Cancer Chemother Pharmacol (2006) 58: 219–228
DOI 10.1007/s00280-005-0158-5

ORIGINAL ARTICLE

Bea Pauwels · Annelies E. C. Korst
Hilde A. J. Lambrechts · Greet G. O. Pattyn
Christel M. J. de Pooter · Filip Lardon
Jan B. Vermorken

The radiosensitising effect of difluorodeoxyuridine, a metabolite of gemcitabine, in vitro

Received: 18 July 2005 / Accepted: 14 November 2005 / Published online: 6 December 2005

Abstract *Purpose*: Gemcitabine is an active antitumour agent with radiosensitising properties. Gemcitabine is rapidly metabolised, intracellularly as well as extracellularly, by deoxycytidine deaminase to difluorodeoxyuridine (dFdU), a compound with little antitumour activity. However, plasma concentrations are maintained for a prolonged period (>24 h) at levels known to cause growth inhibition. This is the first study that investigates the radiosensitising potential of dFdU in vitro. *Methods*: ECV304 and H292, human cancer cells, were treated with different concentrations dFdU (0–100 μM) during 24 h before radiation treatment (RT). The schedule dependency of the radiosensitising effect was studied by varying the interval between dFdU and radiation treatment. In addition, the cell cycle effect of dFdU was investigated with flow cytometry, and the induction of apoptosis under radiosensitising conditions was determined by Annexin V staining and caspase 3 cleavage. *Results*: dFdU caused a clear concentration-dependent radiosensitising effect in both ECV304 and H292 cells. Dose enhancement factor (DEF) increased with an increasing concentration of dFdU: DEFs were 1.10, 1.60 and 2.17 after treatment with 10, 25 and 50 μM dFdU, respectively, in ECV304 cells and 1.08, 1.31 and 1.60 after treatment with 25, 50 and 100 μM, respectively, in H292 cells. DEFs decreased with an increasing interval of 0–24 h between dFdU treatment and radiation. Under radiosensitising conditions, the combination dFdU and radiation resulted in an increased induction of apoptosis. In addition, the cell cycle effect of dFdU, an arrest at the early S phase, is comparable with the cell cycle effect of gemcitabine. *Conclusions*: dFdU, the main metabolite of gemcitabine, causes a concentration- and schedule- dependent radiosensitising effect in vitro. Since the metabolite is present in plasma for a long period (>24 h) after treatment with gemcitabine, it might be partly responsible for the interaction between radiotherapy and gemcitabine. This observation might have important consequences for the optimal schedules of the combination gemcitabine and radiation therapy.

Keywords Gemcitabine · Radiotherapy · Difluorodeoxyuridine · Radiosensitisation

B. Pauwels (✉) · A. E. C. Korst · H. A. J. Lambrechts
G. G. O. Pattyn · F. Lardon · J. B. Vermorken
Laboratory of Cancer Research and Clinical Oncology,
Department of Medical Oncology,
University of Antwerp (UA/UZA), Universiteitsplein 1,
2610 Wilrijk, Belgium
E-mail: bea.pauwels@ua.ac.be
Tel.: +32-3-8202576
Fax: +32-3-8202248

C. M. J. de Pooter
Department of Radiotherapy, St Augustinus Hospital,
Oosterveldlaan 24, 2610 Wilrijk, Belgium

Introduction

Gemcitabine (2′, 2′-difluorodeoxycytidine, dFdC) is a synthetic pyrimidine nucleoside analogue which has a structure very similar to that of deoxycytidine and cytosine arabinoside (Ara-C) [44]. In clinical use, gemcitabine is active against a variety of solid tumours such as cancers of the pancreas, lung, head and neck, bladder, breast, and ovary. The prodrug is phosphorylated to its monophosphate form (dFdCMP) by deoxycytidine kinase. Subsequent phosphorylation yields the active metabolites, gemcitabine diphosphate (dFdCDP) and triphosphate (dFdCTP), which target DNA and RNA and are presumably responsible for the cytotoxic effect. dFdCTP is incorporated into DNA and inhibits DNA polymerase processing, whereas dFdCDP interferes with the enzyme ribonucleotide reductase, causing depletion of deoxynucleotide triphosphates necessary for DNA synthesis. In addition, gemcitabine has several self-potentiating mechanisms that serve to increase intracellular levels of the active compound (Fig. 1) [9, 18, 36, 44].

