Gemcitabine Treatment of Experimental C6 Glioma: The Effects on Cell Cycle and Apoptotic Rate

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Abstract. *Gemcitabine (dFdCyd) is a deoxycytidine analogue showing a broad spectrum of cytotoxic activity; additionally, at non-cytotoxic concentrations, it is a potent radiosensitiser. Its in vitro and in vivo effects were studied on C6 rat glioma. In vitro, dFdCyd induced an increase in S-phase cells at the end of treatment, with a reduction in G1 and G2 cell cycle-phase cells and relevant effects on the apoptotic rate. The in vivo effects of dFdCyd were studied on rats bearing intracranial C6 glioma. The drug was administered at a dose of 120 mg/Kg every 3 days x 4 doses. A significant effect on tumour growth was detected by longitudinal MRI analyses. Furthermore, the drug induced an inhibitory effect on tumour growth, 72 h after a 300 mg/Kg single dose. Analyses performed on tumour specimens showed relevant G1-phase accumulation and about 45% apoptotic cells. The present results justify further studies to determine the potential efficacy of dFdCyd in the treatment of malignant gliomas.*

Malignant glioma is considered to be one of the most aggressive tumours, leading to death within a few months. Cytoreductive surgery followed by radiotherapy (RT) has been considered the standard first-line treatment; systemic chemotherapy (CT) has produced limited clinical benefit. The administration of new cytotoxic drugs, with different modes of action, even concurrent with RT, represents a promising approach, taking advantage of the radiation-sensitising effects of certain chemotherapeutic agents, with the aim of improving local tumour control. Among these drugs, nucleoside analogues such as gemcitabine (2',2'-difluoro–2'–deoxycytidine, dFdCyd) could play an interesting role.

Gemcitabine is a deoxycytidine analogue that has shown a wide range of antitumour activity against different solid tumours (1-8). After transport into the cell, dFdCyd requires intracellular activation through phosphorylation by deoxycytidine kinase, representing the dose rate-dependent step in this process. Two major pathways of cytotoxic activity have been shown:

a. formation of the triphosphate, directly inhibiting DNA synthesis and interfering with DNA elongation;

b. formation of the diphosphate, which inhibits ribonucleotide reductase, depleting biosynthesis of the deoxyribonucleoside triphosphate precursors (9-11).

In addition, dFdCyd has been proven to be a powerful radiation sensitiser at non-cytotoxic concentrations, both in experimental and clinical studies. On this basis, several phase I/II studies are currently being designed on different solid tumours with concurrent dFdCyd and radiation therapy.

The mechanisms of radiation sensitisation have not been completely clarified, but the activation of the apoptotic machinery is believed to be most relevant, depending on tumour cell lines. Most probably, dFdCyd induces cell cycle redistribution into the S-phase, causing tumour cells to accumulate in a more radiosensitive cell cycle phase, and inhibits the repair of DNA damage caused by radiation, leading to increased cell death (12-18). The influence of dFdCyd activity on cell kinetics was also explored in different cancer cell lines, in an attempt to explain its mode of action in relation to key cellular events (19); recent data actually showed early S-phase arrest that was associated with the accumulation of phosphorylated checkpoint kinases (20). Few data are currently available on the effects of

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dFdCyd in primary malignant brain tumours. At clinically relevant concentrations, gemcitabine cytotoxic activity was documented in human malignant glioma cell lines (21); on the contrary, unsatisfactory results with the use of dFdCyd as a single agent were obtained in limited clinical studies (22, 23). Nonetheless, interesting data were recently published on the potential role of dFdCyd as a radio-sensitiser in the treatment of brain metastases (24).

The aims of the present study were to investigate the *in vitro* and *in vivo* effects of dFdCyd on C6 rat malignant gliomas, evaluating, by flow cytometry, the *in vitro* effects on cell cycle phase distribution and induced apoptosis, and observing the growth inhibition induced on C6 brain tumours implanted in Wistar rats by sequential MRI analyses. Furthermore, cell cycle phase distribution and apoptotic rates were analysed by flow cytometry on dissected C6 brain tumour specimens.

