colon cancer lines). For monolayers, 1.2×10^6 cells were grown and exposed in small flasks (25 cm^2 in 5 ml) in duplicate for each concentration at d1. For multilayers, 15 000 cells per well were plated at d0 (two duplicate 96-wells plates with 48 wells/plate per concentration) and exposed for 24 h at d5 (the cells from 48 wells were pooled later, in order to obtain a detectable signal). After 24 h, the cells for the accumulation experiment (two flasks and two plates) were washed with ice-cold phosphate buffered saline (PBS) and harvested by trypsinisation, followed by chilling on ice. The cells were then counted using a haemocytometer and extracted as described by Ruiz van Haperen and associates [12]. The other two flasks and two plates (cells for the retention experiment) were washed with medium and harvested 24 h later in the same way after exposure to drug-free medium. Using the high performance liquid chromatography (HPLC) method, the levels of dFdCTP, CTP, ATP, UTP, GTP and ADP could be measured in one run [12]. Levels were expressed as pmol/10^6 cells.

RESULTS

Chemosensitivity

In our chemosensitivity assays we simultaneously analysed monolayers and multilayers in order to compare the data acquired from the new model with a standard *in vitro* technique. dFdC showed steep dose–response curves for all monolayer cell cultures, with IC_{50}s varying from 6 to 35 nM. A2780 was the most sensitive cell line, followed by WiDr, HT29 and SW620 (Table 1, Figure 1a). Complete growth inhibition was observed over two logs of dFdC dilutions.

The cytotoxicity of the multilayers was expressed as the concentration of dFdC resulting in reduction in OD (reflecting the number of cells) by 50%, when compared with the number of cells in untreated wells (EC_{50}). This effect could only be reached for A2780, for which the EC_{50} was 100 nM. The three colon cancer lines grown as multilayers were quite resistant (Figure 1b). An EC_{50} was not reached at a concentration of 100 µM (WiDr) or even at 1 mM (HT29,

Table 1. Chemosensitivity of mono- and multilayers to gemcitabine (dFdC)

Cell line	IC_{50} mono	EC_{50} multi	Ratio EC_{50}/IC_{50}
A2780	0.006 ± 0.001	0.1 ± 0.04	14
WiDr	0.009 ± 0.002	> 100	> 10 000
HT29	0.026 ± 0.002	> 1000	> 40 000
SW620	0.035 ± 0.003	> 1000	> 30 000

IC_{50} and EC_{50} values (in μM) are means ± standard error of the mean (SEM) of at least three separate experiments.

SW620) [19]. Since the drug was added to the multilayers at d5, relative growth inhibition can be evaluated by comparing the OD at d10 with that at d5. The OD at d5 is approximately 70% of the OD at d10, the day on which the experiment is terminated. Thus, OD values decreasing below the OD of d5 reflect cell kill, whilst a decrease of this OD reflects total growth inhibition. Only a slight growth inhibition was observed for the colon multilayers. At concentrations higher than 1 μM, a plateau was reached for the colon tumour cell lines at the level that equals the amount of cells at the moment of drug addition, meaning no net growth or cell kill