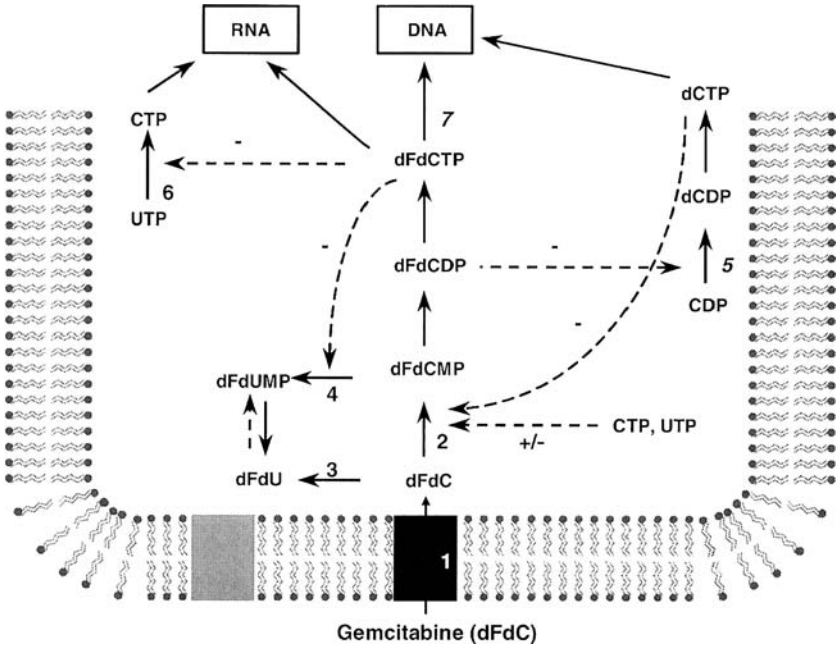

Fig. 1 Metabolism and mechanisms of action of gemcitabine: (*1*) facilitated diffusion human nucleoside transporters (hNT) (*2*) deoxycytidine kinase (dCK) (*3*) deoxycytidine deaminase (dCDA) (*4*) deoxycytidilate deaminase (*5*) ribonucleotide reductase (*6*) CTP-synthetase (*7*) DNA polymerase

In addition to its cytotoxic effect, gemcitabine is a potent radiosensitiser of rodent and human tumour cells in vitro [5, 18, 27, 29, 31, 39–42]. Recent in vivo studies confirmed these observations and have shown significant growth delay with the combination of gemcitabine and ionising radiation in animal models [15, 22, 25]. These results have prompted a variety of clinical trials using gemcitabine as a radiosensitiser [7, 10, 14, 17, 23, 26, 37, 45]. Several investigators have described the radiosensitising effect of gemcitabine in vitro, and studies which have tried to clarify the mechanism(s) of this radiosensitisation have focussed on the changes in cell cycle distribution [5, 18, 28, 34, 38], the induction of apoptosis [20], the role of p53 [5, 29, 35, 38], intracellular levels of dFdCTP [19, 38, 42] and the modulation of the metabolism of deoxynucleotides [19].

An important question is whether catabolites of gemcitabine also have radiosensitisation properties and whether the radiosensitisation observed with gemcitabine in the clinic might be influenced by specific characteristics of these compounds.

Inactivation of gemcitabine occurs through deamination of gemcitabine itself or dFdCMP to the metabolites 2′,2′-difluorodeoxyuridine (dFdU) or 2′,2′-difluoro-2′-deoxyuridine monophosphate (dFdUMP) by the enzymes deoxycytidine deaminase (dCDA) and deoxycytidylate deaminase, respectively (Fig. 1). dFdU is considered to be the main metabolite of gemcitabine. It appears very rapidly in the plasma of patients treated with gemcitabine and, contrary to gemcitabine, remains elevated in the plasma for a prolonged period (> 24 h) at levels known to cause growth inhibition [2]. If indeed dFdU would show radiosensitisation properties this would have major clinical implications with respect to chemoradiation schedules. The present study is the first study that describes the radiosensitising, cell cycle and apoptotic inducing effects of dFdU in vitro.

Materials and methods

Cell lines

The cell lines used in this study were ECV304 (mutant p53), a human bladder cancer cell line [3, 8], and H292 (wild-type p53), a human mucoepidermoid lung cancer cell line. ECV304 was cultured in medium-199 supplemented with 10% fetal calf serum. H292 was cultured in RPMI-1640 medium, supplemented with L-glutamine, sodium pyruvate and 10% fetal calf serum (Invitrogen, Merelbeke, Belgium). Cultures were maintained in exponential growth in a humidified atmosphere at 37°C under 5% CO_2/95% air. The passage numbers of the ECV304 and H292 cells varied for the different cell survival experiments.

Cell survival after treatment with dFdU

Cells were harvested from exponential phase cultures by trypsinisation, counted and plated in 48-well plates. In order to assure exponential growth during the experiments, seeding density was 2,000 cells per well. Following plating and a 24 h recovery period, cells were treated for 24 h with dFdU (0–2,000 μM, kindly provided by Eli Lilly, Indianapolis, USA) dissolved in phosphate buf-

fered saline (PBS; 2.7 mM KCl, 136.9 mM NaCl, 1.5 mM KH_2PO_4, 6.5 mM Na_2HPO_4). PBS was added to control cells. Each concentration was tested six times within the same experiment. After 24 h incubation with dFdU, cells were washed with drug-free medium. Four days after the start of treatment, the survival was determined by the sulforhodamine B (SRB) assay according to the method of Skehan et al. [43] and Papazisis et al. [30] with minor modifications.

Culture medium was aspirated prior to fixation of the cells by addition of 200 µl of 10% cold trichloroacetic acid. After 1 h incubation at 4°C, cells were washed five times with deionised water. Cells were stained with 200 µl 0.1% SRB (ICN, Asse, Belgium) dissolved in 1% acetic acid for at least 15 min and subsequently washed four times with 1% acetic acid to remove unbound stain. The plates were left to dry at room temperature and bound protein stain was solubilised with 200 µl 10 mM unbuffered Tris base [tris(hydroxymethyl)aminomethane] and transferred to 96-well plates for reading the optical density at 540 nm (Biorad 550 microplate reader, Nazareth, Belgium).