Materials and Methods

In vitro

Cells: The polyploid C6 rat cells were maintained as monolayer culture in complete RPMI medium with 10% heat-inactivated FCS.

Gemcitabine (dFdCyd) sensitivity was determined by selecting the dose required to inhibit 50% of cell growth (IC_{50}). Cells ($2x10^4$) were plated in 24-well plates and, after 24 h, were exposed to different doses of dFdCyd (0.5, 1, 2.5, 5, 7.5, 10 nM). Following 24 h of continuous exposure, the viable cells were harvested and counted using the Trypan blue exclusion test. Cell survival in the treated cells was calculated as a percentage of the control. The data are representative of three different experiments.

Cell cycle analysis: Cell cycle analysis was performed after bromodeoxyuridine (BrdU; Sigma, St. Louis, USA) incorporation, as previously described (25). The BrdU was given 30 min before dFdCyd exposure, to prevent dFdCyd interfering with BrdU incorporation into DNA. Briefly, BrdU pulse labelling was performed before dFdCyd treatment, by adding 10 μM BrdU to the medium for 30 min. The cells were then harvested at the end of treatment, as well as 24 and 48 h after drug washout. They were fixed in 70% ethanol and, after rinsing in cold PBS, incubated with 4N HCl for 20 min at room temperature, to partially denature DNA. The cells were washed once in borax-borate buffer (pH=9.1) to neutralise the acid pH and once in PBS. The cells were then permeabilised by incubation with complete medium plus 20% FCS and 0.5% Tween-20 for 10 min. Next, the samples were incubated with mouse monoclonal antibody anti-BrdU (Roche Diagnostics, Milan, Italy) in complete medium containing FCS 20% and 0.06% Tween-20 (Calbiochem, San Diego, USA) at room temperature for 1 h. After washing in PBS, the cells were exposed to FITC-conjugated F (ab') rabbit anti-mouse (Dako, Glostrup, Denmark) in PBS for 1 h. Finally, after washing in PBS, they were stained with a solution containing 5 μg/ml propidium iodide (PI) and 75 KU/ml Rnase in PBS for at least 3 h. Twenty thousand events/sample were acquired using a FACScan cytofluorimeter (Becton Dickinson, Sunnyvale, USA). The analysis was performed using a Cell Quest software package.

The percentages of cell cycle distribution were estimated on linear PI histograms by applying the MODFIT software, a mathematical model able to discriminate two populations with different DNA contents.

Apoptosis: Apoptosis was analysed by flow cytometry (FCM) using the TUNEL assay, as previously described (26). Briefly, cells were fixed in 4% paraformaldehyde, permeabilised in 0.1% Triton X-100 in 0.1% sodium citrate and washed with PBS. Each sample was incubated in 50 μl of reaction mixture (terminal deoxynucleotidyl transferase, TdT and fluorescein-dUTP) for 1 h at 37°C, washed in PBS and then measured by flow cytometry using a FACScan cytofluorimeter (Becton Dickinson).

In vivo

Tumours: The experiments were performed after stereotactic injection of $3x10^5$ C6 tumour cells into the right caudate nucleus of male Wistar rats of approximately 180-200 g (6 to 7 weeks old). The animals were anaesthetised with intraperitoneal (*i.p.*) administration of Equitesin (chloral hydrate 4.2%, sodium penthobarbital 1%), 4.0 ml/Kg. All the procedures involving rats and their care were performed in our animal house (Aut. Min.San. Decreto 104/2003-B, 04-09-2003) in agreement with institutional guidelines in compliance with national and international laws and policies (European Community Council Directive 86/109,OJL 358, 1-12-1987 and the NIH Guide for the Care and Use of Laboratory Animals).

