In vitro studies on radiation effects and radiosensitization: HDR vs LDR and cytotoxic agents
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Chapter Four:

Enhancement of effects of irradiation by gemcitabine in a glioblastoma cell line and cell line spheroids

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Abstract

Background and Purpose: To determine the cytotoxicity of, and radioenhancement by gemcitabine on a glioma cell line grown as a monolayer and as spheroid cultures. Material and Methods: We used a human glioma cell line, Gli-6, which originated from a biopsy specimen of a patient with a glioblastoma multiforme. Spheroids of Gli-6 were prepared by seeding a single cell suspension on agarose coated petri dishes. Clonogenic and growth delay assays were used to determine radio-chemosensitivity of monolayer cultures. The growth delay assay was used to determine that of Gli-6 spheroid cultures. Results: Spheroid cultures were found to be more resistant to irradiation with or without gemcitabine than monolayer cultures. Whereas gemcitabine significantly enhances the radiation effect of exponentially growing Gli-6 monolayer cultures at minimal cytotoxic concentrations (10 nM, 24 hours), no enhancement was seen in confluent monolayer cultures and in large spheroids at the same concentration. In small spheroids no enhancement was observed at a low dose gemcitabine (10 nM for 24 hours), but an enhancement was observed at higher concentrations (100 nM for 24 hours). Conclusion: Gemcitabine can lead to enhancement of the effects of X-irradiation in both monolayer as spheroid glioblastoma cultures. The lack of enhancement in confluent monolayer cultures supports the view that cell cycle distribution of cells is important in radiosensitisation by gemcitabine.
Chapter 4

Introduction

Malignant gliomas are among the most radioresistant tumours (Taghian et al., 1993). Although radiotherapy following surgical resection is the most effective treatment, survival in these patients is unsatisfactory (González González & Hulshof 1993, Hulshof et al., 2001). The median survival is only 10-12 months for glioblastoma multiforme (Prados & Wilson 1997). Radiotherapy is considered the most effective adjuvant therapy to surgery. The modest increase in survival time after radiotherapy treatment has been ascribed to the high intrinsic resistance of the gliomas to X-irradiation.

To improve survival in patients with malignant glial tumours new treatment strategies are required. Further approach includes chemotherapeutic agents as an adjuvant modality or in combination with radiation as a radiosensitiser.

Several different culture models have been used to determine the intrinsic radiosensitivity of gliomas. These include monolayer cultures of glioma lines, both early and late passage after initial isolation, and spheroids derived from these cell lines, the so-called cell line spheroids (reviewed in Taghian et al., 1993). Another culture system used to study gliomas in vitro are the organotypic multicellular spheroids (OMS) (Kaaijk et al., 1995). It is assumed that spheroid cultures can better predict the in vivo response than monolayer cultures, since cell-cell contact, variation in cell cycle, altered metabolism and diffusion of nutrients or drugs may influence the outcome (Kunz-Schunghart 1999, Santini et al., 1999).

A promising cytostatic drug is gemcitabine (2, 2'-difluoro deoxycytidine [dFdC]). Gemcitabine is a nucleoside analog of cytidine with significant cytotoxicity and a radiosensitizing effect on solid tumour cell lines in vitro and in vivo (Arning & Blatter 1997, Mason et al., 1999, Plunkett et al., 1995, Van Bree et al., 2002). In the clinic gemcitabine is mainly used in the treatment of non-small cell lung cancer and adenocarcinoma of the pancreas. Two in vitro studies have reported cytotoxic and radiosensitizing effects on cultured malignant glioma cells (Rieger et al., 1999, Ostruska & Shewach 2000).

Phase II clinical trials in glioblastome multiforme which studied the effect of gemcitabine as a first line therapy before radiation or as a salvage therapy at first relapse (Gertler et al., 2000; Weller et al., 2001) did not demonstrate a survival improvement. The radiosensitizing effect of gemcitabine has not been evaluated clinically.