Cell survival after treatment with dFdU and radiation

Cells were harvested as described above. Seeding density was 100 cells per well. Following plating and a 24 h recovery period, cells were treated with dFdU (0–100 µM), dissolved in PBS, for 24 h immediately before radiation. PBS was added to control cells and each concentration of dFdU was tested six times within the same experiment. After incubation with dFdU, cells were immediately irradiated at room temperature over a dose range of 0–8 Gy, using a ^{60}Co source (Alcyon, St Augustinus hospital, Antwerp), and subsequently washed with drug-free medium. After 7 days, the survival was determined by the SRB assay as described earlier [31]. The SRB test is a suitable test system for in vitro radiosensitivity testing, provided the assay duration is sufficient for the cells to undergo at least six doubling times after radiation treatment [32].

Treatment schedules

Besides the 24 h treatment immediately before radiation, alternative schedules using different intervals between dFdU treatment and radiation were tested. Cells were incubated with dFdU for 24 h and irradiated immediately or 8 or 24 h later (24 + 0, 24 + 8, 24 + 24). In these experiments, ECV304 cells were treated with 50 µM dFdU, H292 cells with 100 µM dFdU.

Cell cycle effect of dFdU

Cells were harvested from exponential phase cultures by trypsinisation, counted and plated in 6-well plates. To ensure exponential growth during the experiments, seeding densities were 50,000 cells per well for ECV304 and 60,000 cells per well for H292. After plating and a 24 h recovery period, cells were treated with different concentrations of dFdU (0–250 µM) for 24 h. PBS was added to control cells. After trypsinisation, cellular DNA content was determined according to the protocol by Vindelov et al. [46]. In brief, cells were resuspended in 100 µl solution A (trypsin) for 20 min at room temperature. Then, 75 µl solution B (trypsin inhibitor, spermine and ribonuclease A) was added and, after 10 min incubation at room temperature, 75 µl solution C (propidium iodide) was added for at least 30 min at 4°C, while the solution was protected from direct light. Samples were analysed in a FACScan flow cytometer (Becton Dickinson, Belgium).

Determination of apoptosis

Apoptosis was determined by Annexin V staining using flow cytometry and caspase 3 cleaving by Western blotting. The cells were harvested from exponential phase cultures by trypsinisation and plated in 75 cm^2 culture flasks to ensure exponential growth during the experiments. After plating and a 24 h recovery period, cells were treated with their IC50 concentration dFdU during 24 h and immediately irradiated (0, 2 and 6 Gy). After irradiation, cells are washed with drug-free medium. Adherent as well as detached cells were collected for both Annexin V staining and western blot analysis of caspase 3, 72 h after treatment with dFdU and/or radiotherapy.

The cells were evaluated using a commercial kit (Annexin V-FITC apoptosis detection kit I, Becton Dickinson Pharmingen) in accordance with the manufacturer's instructions. Briefly, control and treated cells were washed twice with cold PBS, counted and 1×10^6 cells were collected and resuspended in 1 ml 1× binding buffer (10 mM HEPES/NaOH, 140 mM NaCl, 25 mM $CaCl_2$). Hundred microlitres of this solution was mixed with 5 µl fluorescein isothiocyanate (FITC)-conjugated Annexin V (Annexin V-FITC) and 5 µl propidiumiodide (PI). The cells were gently vortexed and incubated for 15 min at room temperature. Then, 400 µl 1× binding buffer was added. Analysis of green (Annexin V-FITC) and red (PI) fluorescence was performed in a FACScan flow cytometer (Becton Dickinson) within 1 h.

For the detection of cleaved caspase 3, 1×10^6 cells were resuspended in loading buffer [950 µl laemmli sample buffer (Biorad) with 5 µl β-mercaptoethanol] and stored at −20°C until analysis. For each sample, 15 µl was loaded on a 12.5% polyacrylamide gel.

After electrophoresis for 45 min at 200 V, the protein was transferred onto Hybond ECL, nitrocellulose membrane (Amersham Pharmacia Biotech, Roosendaal, The Netherlands) at 100 V during 1 h. Blocking of non-specific binding sites was done by incubation of the membrane with a 5% milk solution in Tris-buffered

saline with Tween-20 (TBST, 10 mM Tris–HCl, pH 8, 75 mM NaCl, 0.05% Tween-20) for 1 h at room temperature, whilst shaking. The membrane was then incubated with a 1/1,000 dilution of cleaved caspase 3 (Asp 175) rabbit IgG antibody (Cell Signalling, The Netherlands) in TBST supplemented with 5% milk, at 4°C with gentle shaking, overnight. Subsequently, the membrane was incubated with a 1/1,000 dilution of anti-rabbit IgG peroxidase - conjugated secondary antibody (Cell Signalling) for 1 h at room temperature. After the primary and secondary monoclonal antibody incubations, the membrane was thoroughly washed in TBST with 1% milk. Antibody detection was performed using the Supersignal West Pico chemiluminescent substrate (Pierce, Erembodegem, Belgium) on the Lumi-Imager (Roche Diagnostics, Vilvoorde, Belgium).

Equal loading of protein was controlled by detection of β-actin expression. After stripping and re-blocking, the membrane was incubated with a 1/5,000 dilution of monoclonal anti-β-actin (Clone AC-15; Sigma, Bornem, Belgium) for 1 h at room temperature, followed by the peroxidase-conjugated rabbit anti-mouse IgG antibody (Sigma). Protein bands were quantified using Lumi-Analyst software (Roche Diagnostics), and caspase 3 quantities were corrected for the amount of β-actin present.

