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

*Colour junctions as predictors of radiosensitivity:

*X-irradiation combined with gemcitabine in a lung carcinoma cell line*

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Abstract

Purpose: To determine whether measurement of colour junctions by fluorescent in situ hybridisation (FISH) can predict radiosensitisation of gemcitabine (2'-2'-difluorodeoxycytidine). Methods: Human lung carcinoma cells (SW-1573) were irradiated with X-rays with or without incubation of 10 nM gemcitabine for 24 hours. Cell survival was determined with clonogenic assay. Colour junctions were measured by whole chromosome FISH of chromosome 2 and 18 and were scored according to the PAINT method. Results: A clear radiosensitisation by gemcitabine was observed on cell survival. A significant decrease in the number of colour junctions was observed after gemcitabine treatment compared to radiation treatment alone. The correlation between colour junction induction and cell survival was high for both with and without gemcitabine, but the gemcitabine-sensitised curve did not coincide with the non-sensitised curve. Conclusions: Gemcitabine-induced radiosensitisation is not predicted by induction of colour junctions in cultured SW-1573 cells. This reduces the clinical applicability of this predictive assay for radiotherapy in combination with gemcitabine.

Keywords Gemcitabine · X-irradiation · FISH · predictive assay
Introduction

To optimise individual patient treatment in radiation oncology assays are needed to predict the outcome of radiation treatment. Several authors have tried to develop suitable predictive assays (reviewed by Begg and West 2002). Measurement of cellular radiosensitivity by conventional clonogenic assay or growth assay has proven to correlate well with treatment outcome (Girinsky et al., 1994, West et al., 1997). Unfortunately these techniques are very time-consuming and thus not applicable in the clinic. Another approach to determine cellular radiosensitivity is measurement of chromosome aberrations by fluorescence in situ hybridisation (FISH). This technique has the advantage that it can be performed within a few days and could thus be of more clinical use. Several groups have reported a good correlation between measurement of chromosome aberrations and cell lethality (Coco-Martin et al., 1994, 1996, 1999; Franken et al., 1999; Russell et al., 1995; Castro Kreder et al., 2002).

Radiotherapy is often combined with cytostatic drugs and a predictive assay should ideally be able to predict the combined behaviour of the different treatments. A relatively new anti-cancer agent is gemcitabine (2'-2'-difluorodeoxycytidine, dFdC). It has proven activity in a variety of solid tumours including pancreas and non small cell lung cancer (NSCLC; Hertel et al., 1990). Gemcitabine is a potent radiosensitiser both in vitro and in vivo, but its method of radiosensitisation is not fully elucidated (Rockwell & Grindey 1992; Shewach et al., 1994; Latz et al., 1998; Lawrence et al., 1996; Rosier et al., 1999). Although radiosensitisation may increase the effectiveness of radiotherapy, the radiosensitising effect of normal tissue by gemcitabine warrants caution in its clinical application. An early study in NSCLC patients in which high dose gemcitabine (weekly dose of 600-1000 mg/m2) was given concomitantly with radiation showed high pneumotoxicity that correlated in part with the large volume of radiation given to the lung (Scalliet et al., 1998). Nevertheless preliminary results from more recent trials using lower gemcitabine doses indicate that gemcitabine and radiation can be combined without serious toxicity to the lungs (Blackstock et al., 2001, Vokes et al., 1999, Zinner et al., 1999, Gregor et al., 1999).

Recently we have demonstrated radiosensitisation by gemcitabine in a NSCLC cell line (van Bree et al., 2002). In the same cell line we have shown that radiosensitisation by the halogenated pyrimidine bromodeoxyuridine (BrdU) can be predicted by measurement of colour junctions with FISH (Castro Kreder et al., 2002). Preliminary results cited by Grégoire et al. (1999) showed an increase in chromosomal fragments after combined treatment of radiation and gemcitabine. These results would suggest a correlation with the increase in
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lethality after combined treatment. The aim of this study was to determine whether colour junctions detected by the FISH technique could be used to predict radiosensitisation by gemcitabine.
Chapter 3

Materials and Methods

Cell culture and gemcitabine treatment
The human squamous lung carcinoma cell line SW-1573 was grown in Leibowitz-15 medium (L15; GIBCO-BRL life technologies, Breda, The Netherlands) supplemented with 10 % fetal bovine serum and 2 mM glutamine at 37\(^\circ\)C with no CO\(_2\). The doubling time of the SW-1573 cells in exponential growth is 22-24 h (Haveman et al., 1995).