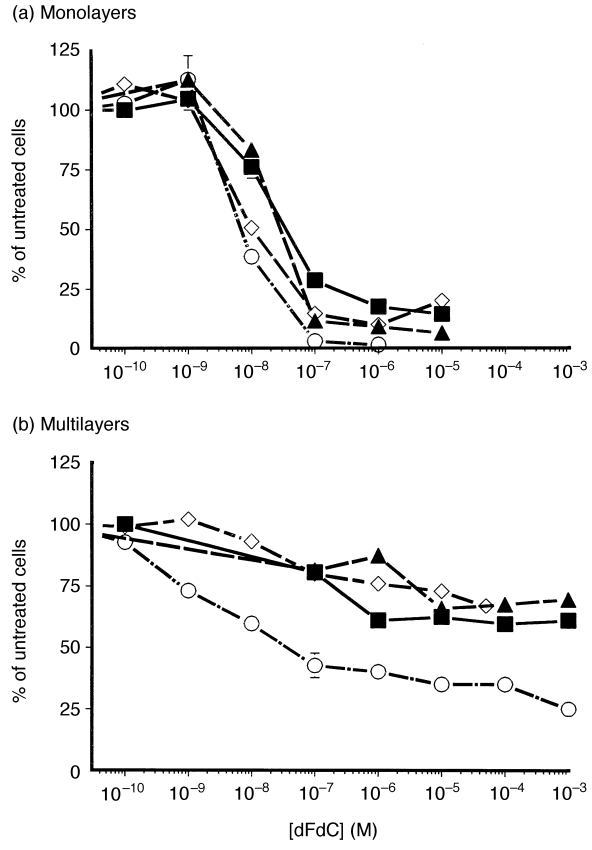

Figure 1. Dose–response curves of four cell lines treated with gemcitabine (dFdC). Cells were exposed for 24 h on (a) day 1 (d1) (monolayers) or on (b) d5 (multilayers). Optical densities from the sulphorhodamine B (SRB) assay were recalculated into percentage of untreated wells (percentage growth without correction for the amount of cells present at the moment of drug addition). One representative experiment of each cell line is shown. The doubling time of the first 5 days is shorter than that of the final 5 days [19]. ■, SW620; ○, A2780; ▲, HT29; ◇, WiDr.

(Figure 1b) [19]. It is clear from Figure 1 that the difference in sensitivity between ovarian and colon carcinoma cell lines is larger in multilayers.

Accumulation

The accumulation of the active metabolite, dFdCTP, was related to the chemosensitivity to dFdC. The steady-state level of dFdCTP was determined after 24 h incubation. dFdCTP accumulation was concentration dependent in all cases, as was expected from previous data. Because of the differences in sensitivity between the ovarian and colon cancer cell lines, we used lower dFdC concentrations for ovarian cancer cells (0.1 and 1 μM) than for colon cancer cells (1 and 10 μM) to study dFdCTP accumulation. In the A2780 cells, no saturation was observed, even at 100 μM [12], but in all colon cancer cells, saturation seemed to be reached at 10 μM, while at 0.1 μM dFdC, dFdCTP was undetectable in our assay (data not shown).

When grown as monolayers, dFdCTP accumulation after exposure to 1 μM dFdC was highest, although not significantly, in A2780, the most sensitive cell line (Table 2). Among the colon tumour lines, WiDr, the relatively sensitive colon cancer cell line, accumulated the highest triphosphate concentrations while SW620 and HT29 contained dFdCTP levels down to 60% of the concentration measured in A2780 cells. At 10 μM dFdC, the highest accumulation was observed in HT29 cells (no data for A2780 for 10 μM).

All multilayered cultures accumulated lower dFdCTP concentrations than the corresponding monolayers after a 24 h exposure to dFdC. The difference was a factor of 2.6 in WiDr (at 1 μM), and varied between 4.9 and 7.7 in the other lines. WiDr multilayers contained the highest accumulated dFdCTP, followed by A2780, SW620 and HT29 (Table 2). There was only a relatively narrow range of variation in the amount of accumulated dFdCTP among these four cell lines. The ratio at 1 μM between the highest and lowest dFdCTP concentration for monolayers was 1.7 (1873/1116) and for multilayers 3.2 (605/187).

Retention

Since clear differences in sensitivity were seen at 1 μM dFdC in multilayers and since the relative retention of

Table 2. Concentration dependency of dFdCTP accumulation in mono- and multilayers of four cell lines

		Concentration of dFdCTP (pmol/10^6 cells)*	
Cell line	dFdC (μM)	Monolayers	Multilayers
A2780	0.1	142 ± 46	46 ± 8†
	1	1873 ± 319	332 ± 64†
WiDr	1	1545 ± 164	605 ± 55†
	10	2919 ± 447	1314 ± 277†
HT29	1	1434 ± 259	187 ± 53†
	10	3999 ± 539	783 ± 174†
SW620	1	1116 ± 89	229 ± 36†
	10	2463 ± 89	789 ± 126†

dFdC, gemcitabine. *Concentration of dFdCTP, means ± standard error of the mean (SEM) of between three and seven experiments, after exposure for 24 h to different concentrations of dFdC. †Significantly different from monolayers with $P < 0.05$.