MRI. The analyses were performed at 4.7 T on the VARIAN INOVA SIS 200/183 system (Varian, Palo Alto, USA). MR images of the tumour area were performed after *s.c.* administration of gadopentetate dimeglumine (Gd-DTPA) 3.0 ml/Kg with T1-weighted multi-slice spin-echo sequence (TR/TE=500/16 ms; NS =4; slice thickness 1.0 mm). The tumour volume was evaluated after each MRI study adopting a dedicated image browser program (Varian); the area of interest was manually defined on each single slice and the total volume automatically measured; two separate measurements were performed, blind, by two independent investigators.

Drug preparation. For the *in vitro* experiments, pure dFdCyd hydrochloride was reconstituted in physiological saline and diluted in the culture medium. For the *in vivo* experiments, dFdCyd was reconstituted in physiological saline and diluted at a concentration of 30 mg/ml in the single-dose experiment and at 12 mg/ml for the four-dose experiment.

Treatments. Two series of experiments with different drug administration schedules were utilised: 120 mg/Kg every 3 days x 4 doses, or 300 mg/Kg single-dose. The drug was *i.p.* injected. The treatment was initiated when the tumour volume, determined by MRI, was in the range of between 10 and 120 mm³, usually on eleventh to thirteenth day of tumour growth. The experiments were performed by selecting pairs of animals with similar starting volumes, one for the control and one for the treated arm.

In the four-dose schedule trial, the potential antitumour effect was evaluated, in five consecutive experiments, comparing the time-course of tumour volume increase in the treated (n=30) with respect to the control (n=25) animals, measured by sequential MRI examinations performed before and every 48 h after the treatment.
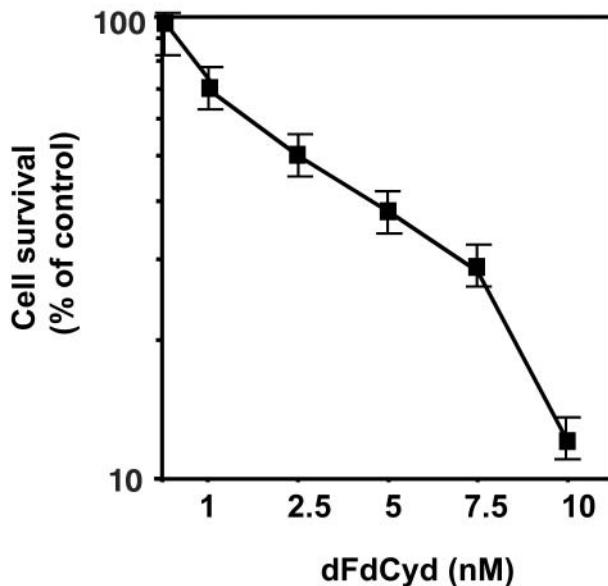
Figure 1. *dFdCyd IC$_{50}$ (dose inhibiting 50% of cell growth) in the C6 rat glioma cell line. Cell survival is reported as a percentage of control. The values are means (bars, SE) of triplicate samples.*

Figure 2. *Flow cytometric bivariate analysis of untreated and dFdCyd-treated C6 cells after BrdU labelling and propidium iodide staining. 2N represents the diploid and 4N the tetraploid population. A), B), C) cytograms of control cells and D), E), F) of dFdCyd-treated cells, at the end of treatment, 24 and 48 h later, respectively. The straight line in each panel separates BrdU-stained cells from unstained cells.*

For the single-dose treatment, the evaluation of dFdCyd activity was performed by MRI measurements of tumour volume, before and 72 h after treatment.

Cell cycle analysis and apoptosis. Seventy-two h after single-dose-treatment, the rats were sacrificed and the brain tumours were minced carefully with sharp blades. The cell cycle and apoptosis analyses were performed by FCM. The cell suspensions obtained from untreated and dFdCyd-treated tumours were fixed in 70% ethanol and stained with a solution containing 50 μg/ml PI and 75 KU/ml Rnase in PBS for 1 h.

