

# Radio sensitization of Colon Cancer Cells Mediated by Gemcitabine through Cell Cycle Synchronization

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## Abstract

We evaluated gemcitabine together with ionizing radiation for improved cell growth inhibition with respect to that by radiation alone in the human colon carcinoma cell lines SW620, HT-29 and SNU-C4. To this end, cells were exposed for 24 h to gemcitabine and then assessed for growth response with the sulphorhodamine B assay. The cell lines, as well as exposed to ionizing radiation and a combination of gemcitabine and ionizing radiation for 24 h, 48 h and 72 h and the radiosensitivity was assessed using a clonogenic assay. Multiple drug effect analysis was used to evaluate the synergistic effect, which was then related to the cell cycle phase distribution. The SNU-C4 cell line showed a greater sensitivity to gemcitabine in comparison to the other two cell lines, while the SW620 cells was more sensitive to damage induced by radiation. Furthermore, gemcitabine increased by 50% the effect of ionizing radiation after 24 h in SW620 cell line, while in the others cell lines, this effect was observed only after 72 h. Moreover, gemcitabine associated with ionizing radiation was synergistic in SW620, HT-29 and SNU-C4 cells. Increased in S phase fraction was seen in gemcitabine treatment in all cell lines studied. While, ionizing radiation only induced an accumulation on G2/M in SW620 and HT-29 cell lines, indicating that SNU-C4 is less sensitive to radiation effect. A significant accumulation of cells in S phase after treatment with gemcitabine followed by radiation was observed in all cell lines. In summary our data indicate that gemcitabine increases the radiosensitivity to radiation in cell lines derived from human colon cancer, and that this effect seems to be associated with the ability of gemcitabine to synchronize cells in S phase of the cell cycle.

**Keywords:** Colon cancer cell lines; Gemcitabine; Ionizing radiation; Radiosensitivity; Cell cycle

## Introduction

Colon cancer is the third most common malign neoplasm worldwide [1]. For the last 10 years, mortality rate from colon cancer has declined by 3%, and the largest drops occurred in adults aged 65 and older [2]. This decline can be attributed to the increase of screening, which detects and allows the removal of precancerous polyps [3]. In contrast, rates increased during this time period among adults younger than 50 years [2]. In Brazil this type of cancer is ranked in third place among other incidences of cancer and it is the third cause of deaths from cancer [4]. In general, the most affected age range is of 40-70 years. The conditions associated to increase a risk to develop colon cancer include a personal history of colorectal cancer or adenomatous polyps; a personal history of inflammatory bowel disease (ulcerative colitis or Crohn's disease); a strong family history of colorectal cancer or polyps; a known family history of a hereditary colorectal cancer syndrome such as familial adenomatous polyposis (FAP) or hereditary non-polyposis colon cancer (HNPCC) [5].

Most colon cancers stem from adenomatous polyps, which are usually asymptomatic and evolve in a quiet long process known as carcinogenesis. It is worth pointing out the importance of primary prevention. Thus, a routine preventive exam for people over 50 years of age must be taken through the colonoscope, which may reduce the average risk of death by colon cancer by 90%. Moreover, most colon tumors are completely cured when diagnosed in early stages [5].

First-line therapy consists of total surgical resection of localized tumor and adjacent lymphonodes, associated with concomitant preoperative or postoperative radiotherapy or with chemotherapy in order to diminish the possibility of relapse. Although a small proportion reveals itself to be incurable, the prognosis becomes extremely poor in

more advanced stages [1]. Thus, it is noted that, although chemotherapy has been showing significant advances in the treatment of the metastatic disease, the responses are still unsatisfactory. These results justify the evaluation of new strategies in the treatment of this neoplasia [6].

Gemcitabine (2',2'-difluorodeoxycytidine, dFdC, Gemzar), a cytotoxic nucleoside analog to the deoxycytidine that triphosphate dFdCTP irreversibly, incorporates into DNA and, subsequently, inhibits exonuclease and DNA repair activity. It is a chemotherapy drug with a broad-spectrum of activity and commonly used either as the single agent or combined with other chemotherapy drugs [7]. According to studies, the efficacy of growth inhibition of human neoplasia obtained in a variety of solid tumors both in vitro and in vivo was successfully confirmed as well [8,9]. Gemcitabine is currently indicated as a single agent in the treatment of patients with metastatic pancreatic cancer and in the combination of chemotherapy drugs in non-small cell lung cancer, bladder cancer, breast cancer, and soft tissue sarcoma [7-11].