In this study we used the Gli-6 glioma cell line cultured as monolayers and as spheroids to determine the cytotoxic and radiosensitizing effect of gemcitabine.
Materials and Methods

Monolayer cell culture
The Gli-6 cell line which originates from a biopsy specimen of a patient with glioblastoma multiforme (Fehlauer et al., 2000), was grown in Dulbecco’s modified Eagle’s medium (DMEM, Gibco BRL, U.K) supplemented with 10% fetal calf serum, 2 mM L-glutamine and 50 μgram/ml gentamycin (Gibco BRL, U.K.) in 25cm² flasks in a 37°C, 5 % CO₂, 95% humidified air incubator. Monolayer cultures were subcultured once a week. The doubling time of the Gli-6 during exponential growth was 36 hours. Prior to irradiation or gemcitabine treatment, cells were trypsinized and plated in appropriate dilutions.

Cell Line Spheroids
Spheroid cultures of Gli-6 were formed by liquid-overlay technique (Carlsson&Yuhas 1984). In short, exponentially growing monolayers were trypsinized and 8x10⁵ cells were seeded in a complete medium into 60 mm petri dishes pre-coated with a thin layer of 3 % agarose solution (Sigma St. Louis, USA), mixed in 1:1 ratio with DMEM supplemented with 20% FCS and 4 mM Glutamine. After 1 to 2 days, cells started to aggregate and after 2 to 3 days, well-rounded, regularly shaped spheroids were formed. After 5-7 days spheroids were transferred and cultured individually in agarose-coated wells of 48-well plates with 500 μl complete culture medium. The culture medium was changed once a week. Treatment groups consisted of 10 spheroids with diameters (Ø) of 250-500 μm (small: Ø 250-400 μm, volume 0.01-0.03 mm³; large Ø 400-500 μm, volume 0.03-0.06 mm³). The volume doubling time of spheroids was 5-6 days.

Irradiation
Cells were irradiated with ¹³⁷Cs at a dose rate of 0.8 Gy/min. Two ¹³⁷Cs sources, one above and one below the petri dish were positioned at a distance of 22.5 cm. Lead flattening filters were used to ensure a homogeneous dose distribution throughout the culture flask.

Gemcitabine treatment and cytotoxicity
Gemcitabine was kindly provided by the Netherlands branch of Eli Lilly&Co, Indianapolis, Indiana, U.S.A) Stock solutions of 1 mM dFdC in PBS were made and kept at -20°C and diluted with medium to appropriate concentrations at the start of the incubation. For
gemcitabine cytotoxicity experiments culture medium was removed and replaced by complete medium containing gemcitabine.

The cytotoxic effect (IC50 value) of gemcitabine on exponentially growing monolayer cultures was determined by MTT assay. The cytotoxic effect of gemcitabine on spheroids was determined after a 24 hours incubation with an increasing concentration of dFdC (in the range of 1-333 nM) with growth delay.

For experiments combining gemcitabine with irradiation, exponentially growing culture were incubated with 10 nM gemcitabine for 24 hours. Confluent monolayer cultures were incubated with 10 nM and 100 nM gemcitabine for 24 hours, spheroid cultures were incubated with 10 nM, 33 nM and 100 nM. At the end of gemcitabine incubation, medium-containing drug was removed and the cells or spheroids were washed with PBS and fresh medium was added. At this point spheroids were transferred to a fresh agarose coated 48-well culture plate.

**Clonogenic Assay**

Cell survival of monolayer cultures after irradiation with or without gemcitabine was determined by clonogenic assay. In brief, for exponentially growing monolayer cultures 2.5 x10⁶ cells were seeded into 60 mm petri-dishes and incubated for 72 hours prior to irradiation. For confluent monolayer cultures 10⁶ cells were seeded into 60 mm petri dishes and incubated until they reached confluence before irradiation (3-4 days). After irradiation, cells were were trypsinised and replated in appropriate dilutions in six-well culture plates (Costar). After 2 weeks, the plates were stained with the solution of 0.05% crystal violet and 6% of glutaraldehyde. Colonies of 50 cells or more were scored as originating from a single clonogenic cell.

The plating efficiency of exponentially growing Gli-6 cells was 13 ± 9 %, the plating efficiency after gemcitabine treatment was 10 ± 9 %. For confluent cultures the control plating efficiency was 47 ± 16 % while after gemcitabine treatment the plating efficiency was 47 ± 12 % for 10 nM and 20 ± 12 % for 100 nM. Surviving fractions (S(D)/S(0)) after dose D, were corrected for toxicity of gemcitabine alone. Analysis of the survival curves was performed using BMDP statistical software (Los Angeles, USA), by fitting the data by multiple regression according to the linear-quadratic model.
Growth delay assay

Growth delay was used to determine the response of spheroids and monolayer cultures of Gli-6 cells to radiation and/or gemcitabine. In our study the comparison between monolayer cultures and spheroid cultures was based on a growth delay assay, because no viable cells could be obtained from the spheroids by trypsination.