dFdCTP in these solid tumour cell lines was not different after exposure to 1 or 10 μM dFdC [12], we used 1 μM for the retention experiments (Figure 2). Elimination of dFdCTP in leukaemic cell lines is, however, concentration dependent, but it is monophasic and linear for cells that accumulated intracellularly less than 100 μM dFdCTP (after incubation to 0.3 μM extracellularly) [15, 24]. Cells were first allowed to accumulate the drug for 24 h, then retention was measured 24 h after washing away the drug. Both in monolayers and multilayers, the ovarian cancer cells retained the highest relative dFdCTP levels. In the monolayers, A2780 continued to accumulate dFdCTP until 1.4-fold higher than the level present at the moment of drug removal. This was probably caused by phosphorylation of the intracellular dFdC or dFdCMP reservoir, after the extracellular drug had been washed away and by potentiation of dCK due to disturbances in nucleotide pools [25]. WiDr monolayers contained 81% dFdCTP after a 24 h drug-free period, while SW620 and HT29 retained less of the active metabolite, 42% or 53%, respectively, compared with the level at the moment of drug removal.

The multilayers retained lower levels than the monolayers, both absolute (4–19 times) and relative (Figure 2). Again, A2780 showed the highest relative retention: 70% of the level at the end of 24 h exposure. Relative retention of dFdCTP was lower in all other cell lines, with the highest retention in WiDr multilayers (47%). The SW620 and HT29 multilayers showed the lowest retention, only 27% and 28% was left, respectively.

In comparison with the accumulation data of dFdCTP (1.7-fold ratio between the lowest and the highest accumulation in monolayers), the ratio between the ovarian cancer cell line and the colon cancer line with the lowest dFdCTP concentration was more evident in the retention experiments (4.2–4.9-fold ratio between mono- and multilayers, respectively).

Effect on other nucleotides

The concentrations of normal nucleotide pools were considerably reduced (2–5 times) in multilayers when compared with the corresponding monolayers. dFdC caused major additional changes in these pools in both cell culture systems, which were cell line and concentration dependent.

Most of the measured ribonucleotides (ATP, ADP, UTP, GTP) increased 1.5–3.5-fold after dFdC exposure, both in mono- and multilayers. This was most pronounced for UTP (maximal 4–6-fold). Figure 3 shows the changes in the ATP and GTP pools in two of the colon cancer lines as examples; the other two cell lines showed the same pattern (data not shown).

The only nucleotide not showing a consistent increase was CTP. Exposure to 10 μM dFdC caused a 3–4-fold decrease in CTP pools in the colon cancer cells which was accompanied by a 2–3-fold increase in UTP pools (Figure 4). This is consistent with an inhibition of CTP synthetase. This effect was concentration dependent: at 1 μM dFdC UTP levels also rose 2–3-fold in all cell lines, but no decrease in CTP was observed.

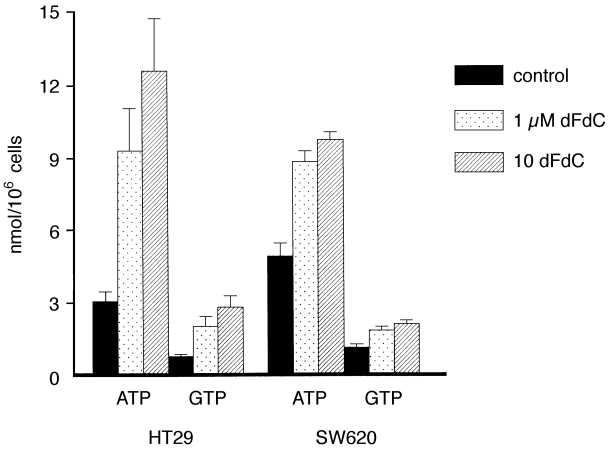

Figure 3. Changes in ATP and GTP pools in monolayered colon cancer cell lines after 24 h exposure to two concentrations of gemcitabine (dFdC). Concentrations of nucleotides are expressed as pmol/10^6 cells and are means ± SEM of at least three experiments.