For a long time, cell radio sensitivity has been the focus of

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investigation due to its clear influence over the result of this therapy. Data in literature show that there are direct proofs that intrinsic tumor cell radio sensitivity is a major key for the response to radiotherapy [12]. Because of these findings, studies have been proving gemcitabine to be a powerful inducing agent of sensitivity to ionizing radiation in several solid tumors [13-16]. These results have been leading to variety of clinical essays that use gemcitabine as a radiosensitizer. Several studies have tried to clarify the mechanisms of action that are concentrated in the cell cycle redistribution, the induction of apoptosis, the role of p53 (inhibiting the repair of chromosome damage induced by irradiation), intracellular dFdCTP levels, the modulation of deoxynucleoside metabolism [17], and damages to DNA [10]. A study has shown that combined gemcitabine and proton radiation enhanced apoptosis in pancreatic cancer cells [18]. In this study, we investigate the radio sensitizing potential of gemcitabine and cell cycle redistribution in intestinal cancer cell lines.

## Material and Methods

### Cell lines and cell lines maintenance

The HT-29 and SW620 human colon cancer cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA). The SNU-C4 human colon cancer cell line was kindly supplied by Dr. GJ Peters (Free University Hospital, Amsterdam, and The Netherlands). Cells were maintained in complete medium consisting of RPMI-1640 (Invitrogen, Grand Island, NY, EUA) containing 2% (w/v) L-glutamine (Sigma-Aldrich, St. Louis, MO, USA) and 10% (v/v) fetal calf serum (Invitrogen), at a temperature of 37°C, a minimum relative humidity of 95%, and an atmosphere of 5% CO<sub>2</sub> in air. For experiments, exponentially growing cells were detached from the culture flasks using ethylenediaminetetraacetic acid (EDTA)-trypsin (Sigma-Aldrich).

### Drug sensitivity assay

Cells (1 × 10<sup>4</sup>) were seeded onto 96-well plates and treated the following day with various concentrations of Gemcitabine (0 to 100 μM) during 24 h. Cytotoxicity was assessed by means of sulforhodamine B assay (SRB; Sigma-Aldrich) [19] involving in situ fixation with trichloroacetic acid (TCA; Sigma-Aldrich), staining with SRB, and solubilization of cell-bound SRB with Trizma base (Sigma-Aldrich). The latter was colorimetrically assessed with a Model Multiskan EX Microplate Reader (Labsystems, USA). Absorbances were read at a wavelength of 540 nm. Three independent experiments were carried out for each treatment. The Gemcitabine concentrations causing a 50% growth inhibition (IC<sub>50</sub>) compared with the controls were calculated from a semi logarithmic dose-response curve by linear interpolation.

### Irradiation and colony formation assay

The cells were irradiated with various single doses (2,5 and 10 Gy) (dose rate 1.14 Gy per minute), using a Telecobalt Theratron Phoenix SR 7510 linear accelerator (Philips, Eindhoven, The Netherlands), at a source-to-target distance of 70 cm, available at Radiotherapy Service from Hospital São Lucas da Pontificia Universidade Católica do Rio Grande do Sul (HSL, PUCRS, Porto Alegre, Brazil). Clonogenic assay was performed as described previously [20]. Briefly, cell lines were seeded into 6-well plates (400 cells/well) after irradiation alone or combination treatment with gemcitabine and irradiation. After incubation for 10 days, the cells were fixed with 70% ethanol and counterstained with 0.5% crystal violet. Only colonies containing 50 or more cells were scored under a microscope. The radiation survival fraction (SF) was then calculated as: SF= (Number of colonies in irradiated cells/Number of colonies in control)/100.