For experiments, the cells were plated in 30 mm or 60 mm petri dishes (Costar Europe LTD, Badhoevedorp, the Netherlands). For sensitising experiments cells were incubated with medium containing 10 nM gemcitabine for 24 hours before irradiation. Before the start of irradiation medium containing gemcitabine was removed, cells were washed two times with PBS and fresh medium was added.

Irradiation
Irradiation was performed with a Siemens Stabilipan 2 X-ray machine (Siemens, Germany). Irradiation were performed at 3.3 Gy/min. Distance between focus and culture dish was 33.5 cm and a 0.5 mm Cu-filter was used. During irradiation cells were kept at 37\(^\circ\)C in a waterbath. Dosimetry was performed with a BF-vat detector and a Farmer electrometer once a month.

Flowcytometric analysis of cell cycle distribution
Cells were plated and treated as described above. At different time points after initial plating, 10 \(\mu\)M Bromodeoxyuridin (BrdU) was administered from a 100x stock. After 2 hours the cells were harvested, fixated in 70% ethanol in phosphate buffered saline (PBS) and stored at \(-20^\circ\)C until immunofluorescent staining. Ethanol-fixated 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\(^\circ\)C. After washing with PB Tb (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 rat anti-BrdU (Harlan Seralab LTD, Loughborough, UK, diluted 1:100 in PB Tb) and incubated at room temperature for 30 min. After washing with PB Tg (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 PB Tg) 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 flowcytometric analysis. Samples were syringed through a 21 gauge needle to reduce cell aggregation before flowcytometry (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.

**Clonogenic Assay**
Cell survival was determined by clonogenic assay. In brief, at 24 hours after irradiation cells were trypsinised and replated in appropriate dilutions in six-well culture plates (Costar). Eight days later the colonies were fixated in 6 % glutaraldehyde and stained with 0.05 % crystal violet. Colonies of 50 cells or more were scored as originating from a single clonogenic cell. The plating efficiency of SW-1573 cells was 89 ± 12 %. After treatment with gemcitabine the plating efficiency was 72 ± 13 %. Surviving fractions \( S(D)/S(0) \) after dose \( D \), corrected for toxicity of gemcitabine alone, were calculated and survival curves were analysed using Graphpad Prism statistical software (Graphpad Software Inc, San Diego, USA) or BMDP (Los Angeles, USA). The data were fitted by multiple regression according to the LQ formula:

\[
S(D)/S(0) = \exp(- (αD + βD^2))
\]

Statistical analysis of the enhancement factor was determined with the student’s t-test.

**Metaphase slide preparation**
For metaphase slide preparation, cells were plated in 60 mm culture dishes. For sensitisation experiment cells were cultured for 24 hours in the presence of 10 nM gemcitabine before irradiation. Twenty-four hours after irradiation cells were transferred to 172 cm² tissue culture flasks. Twenty four hours later, cells were incubated for 2 hours with colcemid (0.1 μg/ml. Sigma) and mitotic cells were shaken off. Mitotic cells were treated with hypotonic KCl for 10 min at 37°C and subsequently washed and fixated in methanol/acetic acid (3:1). Finally the cells were dropped onto moist slides.

**Fluorescence in situ hybridisation**
Directly labelled whole chromosome-specific probes (chromosome 2-Cy3 and chromosome 18-FITC) were obtained from Cambio (Cambridge, UK). Metaphase double staining was
performed with the method described by Pinkel et al. (1986) and Natarajan et al. (1992). Enhancement of the FITC signal was achieved with a commercially available amplification kit (Cambio). Metaphase slides were counterstained with DAPI (2.5 µg/ml) in PBS and embedded in anti-fade solution (Vecta Shield, Vector Laboratories, Burlingame, CA, USA).