For monolayer cultures 2.5 x 10^4 cells were seeded in 60 mm petri dishes to assess for growth delay. After incubation of 72 hours monolayer cultures were irradiated. Growth curves were established by counting cell numbers 2 to 3 times a week until they reached plateau phase. Twice a week the culture medium was changed.

To assess growth delay in cell line spheroids, the spheroids were measured 2-3 times a week by using a CCD-Camera (Sound Vision SV micro) and the computer program Image Pro Plus 4.1.00 for Windows 95/98, 1999. Once a week, the culture medium was changed.

Doubling times and growth delay values were obtained from a linear slope fit to the linear part of the volume time curves obtained from the control and treated spheroids and monolayer cultures. Regression analysis was performed to estimate the doubling time and growth delay. In order to measure growth delay, the average time required to reach 10 times the initial spheroid volume or for monolayer cultures to reach the double amount of cells, was determined. Specific growth delay (SGD) values were calculated by dividing growth delay of treated samples by the corresponding doubling times of control samples.

Flowcytometric analysis of cell cycle distribution

Cells were plated and treated as described above. At different time points after initial plating, 10 μM bromodeoxyuridine (BrdU) was administered from a 100x stock. After 2 hours the cells were harvested, fixed in 70% ethanol in phosphate buffered saline (PBS) and stored at -20°C until immunofluorescent staining. Ethanol-fixed cells were centrifuged (1 min, 2200 rpm), resuspended in 1 ml pepsin solution (0.4 mg/ml 0.1N HCl) and incubated for 30 min at room temperature. Subsequently, the DNA was denatured by a 30 min incubation in 1 ml 2N HCl at 37°C. After washing with PBTb (PBS, Tween-20 0.05% v/v, bovine serum albumin (Sigma) 20 mg/ml, pH 7.4, the pellet was resuspended in 0.1 ml anti-BrdU (Harlan Seralab LTD, Loughborough, UK, diluted 1:100 in PBTb) and incubated at room temperature for 30 min. After washing with PBTg (PBS, Tween-20 0.005 v/v, normal goat serum (Dako, Glostrup, Denmark) 1% v/v, pH 7.4), the pellet was resuspended in 0.1 fluorescein conjugated goat-anti-rat IgG (Jackson, nr 112-015-102, West Grove, Pennsylvania, USA, diluted 1:100 in PBTg) and incubated at room temperature in the dark for 30 min. Propidium-iodine and
ethanol were added to an end-concentration of 1 μg/ml and 30% respectively. Samples were stored at 4°C until flow cytometric analysis. Samples were syringed through a 21 gauge needle to reduce cell aggregation before flow cytometry (FACScan cytometer, Becton Dickenson, San Jose, CA). The distribution of cells over the cell cycle was analysed with Windows Multiple Document Interface Flow Cytometry Application (WinMDI) by placing windows around G0/G1, S and G2/M populations.
Results

Radio sensitivity
The radiosensitivity of exponentially growing monolayer cultures was assessed both by clonogenic assay and the growth delay assay. For spheroids only data on growth delay were obtained as we were unable to obtain single cell suspensions of treated spheroids. Figure 1a.b show the growth delay in monolayer and spheroid cultures after ionizing radiation. After a dose of 10 Gy the survival was 0.2 per cent (%), corresponding with a specific growth delay of 7.1 days. Growth delay and specific growth delay (sGD) for monolayer cultures and spheroid cultures are shown in figure 1c and 1d. From the curves on specific growth delay a Dose Modification Factor (DMF) of 2.5 for spheroids versus monolayer cultures can be derived at an iso-effect level of the sGD of 6 days.