Statistical analyses. Paired and unpaired *t*-tests were used to compare cell cycle phases and tumour volumes. The results were considered significant at *p* level 0.05 (27).

Results

Effects of dFdCyd on cell cycle and apoptosis in vitro. The sensitivity of C6 rat glioma cells to dFdCyd was determined by selecting the dose required to inhibit 50% of cell growth (IC$_{50}$). The C6 rat glioma cells showed relatively high sensitivity to dFdCyd, with an IC$_{50}$ of 2.5 nM (Figure 1). All subsequent *in vitro* experiments were conducted using this dose.

In order to better investigate the mechanism of cell growth inhibition induced by dFdCyd on C6 rat glioma cells, the cell cycle perturbations were studied by DNA-BrdU incorporation and using flow cytometry. Cell exposure to dFdCyd for 24 h impaired DNA synthesis in the C6 cells, as
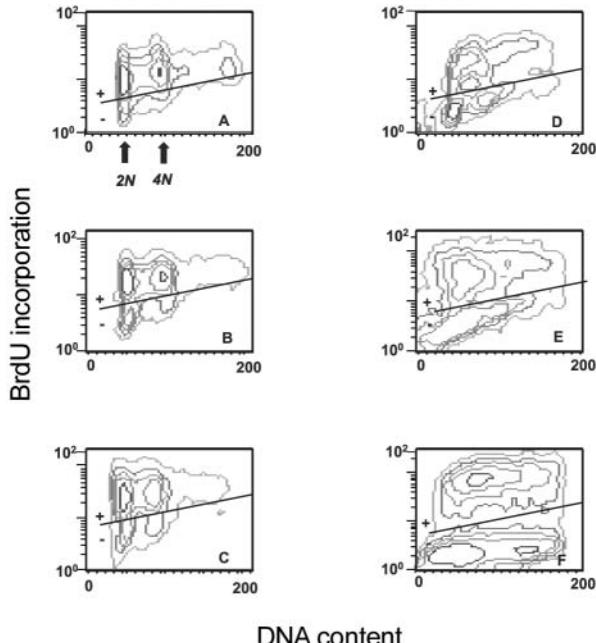
BrdU incorporation was completely inhibited at the end of treatment (data not shown). However, in order to prevent dFdCyd interfering with BrdU incorporation into DNA, a 30-min pulse of BrdU was given before cell exposure to dFdCyd. In this way, the S-phase cells were labelled before dFdCyd induced inhibition of DNA synthesis and the fate of the S-phase cells could be followed afterwards (Figure 2). Analysis of the cytograms clearly showed the presence of two different cellular populations, one diploid (DNA content *2 N*) and the other tetraploid (*4 N*). DFdCyd impaired the progression of the cells through the cell cycle. Even after 24 h of treatment, accumulation of BrdU-labelled cells was evident at the S-phase position (Figure 2 D). The control cells showed, however, the typical cell cycle distribution of exponentially-growing cells, with the presence of BrdU-positive cells at the G1-phase, indicating that the cells proceeded through the cell cycle, completing cell division and re-appeared at the G1-labelled position (Figure 2 A). In order to follow the fate of the S-phase cells, the cell cycle distribution was analysed at 24 and 48 h after dFdCyd washout; 24 h after dFdCyd treatment, tumour cells did not proceed through the S-phase. In addition, an increase of cells in the G1-phase was observed, suggesting that a fraction of cells were able to repair DNA damage (Figure 2 E). The

Table I. *Effect of dFdCyd on the cell cycle distribution of C6 rat glioma cells.*