**Scoring of aberrations**

Slides were examined using a fluorescence microscope (Ortholux; Leica, Wetzlar, Germany) with a green light (552 nm) emission filter (615 nm) to detect Cy3 or a (495 nm) emission filter to detect FITC (519 nm). DAPI-stained metaphases were detected with a UV (372 nm) emission filter (456 nm). A total of 300 to 600 metaphases from three different experiments were scored for each dose and each chromosome. Aberrations were scored according to the PAINT method (Tucker et al., 1995). All aberrations involving a painted chromosome and an unpainted chromosome were scored as colour junctions and thus the sum of stable and unstable aberrations are reported here. Stable-type aberrations include translocations, deletions and insertions. Dicentrics and acentric fragments are examples of unstable aberrations. In general stable type aberrations remain present in cells for several generations and correlate well with cell death, while unstable aberrations lead to cell death within 1 to 2 generations (Coco-Martín et al., 1996). Since scoring was performed in the first metaphase after irradiation both stable and unstable aberrations were scored. When colour junctions are scored the contribution of unstable aberrations (dicentrics etc.) is small (< 5 %) and no corrections were made. Fragments were easily distinguished from colour junctions as they represent small pieces of the painted chromosomes with only one colour and these were counted separately. Three copies of chromosome 2 and two copies of chromosome 18 are present in SW-1573 cells. These chromosomes were selected for our investigations because they showed no colour junctions in controls, have different intrinsic radiosensitivity and predict radiosensitisation of bromodeoxyuridine in a similar experimental set-up (Castro Kreder et al., 2002). In total SW-1573 cells contain between 60 and 67 chromosomes. Dose-effect curves for induction of colour junctions by radiation were analysed using Graphpad Prism statistical software. The data were fitted to a pure linear model:

\[ F(D) = \alpha D \]

Statistical analysis of the enhancement factors was determined with the student’s t-test.

To determine the relative DNA content of chromosome 2 and chromosome 18, the length of all the chromosomes from 10 photographs of well-spread metaphases was measured. For this purpose, DAPI-stained metaphases and metaphases after FISH were photographed with a
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CCD camera (Hi-Sis slowscan cooled, Lambert Instruments, Leutingerwolde, The Netherlands) and the ratio of stained chromosome and total genome was calculated.

Scoring of Apoptosis
To determine the relative number of apoptotic cells after the different treatment schedules, cytospins were made, fixated in 100 % ethanol and air-dried. For staining of the DNA the slides were washed in PBS and incubated for 5 minutes with DAPI (5 μg/μl PBS) and embedded in anti-fade solution (Vecta Shield, Vector laboratories, Burlingame, CA, USA). Fragmented nuclei were counted with a fluorescence microscope (Ortholux: Leica, Wetzlar, Germany).
Results

Cell survival

Previous results have shown that phosphorylation of gemcitabine is requisite for cytotoxicity and for its antitumour activity (Heinemann et al., 1988). To allow phosphorylation to take place it is necessary that cells are incubated some time before combination with another treatment. Haveman et al. (1995), using the same cell line as in this study, showed that 24 hours after 10 nM gemcitabine, the maximum effect was observed in combination with hyperthermia. A clear radiosensitisation by gemcitabine was observed (Fig. 1). Linear quadratic parameters are shown in table 1. Treatment of cells with 10 nM gemcitabine for 24 hours resulted in a relative survival of $0.80 \pm 0.18$ Combining gemcitabine with irradiation resulted in a dose modifying factor of 1.3 at the 10% survival level.

![Graph](image_url)

**Fig. 1.** Radiation dose survival curves of SW-1573 cells after irradiation with and without 10 nM gemcitabine for 24 hours. Cell survival was corrected for the effect of gemcitabine alone. Means with standard errors of at least three experiments are shown.
Cell cycle distribution and apoptosis after gemcitabine treatment

Because gemcitabine induced radiosensitisation is most prominent in S phase (Latz et al., 1998), the effect of gemcitabine on cell cycle distribution was determined (Fig. 2). In this study gemcitabine-containing medium was added 24 hours before irradiation. At this time point cells were in log phase. Treatment of the cells with 10 nM gemcitabine for 24 hours inhibits cell proliferation and induces an early S-phase arrest (76.8 ± 3.1 % S-phase, 19.8 ± 2.9 G1 phase as compared to controls: 48.8 ± 0.7 % S-phase, 40.0 ± 0.3 G1 phase) (Fig. 2). At 24 hours after irradiation when cells are assayed for cell survival and processed for the FISH experiments, cell cycle distribution in gemcitabine treated cells has recovered. After gemcitabine treatment a sub-G1 population arises which suggests the presence of apoptotic cells (Fig. 2A). Analysis of DAPI stained cytospins showed an increased fraction of cells with fragmented nuclei after combined X-ray and gemcitabine treatment and for gemcitabine alone, compared to other treatment schedules (Fig. 3).