Data analysis

The survival rates were calculated by: mean OD (optical density) of treated cells/mean OD of control cells x 100%. The survival curves after treatment with dFdU alone were fitted according to the sigmoid inhibition model: $\exp(\text{survival}) = 1-(C^{\gamma}/C^{\gamma} + \text{IC50}^{\gamma})$. The radiation survival curves were fitted according to the linear–quadratic model: survival = $\exp(-\alpha D - \beta D^2)$, using Winnonlin (Pharsight, USA).

The following parameters were calculated: IC50, concentration of dFdU causing 50% growth inhibition; the linear component α, dominates at low radiation doses; the quadratic component β, causes the curve to bend at higher doses; ID50, the radiation dose causing 50% growth inhibition; SF_2, the surviving fraction at 2 Gy; and the mean inactivation dose (MID), which was calculated by numerical integration of the linear–quadratic curve [11]. The radiosensitising effect was represented by the DEF: ID50(−dFdU)/ID50(+dFdU).

Unless otherwise indicated, all data are presented as the mean ± standard deviation. The experiments were performed at least three times. A two-sample t test was used to investigate significant differences between ID50 values, α and β values, MID and SF_2.

True radiosensitisation can be defined as a synergistic interaction between dFdU and radiation. For the determination of synergism the combination index (CI) was calculated by the Chou–Talalay equation [6, 21, 31], using CalcuSyn (Biosoft, USA and UK). A CI value between 0.9 and 1.1 indicates only additivity. Moderate synergism is depicted by CI values between 0.7 and 0.9, synergism by CI values below 0.7. A CI > 1.1 indicates antagonism.

Results

Cytotoxicity of dFdU

dFdU alone was found to have a cytotoxic effect on ECV304 and H292 cells. The IC50 was 103.8 ± 9.6 μM and 251.7 ± 24.3 μM for ECV304 and H292, respectively, after treatment during 24 h with different concentrations of dFdU (Fig. 2).

Radiosensitisation by dFdU

To investigate the radiosensitising effect of dFdU, cells were incubated with non- or slightly cytotoxic concentrations, i.e. below the IC50 value. A clear concentration-dependent radiosensitising effect of dFdU was observed in ECV304 and H292 cells (Fig. 3). Radiation parameters and DEFs are shown in Table 1. DEFs increased with an increasing concentration of dFdU. The degree of radiosensitisation was cell line dependent and seemed to correlate with the sensitivity of the cell line to the cytotoxic effect of dFdU. The CI analysis showed that after treatment for 24 h with dFdU before radiation, there is synergism with 25 and 50 μM dFdU (CI was 0.582 and 0.428, respectively) and only an additive effect with 10 μM dFdU (CI was 0.951) in ECV304 cells. In H292 cells, 25 and 50 μM results in additivity (CI was 0.998 and 0.904, respectively). There is moderate synergism with the highest concentration (CI was 0.701).

The radiosensitising effect of dFdU was observed at the initial part of the dose–response curve. This was shown by an increasing α value of the linear – quadratic model ranging from 0.14 to 0.35 for ECV304 and from 0.23 to 0.44 for H292 cells. However, the increase in α value was not statistically significant.

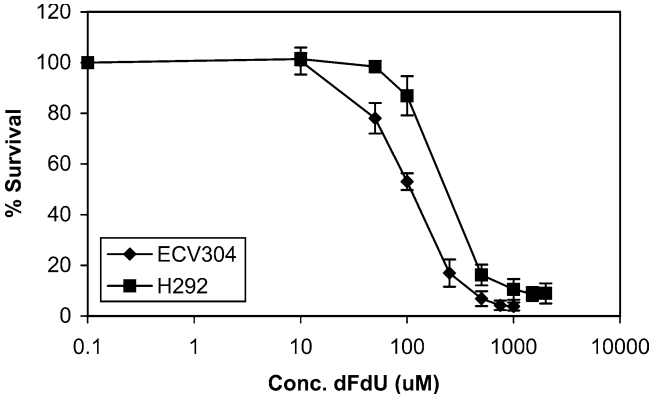

Fig. 2 Dose response curves of ECV304 and H292 cells after 24 h treatment with dFdU