	Time after treatment (h)	Cell cycle phases (% ± SE)					
		2N			4N		
		G1	S	G2	G1	S	G2
control	0	46.5±2.3	43.7±2.1	9.8±0.5	42.1±2.1	46.6±2.3	11.3±0.6
	24	60.1±3.0	33.6±1.7	6.3±0.3	59.9±2.9	31.0±1.6	9.1±0.5
	48	74.5±3.7	22.5±1.1	3.0±0.1	70.0±3.5	23.2±1.6	6.8±0.34
dFdCyd	0	37.8±1.9*	61.4±3.0*	0.8±0.04*	33.4±1.6*	61.9±3.0*	4.7±0.2*
	24	67.4±3.3	30.8±1.5	1.8±0.09*	65.5±3.2	31.4±1.5	3.1±0.2*
	48	76.4±3.8	12.5±0.6*	11.1±0.5*	71.2±3.5	28.2±1.4*	0.6±0.03*

FCM cell cycle analysis of untreated and dFdCyd-treated C6 rat glioma cells at the end of 24-h treatment (time 0) and 24 and 48 after the drug washout. The cell cycle phase percentages were estimated by applying the MODFIT software to the PI/DNA histograms. The data are the means ± SE of three separate experiments with similar results.
*significant difference ($p \leq 0.05$) between treated *vs.* control tumour cells.

control cells still showed the presence of BrdU-positive cells in the G1 position, indicating that the cells proceeded normally through the cell cycle (Figure 2 B, C). These effects were still evident at 48 h after the end of dFdCyd treatment (Figure 2 F). Moreover, the cell fractions at the sub-G1 region, indicative of apoptosis, were also detected 24 and 48 h after the end of dFdCyd treatment, suggesting that at least a fraction of the S-phase-arrested cells, unable to repair DNA damage, underwent apoptosis without proceeding through the cell cycle.

The DNA content histograms were analysed by MODFIT software with the aim of evaluating the percentages of cells in each phase of the cell cycle, albeit conditioned by the presence of two different cell populations (Table I). According to this analysis, the dFdCyd-treated cells significantly accumulated in the S-phase at the end of treatment, with concomitant significant reduction in the G1- and G2-phases in both cell populations, as compared to the control cells. The S-phase population had decreased by 24 h after drug washout, while a fraction of the cells repopulated the G1- and G2-phases, indicating repair of the DNA damage. This was also evident 48 h after drug washout.

The cytotoxic effect produced by dFdCyd in the C6 cells was confirmed by the TUNEL assay, displaying the involvement of apoptosis in cell killing induced by the drug (Figure 3). No apoptosis was observed in the cell culture at the end of the 24-h dFdCyd treatment, whereas approximately 30% of apoptotic cells were found at 24 h after drug washout and about 45% at 48 h.

Effects of dFdCyd treatment on in vivo glioma. MRI sequential evaluations allowed us to determine the profile of brain tumour growth (28). Rats were usually examined
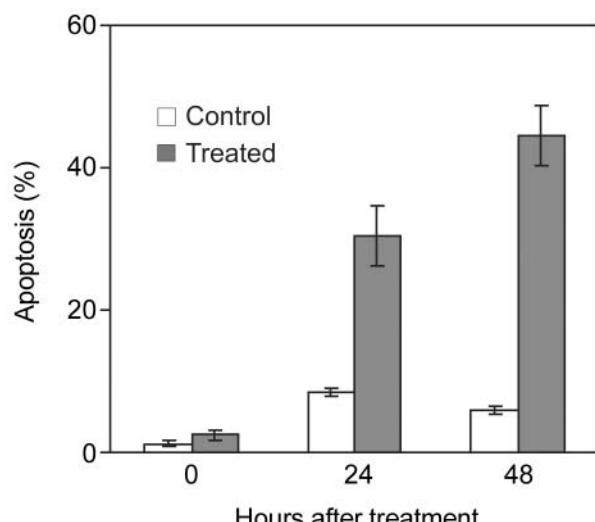