### Analysis of combination treatment effect

Interactions between Gemcitabine and ionizing radiation were assessed by isobologram analysis [21], using a computer programme CompuSyn (ComboSyn, Inc., Paramus, NJ; version 1.0). The programme enables calculation of combination indices (CIs) which, when smaller than 1, equal to 1, or greater than 1, indicate synergism, additivity or antagonism, respectively, between two treatments. CIs were calculated by the formula:  $CI = (D)1 / (Dx)1 + (D)2 / (Dx)2$ ; where (Dx)1, and (Dx)2 are the concentrations of CPT-11 alone or 5-FU alone, giving x% growth inhibition, and (D)1 and (D)2 the drug concentrations in combination inhibiting cell growth also x%. (Dx)1 and (Dx)2 were calculated by the median-effect equation of Chou and Talahay [21],  $Dx = Dm [AF / (1 - AF)]^{1/m}$ ; where Dm is the median-effect dose, FA is the fraction affected, and m the slope of the median-effect plot. Data were evaluated by taking the means of the CIs at FAs of 0.50, 0.75, 0.90 and 0.95.

### Flow cytometric analysis

For cell cycle evaluation, 5 × 10<sup>5</sup> cells were treated with gemcitabine (IC<sub>50</sub>; 24 h), ionizing radiation (5 Gy; 72 h) and association gemcitabine/radiation (72 h). After treatments, cells were harvested and fixed in ethanol 70% overnight. The samples were washed in PBS, resuspended in 0.5 ml PBS and incubated with RNase A 100 μg/ml and propidium iodide 50 μg/ml for 20 min in dark at room temperature [22]. 20,000 cells were analysed by FACS Calibur flow cytometer (Becton-Dickinson, San Jose, CA, USA). The DNA content was analyzed using a ModFit 2.0 software.

### Statistical analyses

All experiments were carried out at least three times in triplicate. When appropriate, Tukey's posttest was applied. All analyses were performed with GraphPad InStat (version 3.05; GraphPad Software Inc., San Diego, CA, USA). Differences were considered significant with p<0.05 values.

## Results

### Effect of gemcitabine treatment on human colon cancer cell proliferation

The sensitivity of colon cancer cells lines SW620, HT-29 and SNU-C4 to gemcitabine was first analyzed to determine which doses are ideals for further studies. Table 1 shows that SW620 e HT-29 cells are more resistant to gemcitabine (IC<sub>50</sub> values of 13 and 10 μM, respectively), whether SNU-C4 cell line presented greater sensitivity to this drug (IC<sub>50</sub> values of 3.5 μM) (Table 1).

### Effect of ionizing radiation on human colon cancer cell proliferation

Ionizing radiation (2,5 and 10 Gy) promoted a dose-dependent decrease in cell proliferation in all cell lines tested (Figure 1). The assays revealed that 2 Gy do not have significant effects in colony formation on HT-29 and SNUC-4 cells lines. However, SW620 cell line showed

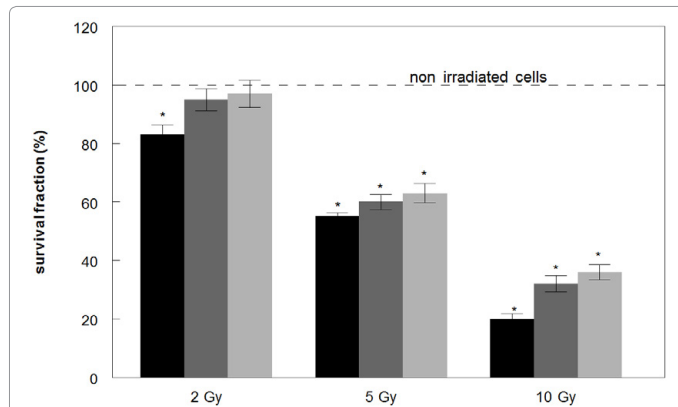
Cell line	Gemcitabine Dose (μM)
	IC <sub>50</sub>
SW-20	13.0 ± 2.4
HT-29	10.0 ± 1.3
SNU-C4	3.5 ± 0.9

**Table 1:** IC<sub>50</sub> values (μM; mean ± standard deviation, n=6) in SW620, HT-29 and SNU-C4 human colon cancer cell lines after 24 h treatment with gemcitabine.