Gemcitabine cytotoxicity

*MTT Assay:* The cytotoxicity of gemcitabine in exponentially growing cells was measured with MTT assay. Gemcitabine showed significant toxicity with steep dose-response curve at nanomolar concentrations when measured with clonogenic assay. IC50 value was 30 nM for 24 hours of incubation. Gemcitabine cytotoxicity appeared to be dependent on both concentration and incubation time. As the drug concentration increased the incubation time necessary to induce a certain amount of cytotoxicity decreased. Incubation of cells for 6 hours induced a biphasic cytotoxicity with an IC50 value of about 200 nM.

*Growth Delay Assay:* Assessment of gemcitabine cytotoxicity by the growth delay assay, showed that spheroids were more resistant than monolayer cultures. Whilst Gli-6 monolayer cultures showed complete growth arrest at 100 nM, the growth delay of small spheroids at the same concentration was only 1.2 day.
Figure 1. Radiosensitivity of Gli-6 monolayer cultures (A) and spheroid cultures (B) measured with a growth delay assay and corresponding relation between radiation dose and growth delay (C) or specific growth delay (D). Figures A and B show a typical experiment.
Enhancement of the effects of radiation by Gemcitabine

Monolayer Cultures: In figure 2 clonogenic survival after irradiation alone or combined with gemcitabine is shown for monolayer exponentially growing and confluent cultures. Cell cycle distribution at the time of irradiation in the exponentially growing cultures was: S-phase 36 ± 1.3 % and G0/G1 phase 55 ± 3.5 %, and in confluent cultures: S-phase 13 ± 4 % and G0/G1 phase 69 ± 2.5 %.

The SF2 value for radiation alone in exponentially growing cells was 0.64±0.27, whilst for gemcitabine (10 nM) treated cells the SF2 value was 0.44 ±0.18. The SF2 value (irradiation alone) for confluent cultures was 0.49 ±0.07, whereas at 10 nM and 100 nM of gemcitabine the SF2 values were 0.47±0.1 and 0.55 ±0.2 respectively. Incubation of exponentially growing cultures for 24 h with 10 nM gemcitabine resulted in a clear enhancement of 1.9 on the α-value (factor determining the initial slope of the radiation survival curve, c.f. Barendsen, (1982)) and 1.3 on the β-value (quadratic term determining the bending of the survival curve at higher dose). Enhancement of radiation effects did not occur when incubation times were longer than 48 hours.

In confluent cultures no enhancement of radiation effects was observed at the same gemcitabine concentration (10 nM). Increasing the gemcitabine dose to 100 nM did not produce enhancement in confluent cultures either.
Figure 2. Cell survival after radiation alone or combined with different doses of gemcitabine. A: exponentially growing cultures (10 nM gemcitabine); B (10 nM gemcitabine) and C (100 nM gemcitabine) confluent monolayer cultures. Means with standard errors of at least three experiments. For the gemcitabine treated samples, the cell survival was corrected for gemcitabine alone.

Spheroids: In figure 3 the effect of irradiation combined with gemcitabine is shown for the cell line spheroids.

Incubation of Gli-6 spheroids with 10 nM of gemcitabine did not induce any additional growth delay, while a significant increase in growth delay was observed after 100 nM gemcitabine. This effect however was found to depend on the size of the spheroids: in spheroids larger than 400 μm no effect of gemcitabine treatment was observed. Monolayer
cultures demonstrated a higher specific growth delay than spheroid cultures, when compared at equitoxic concentrations of gemcitabine (table 1), indicating that spheroids were 3.6 times more resistant to the combined treatment than monolayer cultures.

<table>
<thead>
<tr>
<th>Tumour model</th>
<th>Treatment</th>
<th>Specific Growth Delaya</th>
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<tbody>
<tr>
<td>Spheroid</td>
<td>Rx (5Gy)</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td>dFdCb</td>
<td>1.42</td>
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<tr>
<td></td>
<td>Combined</td>
<td>2.98</td>
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<tr>
<td>Monolayer</td>
<td>Rx (5Gy)</td>
<td>1.16</td>
</tr>
<tr>
<td></td>
<td>dFdCb</td>
<td>0.93</td>
</tr>
<tr>
<td></td>
<td>Combined</td>
<td>10.66</td>
</tr>
</tbody>
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\(^a\text{Specific growth delay} = \frac{\text{growth delay of treated cultures}}{\text{doubling time of control culture}}\)

\(^b\text{Gemcitabine concentrations with a similar cytotoxicity were compared, i.e. 33 nM for monolayer and 100 nM for spheroids}\)