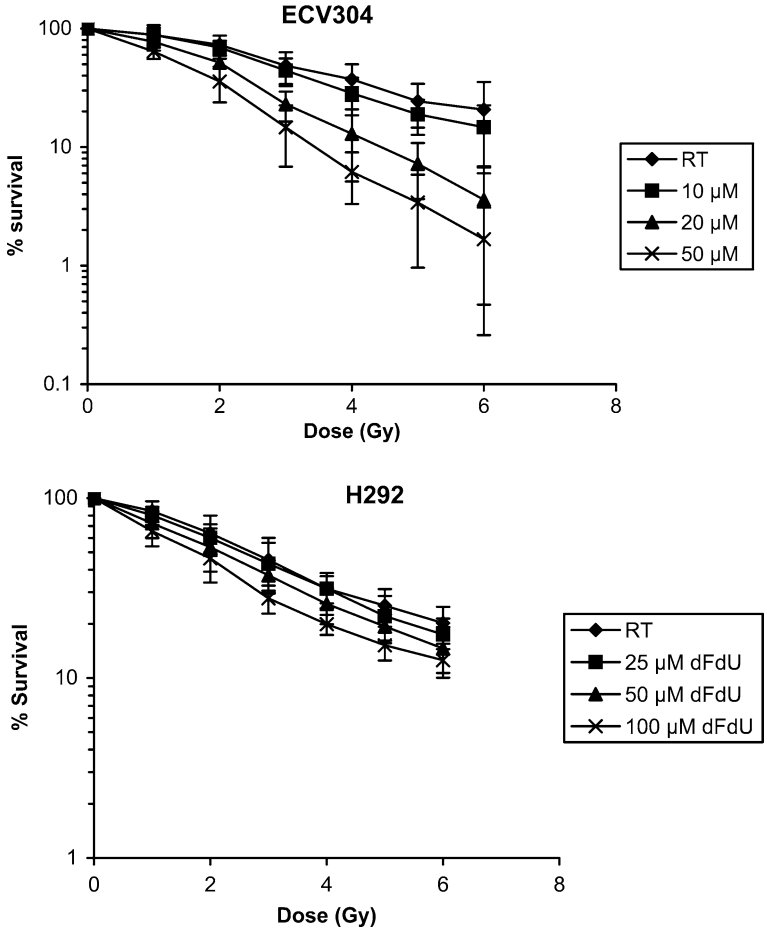

Fig. 3 Radiation dose–response curves of ECV304 and H292 cells after radiation alone and after treatment with dFdU for 24 h before radiation. Experiments were performed at least three times, and the results are expressed as mean ± standard deviation

Schedule dependency of the radiosensitising effect of dFdU

A clear schedule-dependent radiosensitising effect of dFdU was observed in ECV304 and H292 cells (Fig. 4). Radiation parameters and DEFs for the different treatment schedules are summarised in Table 2. In ECV304 cells, dFdU treatment had a significant influence on the ID50, α, MID and SF_2 in comparison to radiation alone using the three treatment schedules. In H292 cells, only when the cells were treated for 24 h immediately before radiotherapy (24 + 0), dFdU treatment had a significant influence on the ID50, α, MID and SF_2 in comparison to radiation alone.

The radiosensitising effect decreases with a longer interval between chemotherapy and radiation; the DEF decreases with an interval of 8 or 24 h (Table 2). However, the three treatment schedules resulted in a synergistic interaction in ECV304 cells (CI was 0.433, 0.478 and 0.679 with 24 + 0, 24 + 8 and 24 + 24, respectively). In H292 cells, synergism was observed with the treatment schedules 24 + 0 and 24 + 8 (CI was 0.650 and 0.694, respectively). Only a moderate synergistic interaction was observed with a 24 h interval between gemcitabine and radiotherapy (CI was 0.817).

Influence by dFdU on the cell cycle

In Fig. 5, the effect of different concentrations of dFdU on the cell cycle is shown. The cells were treated during 24 h with concentrations less than or equal to their IC50 value. dFdU causes a block of cells in the S phase of the cell cycle in both ECV304 and H292 cells. This cell cycle effect is clearly concentration dependent. Besides an increase in the amount of S phase cells with the lowest concentrations, the arrest also seemed to occur earlier in the S phase with increasing concentrations of dFdU, near to the G_1/S border with the highest concentrations.

The radiosensitising effect was maximal, when the cells were irradiated immediately after a 24 h treatment with dFdU, as shown above. At that moment, most of the cells were in S phase (Fig. 5). The greatest DEFs were observed when most cells were blocked in the early S phase of the cell cycle.

Induction of apoptosis by dFdU and radiation

To evaluate the induction of apoptosis after the combination of dFdU and radiotherapy, ECV304 and H292 cells were treated with the IC50 concentration (100 and

Table 1 Radiosensitisation of ECV304 and H292 cells by dFdU

ECV304	RT alone	RT + 10 μM	RT + 25 μM	RT + 50 μM
ID50 (Gy)	3.2 ± 1.1	2.9 ± 0.7	2.0 ± 0.4	1.5 ± 0.4[a]
α (Gy^{-1})	0.130 ± 0.157	0.109 ± 0.104	0.178 ± 0.098	0.346 ± 0.156
β (Gy^{-2})	0.031 ± 0.021	0.048 ± 0.009	0.089 ± 0.013[a,b]	0.098 ± 0.028[b]
MID (Gy)	3.6 ± 0.1	3.2 ± 0.6	2.2 ± 0.3	1.7 ± 0.4[a]
SF_2 (%)	69.5 ± 16.1	67.1 ± 12.0	49.7 ± 9.1	34.8 ± 10.4[a,b]
DEF		1.1 ± 0.1	1.6 ± 0.3	2.1 ± 0.3
H292	**RT alone**	**RT + 25 μM**	**RT + 50 μM**	**RT + 100 μM**
ID50 (Gy)	2.8 ± 0.8	2.6 ± 0.8	2.2 ± 0.7	1.7 ± 0.5
α (Gy^{-1})	0.203 ± 0.156	0.236 ± 0.131	0.334 ± 0.175	0.440 ± 0.210
β (Gy^{-2})	0.021 ± 0.017	0.015 ± 0.016	0.010 ± 0.018	0.014 ± 0.028
MID (Gy)	3.3 ± 0.7	3.2 ± 0.6	2.8 ± 0.7	2.1 ± 0.5[b,c]
SF_2 (%)	62.6 ± 14.8	59.7 ± 12.1	50.8 ± 14.4	40.8 ± 13.4
DEF		1.1 ± 0.1	1.3 ± 0.1	1.7 ± 0.4

Mean values ± standard deviation of radiation parameters and DEF after radiation therapy alone or after prior treatment with various concentrations of dFdU in ECV304 and H292 cells. For abbreviations, see text
[a] $P < 0.05$ vs 10 μM
[b] $P < 0.05$ vs RT
[c] $P < 0.05$ vs 25 μM

250 μM for ECV304 and H292, respectively) dFdU for 24 h, immediately before radiotherapy (0, 2 and 6 Gy). Apoptotic cell death was determined by Annexin V staining (Fig. 6) and caspase 3 cleavage (Fig. 7), 72 h after treatment.