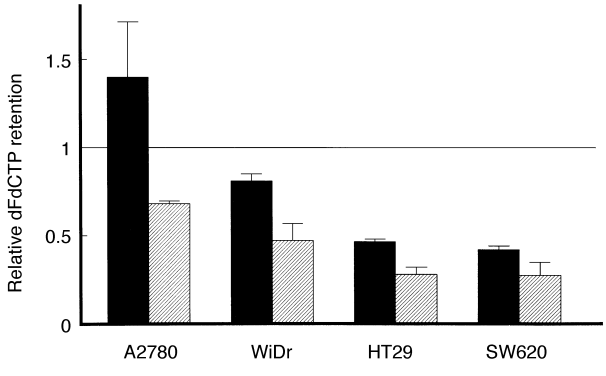

Figure 2. Retention of dFdCTP in mono- and multilayered cell lines. The accumulated dFdCTP concentration at the end of the 24 h exposure is set at 1 for each condition, the relative retention is expressed as a ratio of accumulated dFdCTP ± standard error of the mean (SEM) (data were obtained from at least three separate experiments, see Table 2 for pmol/10^6 cells). Solid bars, monolayers; hatched bars, multilayers. For HT29 and WiDr, the differences between mono- and multilayers was significantly different ($P < 0.05$). The percentage of retained dFdCTP was significantly higher in A2780 cells compared with HT29 and SW620, for both mono- and multilayers.

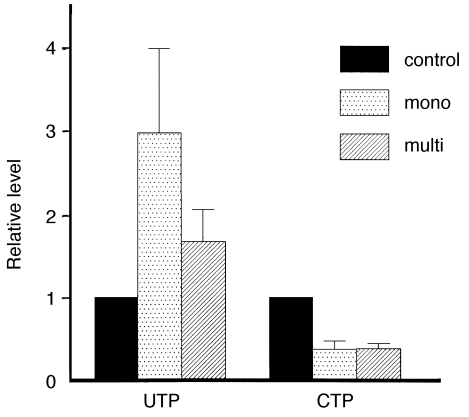

Figure 4. Changes in UTP and CTP pools by 10 μM gemcitabine (dFdC) in mono- and multilayers of the colon cancer cell line HT29. The levels in normal cells (without dFdC) are set as 1. Relative levels are depicted as means ± SEM from between three and five experiments. The absolute values for the control levels were: 1032 ± 165 pmol/10^6 cells for UTP in monolayers and 643 ± 252 pmol/10^6 cells for UTP in multilayers; 285 ± 64 pmol/10^6 cells for CTP in monolayers and 119 ± 58 pmol/10^6 cells for CTP in multilayers.

DISCUSSION

In this paper, we describe the selectivity of a three-dimensional *in vitro* culture system as a model for solid tumours in predicting *in vivo* sensitivity towards the novel antimetabolite dFdC. In the conventional model, with exponentially growing monolayers, ovarian cancer cells were only slightly more sensitive than colon cancer cells. However, in the multilayered cultures, the selectivity of the drug for the ovarian cancer cell line was much more evident, with a 1000-fold higher sensitivity. This selectivity for ovarian cancer cells over colon cancer cells has been described previously in cell lines [12] and reflects sensitivity in both human tumour xenografts and in patients [5, 7]. Human tumour xenografts of ovarian carcinoma and head and neck squamous cell carcinoma were reasonably responsive to various schedules; even complete remissions were seen [3, 4, 26], while colon cancer xenografts were less sensitive [12].