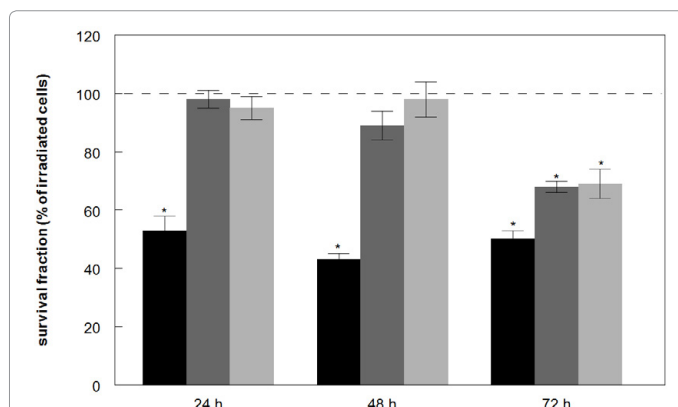
reduction in 17% in colony formation after irradiation with 2 Gy. After irradiation of 5 Gy, all three-cell lines exhibited reduction about 40% on survival cell rate, when compared with control cells. Doses of 10 Gy reduced survival cell rate approximately 70% on HT-29 e SNU-C4 cells line and 80% on SW620 cells line (Figure 1).

### Gemcitabine effect on cellular radiosensitization

In order to determine whether gemcitabine could increase radiation



**Figure 1:** Survival fraction of human colon cancer cell lines SW-620 (■), HT-29 (■) and SNU-C4 (■) irradiated with 2, 5 or 10 Gy. They were quantified after 10 days in colonies containing at least 50 cells. Data were expressed as the percentage of the survival fractions of cells versus the different treatments and were plotted as the mean + SD of three different experiments. \*Significantly different from control (p<0.05).



**Figure 2:** Survival fraction (SF) of the human colon cell lines SW-620 (■), HT-29 (■) and SNU-C4 (■) treated with gemcitabine (IC<sub>50</sub>) followed by ionizing radiation (5 Gy). They were quantified after 10 days in colonies containing at least 50 cells. The data were presented as percentage of cells relative to cells treated with irradiation. Data were plotted as the mean ± SD of three different experiments. \*Significantly different from cells treated with radiation alone (p<0.05).

sensitization of SW620, HT-29 and SNU-C4 cell lines, cells were treated with IC<sub>50</sub> gemcitabine dose and then irradiated with 5 Gy (dose which presented inhibition of 40% in survival cell rate in all three cell lines for 24 h, 48 h and 72 h. After treatment cells were seeded in a 6-wells plate in a density of 300 cells/well and maintained in culture for 10 day (Figure 2). Gemcitabine potentiates radiation effect only after 72 h in HT-29 and SNU-C4 cells lines. On the other hand, in SW620 cell line, this effect was seen already within 24 h of treatment and persisted during the period of the study (until 72 h) (Figure 2).

Combination Index analyses shown that the association of gemcitabine (IC<sub>50</sub>) with ionizing radiation (5 Gy) acted synergistically in all three cells lines (CIs values of 0.53 ± 0.12, 0.65 ± 0.11 and 0.70 ± 0.15 for SW620, HT-29 and SNU-C4, respectively). Moreover, SW620 cell line presents a stronger synergistic effect when compared to the other cells.

### Cell cycle distribution of human colon cancer cells in response to treatment

To determine whether the inhibition of cell proliferation in cell lines correlated with differences in cell cycle response to irradiation and/or gemcitabine, we analyzed cell cycle distribution after 72 h (Table 2). Overall, the non-treated cells demonstrated differences in cell-cycle distributions as determined by FACS analysis.

5 Gy irradiation induces distinct responses in cell cycle phases distribution among the cell lines. Cell accumulation in G2/M phase was observed only in SW620 and HT-29, with an increase of 30% and 20%, respectively, when compared with untreated cells. SNU-C4 cell line did not demonstrated difference when compared with control (Table 2).