For both ECV304 and H292 cells, the amount of early apoptotic cells (Annexin V positive) cells increases with the combination of dFdU and radiotherapy (Fig. 6). More apoptotic cells were observed with a higher radiation dose. In ECV304 cells this was significantly different, while in H292 cells this was not the case. The induction of apoptosis under radiosensitising conditions of dFdU therefore seemed also cell line dependent.

The induction of apoptosis was confirmed by caspase 3 cleavage shown by Western blot.

Discussion

In the current study, a clear concentration- and schedule-dependent radiosensitising effect of dFdU, the main metabolite of gemcitabine, was observed in the human cancer cell lines ECV304 and H292. In addition, this radiosensitisation resulted in an increase of apoptotic cell death. dFdU also caused an early S phase block, comparable with the cell cycle effect of gemcitabine, which is an important factor for the radiosensitising effect.

Table 2 Schedule dependency of the radiosensitising effect of dFdU in ECV304 and H292 cells

ECV304	RT alone	24 + 0	24 + 8	24 + 24
ID50 (Gy)	5.0 ± 0.8	1.8 ± 0.6[a]	2.1 ± 0.2[a]	3.0 ± 0.3[a,b,c]
α (Gy^{-1})	0.059 ± 0.030	0.338 ± 0.190	0.214 ± 0.082	0.191 ± 0.044[a]
β (Gy^{-2})	0.017 ± 0.008	0.038 ± 0.037	0.055 ± 0.055	0.015 ± 0.013
MID (Gy)	5.6 ± 0.9	2.2 ± 0.6[a]	2.6 ± 0.4[a]	3.8 ± 0.5[b,c]
SF_2 (%)	83.1 ± 4.6	44.9 ± 13.2[a]	52.5 ± 4.6[a]	64.4 ± 2.9[a,c]
DEF		2.8 ± 0.4	2.4 ± 0.4	1.7 ± 0.1
H292				
ID50 (Gy)	4.0 ± 1.4	2.1 ± 0.6	2.6 ± 0.7	3.4 ± 0.6[b]
α (Gy^{-1})	0.146 ± 0.051	0.374 ± 0.110[a]	0.278 ± 0.102	0.188 ± 0.086
β (Gy^{-2})	0.013 ± 0.008	0.001 ± 0.002	0.007 ± 0.006	0.011 ± 0.013
MID (Gy)	4.7 ± 1.5	2.7 ± 0.8	3.2 ± 0.8	4.0 ± 0.8
SF_2 (%)	71.5 ± 9.5	47.9 ± 10.5[a]	56.4 ± 10.9	66.0 ± 8.8
DEF		1.9 ± 0.1	1.6 ± 0.3	1.1 ± 0.3

Mean values ± standard deviation of radiation parameters and DEF after radiation therapy alone or after prior treatment of dFdU using different treatment schedules in ECV304 and H292 cells. ECV304 cells were treated with 50 μM, H292 with 100 μM. For abbreviations, see text
[a] $P < 0.05$ vs RT
[b] $P < 0.05$ vs 24 + 0
[c] $P < 0.05$ vs 24 + 8

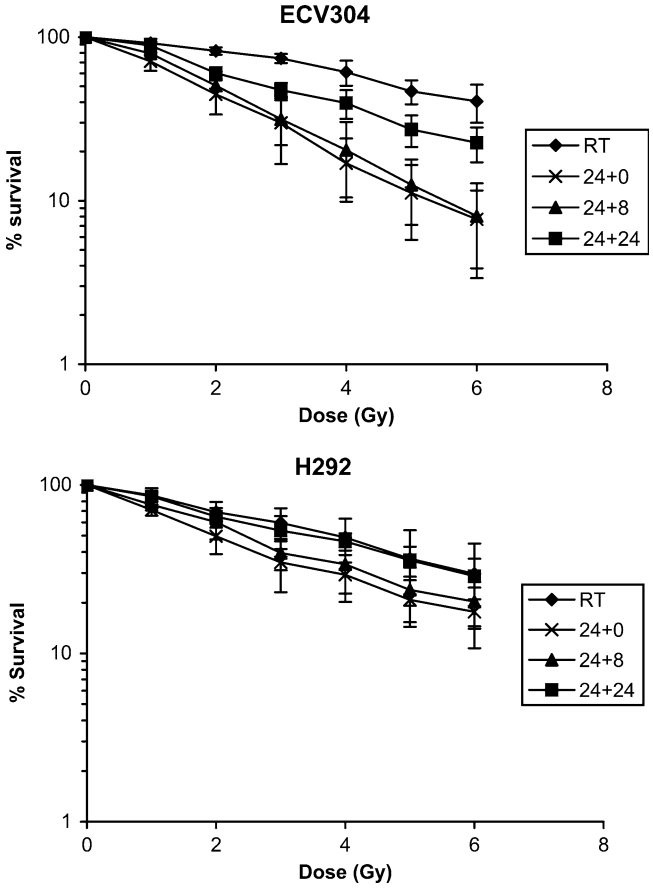

Fig. 4 Radiation dose–response curves of ECV304 and H292 cells after radiation alone and after treatment with dFdU using different schedules. ECV304 cells were treated with 50 μM and H292 with 100 μM dFdU