\(\text{Rx} = \text{Irradiation}\)

*Table 1. Growth delay after radiation and gemcitabine in Gli6 spheroid and monolayer cultures.*
Figure 3. Growth delay in speriods after irradiation combined with different doses of gemcitabine (A: 10 nM, B: 33 nM, C 100 nM and D: 100 nM). Figure D shows the effect in large speriods (Ø 400 -500 µm). Figures show a typical experiment.
Discussion

Three different in vitro culture systems are often used in glioma research. These include monolayer cultures of single cells with an early or late passage number after initial isolation, cell line spheroids and organotypic multicellular spheroids (OMS). Most experimental data on the response of glioblastoma to radiation is based on monolayer cultures. The radiosensitivity of glioma cells as determined in monolayer cultures varies widely as reviewed by Taghian et al. (1993). The SF2 values range from 0.12 to 0.87. These data as well as the data presented in this article do not explain the high radioresistance observed for these tumours in the clinic.

Tumourgenesis is a multistep process, in which through a succession of genetic changes, each conferring one or another type of growth advantage, normal cells are transformed into highly malignant derivatives (Hanahan&Weinberg 2000). Even with all these genetic changes not all cells from the tumour shall grow in vitro, and as such monolayer cultures still represent a further selection compared to cells from the original tumour. Many early passage cell lines do not form colonies, so survival data can not be obtained by clonogenic assay. In these cases the MTT assay may be applied, as was shown by Fehlauer et al. (2000). Fehlauer et al. (2000) showed survival data for 2 primary glioma cell lines and one reference cell line which were determined both by clonogenic assay as by MTT assay. The SF2 values determined by MTT assay were consistently higher than those obtained by clonogenic assay, but both assays indicated a high radioresistance of the glioma cell lines (SF 2 MTT assay > 0.95, SF2 clonogenic assay 0.61-0.72). These results confirm data by Ramsay et al. (1992) who showed SF2 values for 13 glioma cell line of 0.63 (MTT assay) and 0.47 (clonogenic assay). Late passage cell lines represent an even further selection as a result of further genetic changes. According to Taghian et al. (1993) late passage glioma cell lines did not display a different radiosensitivity when compared to early passage cell lines.

Another culture model used, are OMS which are not selected for immortality. OMS maintain orginal tumour heterogeneity and tumour architecture including tumour vessels and extracellular matrix (Bjerkvig et al., 1990). These OMS which are subcultured from fresh surgical tumour material, were shown to be very radioresistant (Kaaijk et al., 1997): hypofractionated radiation of 40 Gy in 8 fractions of 5 Gy resulted in a significant decrease in cell proliferation but no histological damage and even OMS which were treated with 50 Gy single dose did not show major histological damage.

In our hands OMS behaviour varied widely (data not shown). Not all tumour biopsies gave rise to suitable OMS and large differences were also observed in growth rate, which may be
dependent on the differences in initial genetic changes in these cells, which led to malignancy (c.f. Hanahan & Weinberg, 2000).

Another model system is the cell line spheroid. These have the advantage that they are relatively easy to obtain and to maintain in culture compared to OMS. They lack the cellular heterogeneity of the OMS as they are derived from a single immortal cell line, but several studies have shown that these cell line spheroids still have some characteristics of the original tumour, which include aspects of tumour morphology and behaviour (Desoize et al., 1998, Santini et al., 1999). In cell line spheroids, as in OMS, cell-cell-contact, variation in cell cycle distribution, diffusion effects, altered metabolism and hypoxia are presumed to influence the outcome of radiation treatment (Olive & Durand 1994). And as such they resemble the in vivo situation.

In this study we used both monolayer cultures and spheroids cultures to determine radiosensitivity and the effect of gemcitabine on Gli-6 cells. The SF2 value for radiation alone in confluent monolayer cultures (0.49±0.07) observed in this study differs a bit from the previously published SF2 value (0.61) by Fehlauer et al., (2000), maybe as a result of the increased passage number and the genetic changes connected with this increase.