<table>
<thead>
<tr>
<th></th>
<th>LQ parameter*</th>
<th>Control</th>
<th>Gemcitabine</th>
<th>Enhancement Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell survival</td>
<td>α</td>
<td>0.16 ± 0.02</td>
<td>0.46 ± 0.04</td>
<td>2.9 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>β</td>
<td>0.03 ± 0.004</td>
<td>0.03 ± 0.007</td>
<td>1 ± 0.3</td>
</tr>
<tr>
<td>Chromosome 2</td>
<td>α</td>
<td>0.059 ± 0.002</td>
<td>0.04 ± 0.002</td>
<td>0.67 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Chromosome 18</td>
<td>α</td>
<td>0.031 ± 0.001</td>
<td>0.021 ± 0.001</td>
<td>0.67 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total genome relative to 2</td>
<td>α</td>
<td>0.75 ± 0.03</td>
<td>0.51 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>Total genome relative to 18</td>
<td>α</td>
<td>1.24 ± 0.04</td>
<td>0.84 ± 0.04</td>
<td></td>
</tr>
</tbody>
</table>

*α (Gy<sup>-1</sup>) and β (Gy<sup>-2</sup>)
<sup>a</sup> Significantly different from control p<0.01

Table 1. Linear quadratic (LQ) parameters for cell survival and colour junction induction after irradiation with and without 10 nM gemcitabine for 24 hours. Analysis performed with BMDP statistical software for the cell survival data, for colour junctions the analysis was performed with Graphpad Prism software.
Fig. 2a Bivariate flow cytometry with propidium iodide-fluorescence indicating the amount of DNA on the X-axis and FITC-fluorescence indicating BrdU-incorporation on the Y-axis. Representative examples of control samples and gemcitabine treated samples at the time of irradiation (t=0 h), of cell survival assessment (t=24 h) and of harvest of metaphases (t=48 h). b. Percentage of cells in the early and late S-phase at different time points after gemcitabine treatment.
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*Fig. 3.* Percentage of fragmented nuclei at different time intervals after treatment with 10nM dFdC for 24h, with 4 Gy or with the combination of treatment modalities. Mean and standard deviation are given of at least two separate measurements. At least 200 nuclei were scored for each point. Survival of dFdC alone in this experiment was 0.65 which may be related to the high cell number at the time of dFdC exposure.

**Colour junctions**

Fig. 4 shows the induction of colour junctions in chromosome 2 and 18 after irradiation. Gemcitabine alone did not induce colour junctions and no chromatid types of aberrations were observed after radiation alone or in combination with gemcitabine. After the combined treatment a significant decrease in the number of in colour junctions in both chromosomes was observed (p<0.01). Corresponding linear quadratic parameters and enhancement factors can be found in table 1. Based on their physical length chromosome 2 and 18 represent 7.8 ± 0.6 % and 2.5 ± 0.1% respectively, of the entire genome. Extrapolation of the values of LQ parameters for the entire genome resulted in α values for chromosome 2 and chromosome 18 of 0.75 ± 0.03 and 1.24 ± 0.04 respectively.
As gemcitabine is implicated in inhibition of chromosome damage repair (Grégoire et al., 1999) which may lead to more chromosomal fragments, we also scored the number of painted fragments after treatment (Fig. 5). No clear difference in the number of fragments was observed between control and gemcitabine treated cells.
Fig. 5. Number of linear acentric fragments in chromosome 2 and 18 with or without 10 nM gemcitabine for 24 hours. Means with standard errors of at least two experiments are shown.

**Correlation between Colour junction induction and cell survival**  
In Fig. 6 the correlation between cell survival and colour junctions is shown. The correlation between cell survival and colour junctions was high for each individual curve ($R^2 0.90-0.98$). The gemcitabine sensitised curve clearly does not coincide with the non-sensitised curve. This leads to the conclusion that the number of colour junctions does not adequately predict the increased cell death induced by gemcitabine treatment.