The clinical importance of this metabolite is still unclear. dFdU is usually considered an inactive metabolite of gemcitabine because of its much lower antitumour activity. dFdU was found to be 1,000-fold less active than gemcitabine in the ovarian cancer cell line A2780 cells [2]. In our study, the IC50 of dFdU was even 3×10^4 times higher than that of gemcitabine (100 μM dFdU vs. 3.1 nM dFdC for ECV304 and 250 μM dFdU vs. 8.0 nM dFdC for H292) [31]. In patients, dFdU is rapidly formed in plasma. Peak dFdU concentrations (C_{max}) are generally observed 5 – 15 min after the end of a gemcitabine infusion and are proportional to the given dose. These C_{max} values ranged from 45 to 188 μM after 1,000 mg/m^2 of gemcitabine in the phase I study described by Abbruzzese et al. [1]. We found a C_{max} of 89.8 ± 10.8 μM with 800 mg/m^2 of gemcitabine [16], which is in line with that finding. The median $t_{½}$ α of dFdU in Abbruzzese et al.'s study was 27 min. However, in contrast to gemcitabine, the terminal elimination phase of dFdU was quite long (range 2.5–24 h). We observed 24 h after 800 mg/m^2 of gemcitabine, concentrations of dFdU ranging from 9.9 to 30.4 μM [16]. Although higher concentrations were needed to obtain a cytotoxic effect in the cell lines we used for our experiments, Bergman et al. [2] indicated that these concentrations are sufficient to cause growth inhibition.

The combination of gemcitabine and radiotherapy has been associated with a marked increase in toxic effects in clinical studies. Therefore, standard doses of gemcitabine cannot be administered in combination with radiotherapy. Several investigators reduced the gemcitabine dose to below 500 mg/m^2 [4, 10, 14, 47]. Abbruzzese et al. [1] published peak plasma concentrations of dFdU ranging from 3 to 99 μM after treatment of patients with 53 to 525 mg/m^2 gemcitabine. Since the radiosensitising effects of dFdU in our study were observed using concentrations which can be reached in plasma of patients treated with doses of gemcitabine less than 500 mg/m^2, they might play a role in the interaction between gemcitabine and radiotherapy as observed in clinical studies.

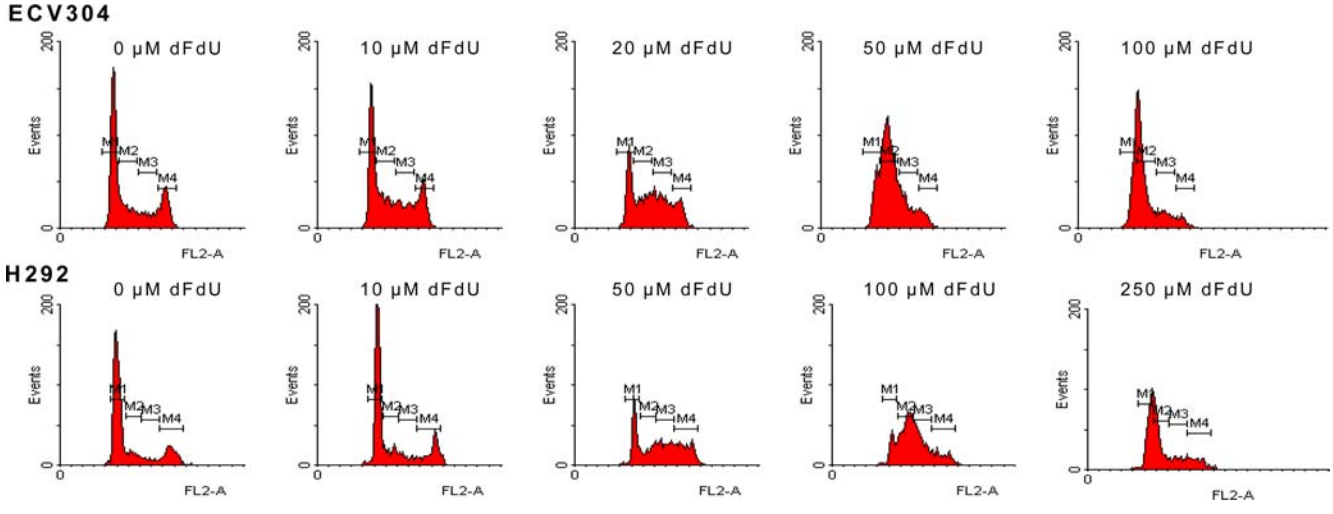

Fig. 5 Cell cycle analysis of ECV304 and H292 cells after treatment with different concentrations dFdU during 24 h. Flow cytometry was performed at the end of dFdU treatment after DNA staining with propidium iodide (M1 = G1, M2 = early S, M3 = mid S, M4 = G_2/M)