Phosphorylation of dFdC is essential for its biological activity: cells that lack dCK are not affected by dFdC [11, 27]. It is presumed that clinical activity of dFdC resides in the enhanced ability of tumour cells to accumulate and retain dFdC-nucleotides for a long period of time [16]. The amount of cellular dFdCTP was directly correlated with the incorporation of dFdCTP in DNA, a process which was followed by inhibition of DNA synthesis and cell growth inhibition or cell kill [9, 15, 17]. Elimination of the triphosphate in leukaemic cells was concentration dependent [15, 17], as was the accumulation. Gandhi observed saturation of dFdCTP accumulation at 3 h exposure to 10 μM in K562 leukaemia cells [28] and in the colon cancer cell line HT29, accumulation increased up to 10 μM exogenous dFdC [29]. However, this pattern is not universal, as dFdCTP accumulation still increased in other colon cancer cell lines at extracellular dFdC concentrations as high as 100 μM [12]. At higher concentrations, dCK was inhibited by dFdCTP. We also found a concentration dependency in the dFdCTP accumulation (Table 2).

Differences in chemosensitivity can be partly explained by the accumulation and retention of this active metabolite of dFdC in the cells, as was also found by Hertel and colleagues [1]. The clinical importance of the accumulation and retention of ara-C, another cytidine analogue, has been established by Rustum and Preisler [30]. In our study, the highest dFdCTP concentration was observed in A2780 monolayers, although the differences between the four cell lines were not large. More importantly, the A2780 monolayers retained more dFdCTP, when compared with the colon cancer cell lines (Figure 2). The differences were larger than for the initial accumulation experiments. All cell lines were more sensitive to dFdC as monolayers than the multilayers, which is also in line with the higher amount of the active metabolite that was detected in monolayers.

When considering the absolute numbers, the retention of dFdCTP in WiDr multilayers was exceptionally high, which did not result in a sensitive profile. However, dFdC is an S-phase specific drug [1]. Previously we observed that colon cancer multilayers contain a 2-fold lower S-phase fraction than monolayers, which is characteristic for most solid tumours [19]. This lower growth fraction is associated with decreased DNA synthesis, which will limit incorporation of dFdCTP, resulting in resistance. The S-phase fraction of multilayers in the ovarian cancer cell line is even higher than in monolayers. This, in addition to the effect of dFdCTP accumulation and retention, could explain the resistance of the colon cancer multilayers and the relative sensitivity of the ovarian cancer multilayers.

The effect of dFdC on the nucleotide pools was comparable to the data of Ruiz van Haperen and associates [12] who showed the increase of all NTP levels in A2780 after 24 h exposure. A decrease in CTP levels was also found in colon cancer cell lines and in CHO cells after exposure to dFdC concentrations higher than 1 μM, while *in vivo* all nucleotide pools increased [11, 12]. The decrease in CTP pools is accompanied by an increase in UTP pools. This phenomenon can be explained by an inhibition of CTP synthetase by dFdCTP; a subsequent increase in both CTP and UTP reflects a recovery of the cells from this inhibition. Other studies on deoxynucleotides revealed that dATP, dCTP and dGTP pools decrease, while dTTP is less affected [28, 29]. This decrease in dNTPs is held partly responsible for the inhibition of DNA synthesis.

Thus, when relating the sensitivity profile of the monolayers with the clinical response rates, the data do not fit. However, when the same is done with the multilayer data, it could be expected that pharmacologically relevant and achievable dFdC concentrations could inhibit or even kill cell clusters of ovarian cancer cells, while growth of colon cancer cells could only just or not even be stopped. In previous studies we observed that more drugs, such as cisplatin, which are effective against clinical ovarian cancer, were also very active against ovarian cancer cell lines cultured as multilayers [19].

Retrospectively, using the multilayer model we could demonstrate a selectivity of dFdC for ovarian cancer over colon cancer, while this was less obvious with monolayers. This kind of study should be expanded by using more tumour cell types and other established drugs which are in use against solid tumours. Eventually, it is anticipated that this three-dimensional *in vitro* model will have such a predictive value for solid tumour sensitivity that it may be used in the evaluation of which new drugs will or will not be tested further *in vivo*. In that sense, this model can contribute to the reduction or refinement of animal experiments.

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