Fig. 6. Correlation between colour junction induction and cell survival after treatment for chromosome 2 and 18 with and without 10 nM gemcitabine for 24 hours.
In this study, treatment with gemcitabine resulted in a clear radiosensitisation on cell survival. Our results concur with previous reports of radiosensitisation of gemcitabine after irradiation (reviewed by Grégoire et al., 1999; van Bree et al., 2002). In this study radiosensitisation of gemcitabine was determined after a 24 hour incubation with 10 nM. This concentration and time schedule were chosen to allow optimal phosphorylation of gemcitabine. Furthermore a 24 hours incubation of 10 nM gemcitabine was previously shown to induce maximum effect in this cell line (Haveman et al., 1995). However other groups (Rosier et al., 1999, Van Putten et al., 2001, Weiss et al., 2003) have demonstrated radiosensitisation of gemcitabine after shorter incubation intervals, but in such cases a much higher dose of gemcitabine is required. Several features of gemcitabine cytotoxicity such as cell cycle redistribution (Shewach & Lawrence 1996; Latz et al., 1998) and inhibition of DNA repair (Grégoire et al., 1999) have been suggested to play a role in radiosensitisation.

Although a significant radiosensitising effect was observed after gemcitabine treatment, a significant decrease in colour junctions was observed. As was reported for another deoxycytidine analogue Ara-C (Preston 1980), Grégoire et al. (1999) assume that gemcitabine inhibits chromosome damage repair after irradiation. As consequence of this assumption, the misrepair leading to aberrations may be inhibited and no increase in colour junctions will be observed. If this is the case more non-rejoined aberrations (fragments) should be observed in the gemcitabine treated cells. An increase in fragments after radiation treatment combined with gemcitabine was reported (cited by Grégoire et al., 1999); however in our study, there is no clear increase in fragments after radiosensitisation by gemcitabine. This implies that inhibition of chromosome damage repair is not responsible for the reduction in colour junctions.

Our flowcytometry data indicate that the cell cycle distribution has almost recovered from the gemcitabine treatment at the time of sampling for colour junction measurement. However, some of the most severely damaged cells died from apoptosis in the mean time (Fig. 3) and are thus not available for analysis of chromosome aberrations. We observed that gemcitabine treatment alone and the combined treatment of gemcitabine with X-rays, both led to an increased level of apoptosis. However, apoptosis can not solely explain the reduction in colour junctions after combined X-rays and gemcitabine treatment.

Application of the premature chromosome condensation (PCC) technique in principle might permit an earlier scoring of colour junctions after treatment without the need to grow the cells.
to the next metaphase (Coco-Martin et al., 1997). Unfortunately PCC-analysis can not be performed in S-phase cells, which makes the technique not useful after gemcitabine treatment. Gemcitabine arrests the exponentially growing cell population in S phase, which renders them more radiosensitive as compared to controls.

Resuming the data, no clear explanation can be given for the decrease in number of colour junctions following combined therapy. Of course, the ultimate purpose of this study is to predict the sensitivity of cells in tumours. We demonstrated that the method is applicable in a situation in vitro, which resembles the situation in the clinic in which patients are treated with gemcitabine before receiving radiotherapy. We show that the number of colour junctions is able to predict the survival after radiation alone as well as after combined radiation plus gemcitabine. However, the present results on gemcitabine show that data on the number of colour junctions do not predict cellular survival unless the given treatment combination is known. This in contrast to earlier observations on radiosensitisation induced by BrdU (Castro Kreder et al., 2002).

Conclusion

Radiosensitisation by gemcitabine, a drug with cell cycle specific inhibitory action and a relatively high level of cytotoxicity, cannot be predicted by measurement of colour junctions with FISH. Whether this is a general phenomenon, also for other types of chemotherapeutic agents (e.g. CisPt) in combination with X-irradiation, remains to be investigated.

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Chapter 3

References


Ostruszka LJ, Shewach DS. 2000 The role of cell cycle progression in radiosensitization by 2',2'-difluoro-2'-deoxycytidine. Cancer Res 60:6080-6088


Vokes EE, Leopold KA, Herndon JE I, Crawford J, Perry MC, Miller AA, Green MR. 1999 A Randomized Phase II Study of Gemcitabine or Paclitaxel or Vinorelbine with Cisplatin as Induction Chemotherapy (Ind CT) and Concomitant Chemoradiotherapy (XRT) for Unresectable Stage III Non-Small Cell Lung Cancer (NSCLC) (CALGB Study 9431). Proc ASCO; 18:459a (Abstr)
West CM, Davidson SE, Roberts SA, Hunter RD. 1997 The independence of intrinsic radiosensitivity as a prognostic factor for patient response to radiotherapy of carcinoma of the cervix. Br J Cancer 76:1184-1190

Weiss C, Grabenbauer GG, Sauer R, Distel L. 2003 Significant increase in residual DNA damage as a possible mechanism of radiosensitization by gemcitabine. Strahlenther Onkol. 179:93-8