Figure 3. *dFdCyd-induced apoptosis in the C6 rat glioma cell line at the end of treatment (time 0) and 24 and 48 h after drug removal. Apoptosis was evaluated by the TUNEL assay. dUTP-FITC fluorescence was reported as a percentage of the FITC-fluorescent cells. The values are means (bars, SE) of three separate experiments. Grey columns represent treated cells; empty columns control cells.*

on the eleventh to thirteenth day from tumour cell inoculation; in this period, measurable tumours were detected regularly (29). Considering the animals' lifespan, dFdCyd did not determine any statistically significant difference in the survival curves of treated with respect to control animals; nevertheless, in one single-treated animal, complete remission of a voluminous tumour mass was observed, with consistent extension of the total survival.
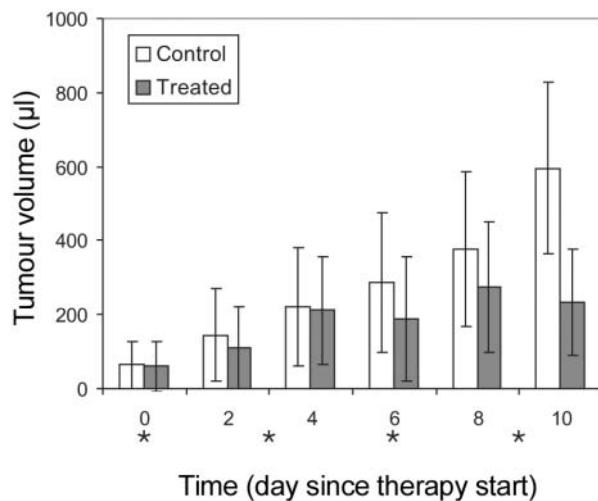
Figure 4. *Differences in C6 tumour volumes, evaluated with sequential MRI analyses, between control (white boxes) and treated (grey boxes) tumours. dFdCyd administration (120 mg/Kg every 3 days for four doses) is indicated by stars.*

Figure 5. *Contrast-enhanced coronal T1W MRI of typical rat C6 brain tumours. (A) Tumour on day 13 of growth and (B) 72 h after a single dose of dFdCyd. (C) Similar-sized control tumour, on day 13 of growth, and (D) 72 h after.*

In the first series of experiments, tumour growth was monitored during a four-dose schedule (120 mg/Kg every 3 days x 4), measuring, with sequential MRI examinations, absolute tumour volume, as well as incremental tumour growth in the treated and control animals. The starting tumour volumes were not statistically different in the control (n=23) and treated (n=25) arms [mean value±SE: 64.7±13.2 *vs.* 60.8±13.6 (*p*=0.84)]. Significant reduction in the absolute tumour volume of the treated *vs.* control group was documented at 72 h after second-dose administration [*t6*] (*p*=0.03) (Figure 4). Furthermore, a significant difference in tumour growth was detected between the initial observation [*t0*] and t6 (*p*=0.004) and analogous effects were observed even when considering tumour volume after the first administration [*t2*] and at t6 (*p*=0.05). Considering incremental tumour growth from the initial to the final MRI measurements [*t10*], a significant effect of dFdCyd on tumour growth was observed (233.7±50.8 *vs.* 596.1±104.3) in this setting (*p*=0.005). (Figure 4)

The second series of experiments was carried out after single-dose administration of dFdCyd (300 mg/Kg); MRI analyses were performed before and 72 h after treatment (Figure 5) The initial tumour volumes were not statistically different among the treated and control groups [mean value ± SE=56.2±8.0 *vs.* 57.5±8.1 (*p*=0.91)]. A significant difference in the volumes was, however, observed in the treated animals after 72 h [(100±16.7 *vs.* 140±22.2) (*p*=0.03)], confirming the relevant activity of dFdCyd in our tumour model, even after single-dose administration (Table II).