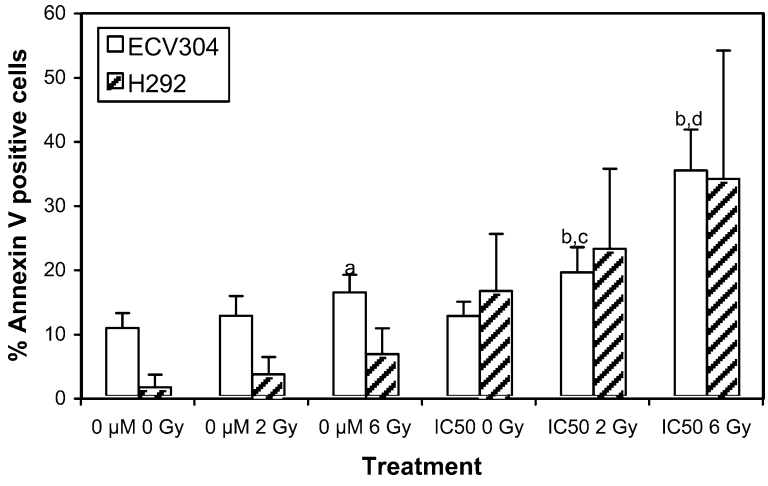

Fig. 6 Percentage of early apoptotic cells (Annexin V positive) 72 h after treatment with the combination dFdU and radiation. [a]$P < 0.05$ vs 0 µM–0 Gy, [b]$P < 0.05$ vs IC50-0 Gy, [c]$P < 0.05$ vs 0 µM–2 Gy, [d]$P < 0.05$ vs 0 µM–6 Gy

The schedule dependency we observed in the radiosensitising effect of dFdU is quite comparable with the schedule dependency we observed in the radiosensitising effect of gemcitabine [34]. Both gemcitabine and dFdU have the same effect on the initial part of the radiation dose–response curves resulting in an increase of the α value.

The cytotoxic effect of gemcitabine is a result of an induction of apoptosis [12, 13]. We and others have shown that the combination of gemcitabine and radiation induces increased apoptosis. However, this effect was clearly cell line dependent [20, 33]. The present study indicates that this also seems to be the case for the combination of dFdU and radiation. As was seen with gemcitabine [31, 33], the apoptotic induction with dFdU and radiation was most pronounced in the most sensitive cell line (ECV304), with at the same time the greatest radiosensitising effect. The absolute contribution of apoptosis in the toxic and radiosensitising effect of dFdU is therefore a matter of further research.

In previous studies, it has been shown also that gemcitabine's effect on the cell cycle plays an important role in its radiosensitising effect [28, 34]. Gemcitabine causes an arrest in the early S phase of the cell cycle [18, 29, 34]. This cell cycle effect was concentration dependent. Low doses of gemcitabine ($<$IC50 value) caused an accumulation of cells in S phase. Increased concentrations of gemcitabine ($\pm$ IC50 value) resulted in a shift of this arrest to early S phase and finally a blockade of cells at the G_1/S border [24, 34]. The cell cycle effect of dFdU is quite the same as the cell cycle effect of gemcitabine. The lowest concentrations used only resulted in an accumulation in S phase. Higher dFdU doses blocked the cells earlier in S phase and the IC50 doses caused an arrest at the G_1/S border. This cell cycle arrest of dFdU could also play a role in its radiosensitising effect. However, the importance of this phenomenon requires further investigation.

In conclusion, dFdU, the main metabolite of gemcitabine, caused a clear concentration- and schedule-dependent radiosensitising effect in vitro and might be partly responsible for the interaction between radiation and gemcitabine observed in vivo. Since concentrations of dFdU in the plasma of patients treated with gemcitabine remain for a long period ($>$24 h) at a level at which radiosensitisation occurs, this observation might have important consequences for optimal chemoradiation scheduling, both in terms of efficacy and toxicity.

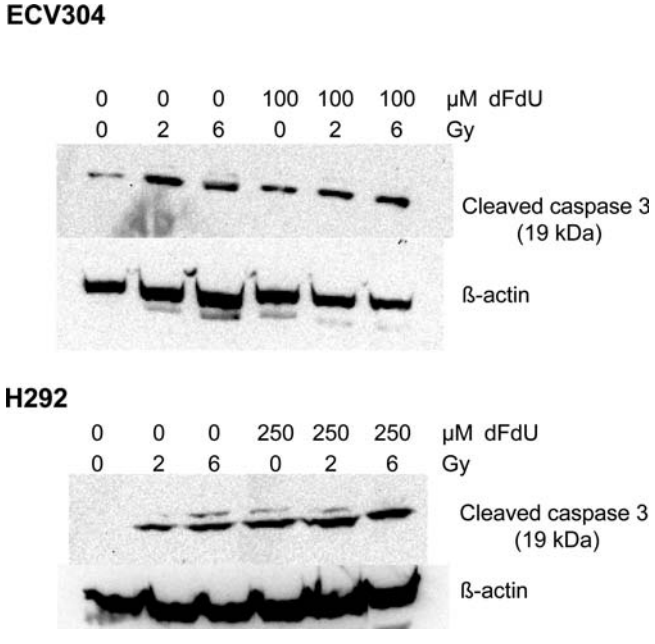

Fig. 7 Western blot of cleaved caspase 3, 72 h after treatment of ECV304 and H292 cells with the IC50 concentration of dFdU and radiation

Acknowledgements The authors wish to thank Dr. Paul Meijnders and Dr. Gerard Van den Aardweg for carefully reviewing this manuscript. This study was financially supported by a grant from the foundation Emmanuel van der Schueren.

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