As depicted in Table 2, cells treated with gemcitabine alone induced a strong S cell cycle arrest (p<0.05) when compared with untreated cells in all cell lines. The combination treatment with gemcitabine and ionizing radiation induced a significant increase in the number of cells in S phase when compared to radiation alone. This effect was more pronounced in SW620 cell line (44.7%).

### Discussion

Colon cancer is one of the most frequent type of human cancer and the third cause of death worldwide [1]. Systemic chemotherapy, therefore, plays an important role in patients with advanced disease. Unfortunately, cytotoxic drug therapy generally produces partial clinical responses of short duration, making this form of treatment only of palliative value, which justifies the evaluation of new treatments strategies for this disease [5]. Several chemotherapeutic drugs have obtained success in cancer treatment, among these gemcitabine has shown to be effective against several solid tumors [6,10]. Among these strategies, one of the most explored is the radiosensitization of tumor cells in order to improve cellular response to radiotherapy [12].

Treatment	SW-620			HT-29			SNU-C4		
	G0/G1	S	G2/M	G0/G1	S	G2/M	G0/G1	S	G2/M
Control	67.2 ± 6.5	21.5 ± 3.9	12.9 ± 2.8	83.3 ± 8.9	6.2 ± 1.6	10.5 ± 2.4	60.1 ± 5.2	24.2 ± 2.9	16.3 ± 2.4
Gemcitabine IC <sub>50</sub>	45.5 ± 2.5	41.0 ± 2.9	10.6 ± 1.7	56.3 ± 4.1	31.4 ± 3.5	13.1 ± 2.3	50.2 ± 4.8	43.0 ± 5.2	7.6 ± 2.9
Radiation 5 Gy	27.7 ± 1.9	33.8 ± 5.0	41.7 ± 2.3	36.2 ± 3.4	28.4 ± 5.3	35.3 ± 6.3	55.4 ± 7.3	28.8 ± 2.3	16.9 ± 3.3
Gemcitabine IC <sub>50</sub> + Radiation 5 Gy	43.1 ± 3.6*	44.7 ± 4.9*	12.1 ± 3.6	47.5 ± 1.5	35.7 ± 0.7	12.1 ± 7.1	52.1 ± 7.1	36.3 ± 0.7	10.8 ± 7.4

**Table 2:** Effect of gemcitabine at IC<sub>50</sub>, ionizing radiation at 5 Gy and gemcitabine (IC<sub>50</sub>) and ionizing radiation (5 Gy) combination on cell cycle distribution of SW620, HT-29 and SNU-C4 human colon cancer cells. Data were presented as mean ± standard deviation of percentage of cells of three independent experiments. \*Different from irradiated cells (p<0.05).

Other antimetabolites that deplete cellular dNTP pools have been shown to act as radiation sensitizers in solid tumor cells [23,24], and thus, it was important to assess the ability of gemcitabine to enhance the sensitivity of colon cancer cell lines to radiation. Several studies have been published concerning the gemcitabine ability to induce radiotherapy sensitivity in different type of cancer cells [8,9,11-14,25]. On this study we evaluated if gemcitabine could increase radiosensitization in colon cancer cell lines.

We found that in response to gemcitabine treatment, all cell lines demonstrated a dose-dependent inhibition on cell proliferation. Furthermore, SNU-C4 cells were considered, after drug exposure, gemcitabine sensitive cells. Indeed, in other study with this cell line, we found that SNU-C4 is a 5-fluorouracil (other antimetabolite agent) sensitive cells [26]. Although the three cell lines differed significantly (up to 4-fold) in their sensitivity to gemcitabine they displayed much smaller differences (less than 1-fold) in their response to ionizing radiation. These findings are in agreement with the absence of cross-resistance between both treatments, as well as with the demonstrated efficacy of ionizing radiation in gemcitabine-resistant colon carcinoma [27].