In this setting, the cell cycle analysis was performed on dissected brain tumour specimens, obtained 72 h after
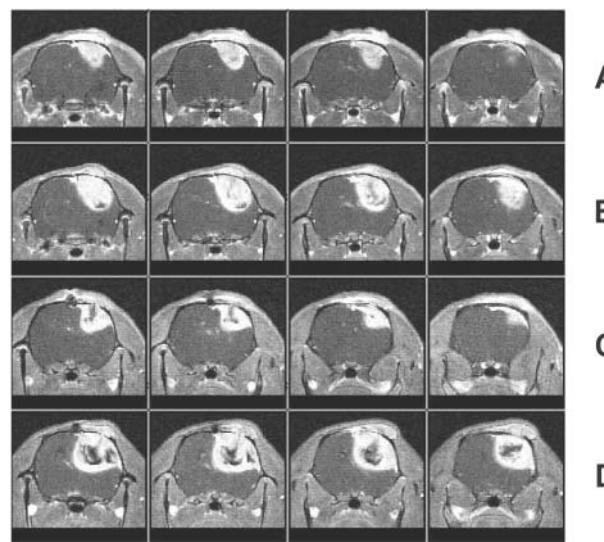
Table II. *C6 tumour volume μl before and after single dose of gemcitabine treatment.*

Animal group (#)	Before	After
Control (17)	56.2±32.9	140±91.4
Treated (19)	57.5±35.3*	100±72.8**
	p=0.91	**p*=0.03

#number of animals

Tumour volume was evaluated with MRI examinations performed before and 72 h after treatment with a single dose administration of dFdCyd (300 mg/Kg).

treatment both in eight treated animals and in eight controls. Cell cycle analysis showed a relevant G1 increase in the treated arm, associated with concomitant depletion of the S and G2 cell populations (Table III). These variations were significant in the *4N* population (*p*≤0.006), suggesting higher sensitivity in the former. As already observed in C6 cell cultures, the substantial decrease in S-phase was associated with the presence of a significantly higher apoptotic rate (*p*<0.001), with more than 45% of apoptotic cells being detected by TUNEL assay on fresh tumour biopsies.

Discussion

Malignant gliomas represent a heterogeneous group of aggressive brain tumours with a gloomy outcome, despite serious efforts to influence the natural course of this disease

Table III. *C6 tumour cell cycle and apoptosis before and after single-dose gemcitabine treatment.*

Animal group (#)	Cell cycle phases (%±SE)						Apoptosis (%)
	2N			4N			
	G1	S	G2	G1	S	G2	
Control (5)	76.0±3.8	20.1±1.0	3.9±0.2	66.9±3.3	26.7±1.3	6.4±0.3	6.5±0.3
Treated (5)	81.6±4.1	16.7±8.3	1.7±0.1*	87.4±4.4*	8.4±0.4*	4.6±0.2*	47.0±2.3*

FCM cell cycle and apoptosis analyses in C6 brain tumour tissues obtained from Wistar rats treated with a single-dose dFdCyd administration (300 mg/Kg). The analysis was performed 72 h after treatment. The cell cycle phase percentages were estimated by the MODFIT software on the PI/DNA histograms. Apoptosis was evaluated as the fraction of cells in the sub-G1 region. The data are representative of three separate experiments with similar results. The values are means±SE of five rats.
*significant difference ($p \leq 0.05$) between treated *vs.* control brain tumour tissue.
#number of animals.

with combined modality treatments (30, 31). Many new drugs are currently under study, also with the aim of adopting concomitant chemo-radiation treatment strategies.

Few data are available in the literature concerning gemcitabine activity on primary brain tumours, without any significant survival advantage of utilising different schedules having been found (22, 23). Nevertheless, its radiosensitising properties at non-cytotoxic concentrations, as well as its tolerability, make dFdCyd a possible candidate for clinical trials of concurrent radio-chemotherapy in malignant glioma (32).

Rat brain tumour models can supply relevant information for the evaluation of novel therapeutic approaches, even if the currently utilised model fails to emulate human malignant glioma growth. Moreover, non-invasive tumour imaging with sequential MRI examinations enables us to follow tumour growth, as well as to detect the effects of candidate tumour drugs. C6 glioma has been criticised as being immunogenic in different immunocompetent rat systems, favouring misleading evaluation of the eventual *in vivo* therapeutic activity. In the present study, we report analyses of dFdCyd activity, carried out both *in vitro* on C6 glioma cells and *in vivo* on rat brain tumours obtained after C6 tumour cell implantation. The *in vitro* and *in vivo* results were constantly compared, utilising dissected brain specimens.