The comparison between monolayer cultures and spheroid cultures was based on a growth delay assay. Growth delay is the function of both surviving cells and regrowth rate and it reflects both radiosensitivity and radiocurability. In this study “small” Gli-6 spheroids (Ø 250-400 µM) were found to be substantially more resistant to radiation than the monolayer cultures, with a DMF of 2.5. This is probably due to factors characteristic for spheroids as e.g. presence of hypoxic cells and a high proportion of non-proliferating cells (table 1; Olive & Durand 1994, Dertinger & Hulser 1981). Since hypoxia was first observed by Sutherland and Durand (1973), it has been observed in many types of spheroids (reviewed by Gorlach & Acker 1994). As hypoxia is thought to be a major factor responsible for radioresistance of tumor cells (Sutherland 1998), it is assumed that hypoxic regions in spheroids influence effects of radiation. “Radiobiological” hypoxia was observed in older studies (Durand & Biaglow 1977; Biaglow et al., 1983). However in more recent studies the correlation between radioresistance and hypoxia was not evident (Gorlach & Acker 1994, Sminia et al., 2003, Buffa et al., 2001), suggesting that other factors like cell-cell contact and the fraction of non-proliferating cells might play a more important role in radiation outcome.

As spheroids grow the number of proliferating cells decreases and the number of quiescent cell increases. Proliferation becomes limited to the outer rim of the spheroid (Carlson et al., 1983, Sutherland 1988, Nirmala et al., 2001).
Most studies on the effect of gemcitabine use monolayer cell cultures. However, Smitskamp-Wilms et al. (1998) found that gemcitabine was far less effective in human colon tumour cell lines when these were grown as multiple layers, than when they were cultured as exponentially growing monolayers. Our results show that spheroids are indeed rather resistant to gemcitabine. This is probably caused by the fact that spheroids contain more cells in insensitive cell cycle phases and moreover the effect of gemcitabine in spheroids may be diffusion limited. The results of Neshateh-Riz et al. (1997) with radiolabelled deoxyuridine ($^{125}\text{IudR}$) support this view. These authors show a decreased incorporation of $^{125}\text{IudR}$ in glioma spheroids as the spheroid size increases. Maximal incorporation was observed at the proliferating outer rim, while the incorporation decreased with depth.

The mechanism of radiosensitisation or radioenhancement by gemcitabine is studied by many authors. Its radiosensitisation has been associated with redistribution of cells into S phase and depletion of the deoxynucleotide triphosphate pools (Lawrence et al., 1997, Van Bree et al., 2002). In the present study, exposure of exponentially growing Gli-6 cells to 10 nM gemcitabine for 24 hours produced minimal cytotoxicity but significant radioenhancement was observed. Gemcitabine induced radiosensitisation in exponentially growing cells and small spheroids (Ø 250-400 μM) at concentrations of 10 and 100 nM respectively. Radiosensitisation did not occur in confluent cell cultures and large spheroids (Ø 400-500 μM). The difference in volume between the small and large spheroids used in this study is not large, about a factor of two. Still a large difference in response is observed. This suggests the existence of a 'critical' size (above approximately Ø 400 μm), beyond which apparently changes in cell cycle distribution, drug and nutrient diffusion significantly alter the response.

The findings reported above support the view that cell cycle distribution of cells is important in radiosensitisation by gemcitabine (Latz et al., 1998). The fact that the cytotoxic action of gemcitabine is largely S-phase dependent may explain the observation that no clinical benefit was observed in the two phase II clinical trials mentioned earlier (Gertler et al., 2000, Weller et al., 2001). It is very likely that the glioblastoma tumours in situ have a large population of non proliferating cells, G0 and G1 cells (cf. Barendsen et al., 2001). This will severely hamper the direct cytotoxic action as well as radiosensitisation of gemcitabine.

In summary, we showed that gemcitabine can enhance the effects of irradiation in both exponentially growing cell monolayers as well as in small spheroids. In confluent monolayer cultures and large spheroids no enhancement of radiation effects was observed and this strongly supports the view that cell cycle distribution of cells is important in radiosensitisation
by gemcitabine. The results presented in this study also have clinical implications. The cell cycle dependency of gemcitabine obviously reduces the effectiveness of this drug in tumours with a large population of non-proliferating cells. The optimal cytotoxic effect of gemcitabine will be achieved in rapidly growing tumours, for example in glioma tumours after "debulking" surgery, where it is known that local recurrences grow rapidly.
Enhancement of effects of irradiation by dFdC in glioblastoma cells

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