Considering that many of cellular damage initiated by radiation are repaired, determining its long-term consequences over tumor cells growth is primordial [28]. With this goal, late effect of radiation over cells culture was evaluated by counting the number of cells colonies developed 10 days after radiation treatment. Taking together our results suggested that SW620 cell line is more sensitive to radiation-induced damage when compared to the two other cell lines. However, response to treatment was observed in all three cell lines tested after 72 h. Previous studies with different cancer cells, including HT-29 cell line, demonstrate that radiation effect is dose-dependent, exposure time and molecular characteristics of cell lines [29,30]. Similar to our result, it was demonstrated that SW620 is a radiosensitive cell line [31]. Different responses to ionizing radiation probably occur due to distinct features of each cell line. Irradiation of cells may not only lead to cell death but to other changes as well. Many factors affect radiation response including the position of tumor cells within the cell cycle, which may confer radiosensitivity or resistance. For instance, the late G2 and M phases are the most radiosensitive [32].

Studies with soft tissue sarcoma demonstrate that gemcitabine presents radiosensitivity effects using doses established by IC50 [29]. Besides, Lawrence et al. (1997) demonstrate, in HT-29 cell line, a radiosensitivity effect after 72 h of gemcitabine exposure [30]. Here we demonstrated a radiosensitization effect of gemcitabine in three colon cancer cell lines. In this line, Pauwels et al. (2006), demonstrate a pronounced synergism in human colon cell lines when combined gemcitabine and radiation effects [33]. Several studies suggest that radiosensitization grade is dependent of molecular-characteristics in each different cell line, including apoptosis activation genes, DNA-repair genes and cellular distribution on cell cycle phases [33,35]. Many hypotheses were proposed in order to explain the radiosensitization gemcitabine effect [33]. Among others, the most presumable includes DNA-repair and cell synchronization in cell cycle phases [33-36].

To elucidate the factors involved in the radiosensitization gemcitabine effect observed we analyzed cell cycle distribution after treatments. Initially, we observed that untreated cells demonstrated equivalent cell-cycle distributions as determined by FACS analysis. Treatment with 5 Gy radiation induced different responses among the cell lines. SW620 and HT-29 cell lines demonstrated cell-cycle arrest in the G2/M phase, while in SNU-C4 cell line it is not observed any

changes when compared to the untreated control. This accumulation of cells in G2/M, contributes to the suggestion that the SW620 and HT-29 cell lines is promoting the radiation blocking of cells in G2/M, with subsequent induction of apoptosis and consequently decrease in the number of cells and cell colonies. Induction of cell arrest in G2/M is an effect rather described in the literature as damage induced by ionizing radiation [31,34-36].

Oxidative stress caused by radiation induces cell impairment and apoptosis through peroxidation of DNA and activation of the apoptosis pathways [37,38]. Moreover, Hussain et al. identified a novel mechanism of p53 dependent apoptosis in which p53 mediates up regulation of manganese-containing superoxide dismutase (Mn-SOD) produces an imbalance in antioxidant enzymes and oxidative stress [39]. Thus, the Mn-SOD isoform, which is inducible and localized in the mitochondria, has been implicated in apoptosis induction and could be involved in mechanisms of radioresistance. Indeed, we have found that Mn-SOD activity was approximately, 2.5-fold higher in the SNU-C4 when compared to HT-29 [40]. This could explain the more radioresistance observed in SNU-C4 cell line.

The mechanisms of action of gemcitabine include inhibition of DNA synthesis and induction of apoptosis [17]. The dFdCTP is incorporated into DNA, inhibiting the polymerase by competition, interfering with the enzyme ribonucleotide reductase, causing depletion of deoxynucleotide triphosphates necessary for the DNA synthesis, therefore inhibiting its formation [33]. Thus, the maximum radiosensitization induced by gemcitabine is associated with an accumulation of cells in S phase [30,35]. In our experimental conditions we observed a significant accumulation of cells in S phase after treatment with gemcitabine followed by radiation. The highest percentage of cells in S phase was observed in the SW620 cell line, which showed greater radiosensitization. In fact, several studies have reported that the effect of gemcitabine is dependent on the stage of the cell cycle [11,33,35] and that this effect is important for its ability to radiosensitization [8,11,13,33].

Basically our data indicate that gemcitabine increases radiosensitivity to ionizing radiation in three cell lines derived from human colon cancer and that this effect seems to be associated with the capacity of gemcitabine to synchronize cells in the S phase of the cell cycle.

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