The cell cycle analyses on C6 tumour cells documented the presence of two different populations, diploid and tetraploid, with specific sensitivity to the action of dFdCyd; the drug was active on both cell populations, with a prevalent effect on the tetraploid cells.

At the end of treatment with dFdCyd, treated cells accumulated in the S-phase, with concomitant reduction in the G1- and G2-phases. The S-phase population decreased by 24 h after drug washout, while a fraction of cells was present in the G1-phase, indicating possible repair of the DNA damage induced by dFdCyd treatment. This recycling was also more evident by 48 h after drug washout, when the cells apparently proceeded through the S- and G2-phases. Moreover, the increase of apoptosis, detectable 24 and 48 h after the end of treatment, suggested that a fraction of the S-phase arrested cells were unable to repair DNA damage, with consequent activation of their apoptotic machinery. Similar data on a delayed detection of the maximum rate of apoptotic cells were also reported by Milas *et al.* and Cappella *et al.*, documenting a relevant and prolonged perturbation of cell cycle progression (15, 20).

As described by Pauwels *et al.*, different dFdCyd-induced cell cycle modulations, mainly the S-phase arrest and/or blockade of cells at the G1/S border, were observed depending on the exposure time and dose schedule (18). These cell cycle perturbations were considered to be a possible explanation for the dFdCyd-radioenhancing activity; the inhibition in cell cycle progression could favour cell synchronisation after the end of drug activity, with the result of potentially increasing the sensitivity of brain tumour cells to radiation, by exposing these cells in more radiosensitive phases, and reducing the ability for DNA sub-lethal damage repair. Other authors suggested that the synchronised progression of cells through the S-phase, after dFdCyd exposure and radiation treatment, could result in an increase of pro-apoptotic signals and, consequently, interfere with the potential lethal damage repair/fixation pathway, finally resulting in cell death (13, 16).

In order to evaluate whether similar effects could be observed in the *in vivo* brain tumour system, we utilised both MRI evaluations on measurable C6 brain tumours and flow cytometric cell cycle analyses on dissected C6 tumour specimens. The MRI examinations detected a significant

reduction in tumour volume, with two different drug administration modalities. Sequential MRI analyses, performed after a more effective and conventional four-dose schedule (33), displayed significant incremental tumour volume growth delay and a significant difference between the final tumour volumes. Cell cycle analyses on brain tumour specimens confirmed the *in vitro* data, showing a significant alteration in cell cycle progression, with a selective decrease of the S-phase cell population. dFdCyd activity was also confirmed by evaluating the apoptotic rate; the TUNEL assay showed more than a 45% increase in apoptosis.

These data indicate that, in the present experimental model, dFdCyd treatment could have a significant effect on brain tumour growth, through relevant perturbation of the tumour cell cycle phases and a greatly increased apoptotic rate. Although the radiosensitising effect of dFdCyd seems to represent a multifactorial, time- and dose-dependent process, the role of the cell cycle modulations observed in our experimental brain tumour model could support the potential interest in associating dFdCyd and fractionated radiation therapy in the clinical setting. The recent phase I study by Maraveyas *et al*. documented the potential role of dFdCyd as a radiosensitiser in the combined management of brain metastases and the safety of a twice-weekly schedule (24).

On this basis, further studies utilising other brain tumour cell lines endowed with different apoptotic machinery are warranted, as well as new phase I-II clinical trials utilising dFdCyd as a radioenhancing drug concurrent with radiation therapy. The preliminary clinical results obtained in other solid tumours and brain metastases, associating dFdCyd with radiotherapy, justify the interest in this therapeutic association, with the aim of delineating new and more active combined treatment strategies against human malignant gliomas.

Acknowledgements

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