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Original Research Article

Overcoming radioresistance in WiDr cells with heavy ion irradiation and radiosensitization by 2-deoxyglucose with photon irradiation

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ABSTRACT

Background and purpose: Radiosensitizers and heavy ion irradiation could improve therapy for female patients with malignant tumors located in the pelvic region through dose reduction. Aim of the study was to investigate the radiosensitizing potential of 2-deoxy-D-glucose (2-DG) in combination with carbon ion-irradiation (^{12}C) in representative cell lines of cancer in the female pelvic region.
Materials and methods: The human cervix carcinoma cell line CaSki and the colorectal carcinoma cell line WiDr were used. 2-DG was employed in two different settings, pretreatment and treatment simultaneous to irradiation. Clonogenic survival, α and β values for application of the linear quadratic model and relative biological effectiveness (RBE) were determined. ANOVA tests were used for statistical group comparison. Isobolograms were generated for curve comparisons.
Results: The comparison of monotherapy with ^{12}C versus photons yielded RBE values of 2.4 for CaSki and 3.5 for WiDr along with a significant increase of α values in the ^{12}C setting. 2-DG monotherapy reduced the colony formation of both cell lines. Radiosensitization was found in WiDr for the combination of photon irradiation with synchronous application of 2-DG. The same setup for ^{12}C showed no radiosensitization, but rather an additive effect. In all settings with CaSki, the combination of irradiation and 2-DG exhibited additive properties.
Conclusion: The combination of 2-DG and photon therapy, as well as irradiation with carbon ions can overcome radioresistance of tumor cells such as WiDr.

1. Introduction

According to recent estimates, colorectal cancer is the second, and cervix and uterus cancer the fourth most common female cancers worldwide [1]. Even with modern treatment, the 5-year-survival for both rectal and cervical cancer is generally still below 70% [2]. Irradiation of the pelvic region is often necessary to achieve locoregional control for these cancer entities, which leads to a series of symptoms that are not tumor-related but treatment-related, best summarized by the term 'pelvic radiation disease' [3]. Examples of pelvic radiation disease include chronic diarrhea, bleeding and sexual dysfunction, all of which lead to a serious loss of quality of life [4–6]. Both types of cancer discussed here are commonly irradiated with photons.

Overcoming radioresistance of the targeted tumor entities is the key point for reducing radiation dose and achieving better tumor control. One approach in this endeavor is carbon ion irradiation. Heavy ions have much better physical qualities when it comes to avoiding side effects. This is derived from the dose-depth profile of heavy ion beams which avoids applying much of the unwanted dose between the source of the beam and the target [7]. In the target region heavy ions like ^{12}C have a high linear energy transfer (LET). This high LET in turn is responsible for a high RBE, caused by DNA double strand breaks (DSB), which are crucial for clonogenic cell death [8]. The details of tissue response in heavy ion irradiation have yet to be studied [9]. To date, only a few trials with small patient numbers have examined carbon ions in relation to cervical and colorectal cancer [10–13].

Apart from changing the modality of irradiation itself, the effects of irradiation can be altered to improve response in malignant cells. 2-DG is an example of an altering substance which can make certain tumor cells more susceptible for irradiation in the sense of a radiosensitizer [14,15]. The use of the anti-metabolite

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2-DG utilizes the differences between the metabolism of healthy human cells and that of tumor cells. 2-DG has been long known for its ability to inhibit glycolysis [16], which strongly affects tumor cells because of the Warburg effect [17]. 2-DG is phosphorylated but cannot be further metabolized and inhibits glucose phosphorylation upon accumulation [18]. A well-known mechanism of action of 2-DG is the induction of ER-stress and consecutively UPR-upregulation which in turn promotes apoptosis [19,20]. Furthermore oxidative stress due to alteration of the thiol metabolism is thought to play a major role in the cell toxicity of 2-DG [21–23]. The inhibition of DNA repair, which includes the repair of DSB, is a proven cause of clonogenic cell death observed under 2-DG treatment [24,25]. Many properties of the substance have just recently been discovered and are not yet fully understood [26]. Inhibition of glycolysis and cell proliferation have been described for numerous cancer cell lines in a 2-DG dose range from 0.01 mM (48 h treatment) up to 20 mM (24 h treatment) [27,28]

In this in vitro study we investigated the radiosensitizing potential of 2-DG in combination with photon as well as heavy ion therapy for treatment of malignancies within the female pelvic region.

2. Methods

2.1. Clonogenic assay

The cell lines of HPV-transformed cervical carcinoma (CaSki) and colorectal adenocarcinoma (WiDr) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). For CaSki cells RPMI 1640 medium (11 mM Glucose) was used, while WiDr cells were cultivated in DMEM F 0415 (Dubelcco's Modified Eagle Medium, Biochrom, Berlin, Germany, 5.6 mM Glucose). Each medium was supplemented with 10% fetal bovine serum (Gibco, Life Technologies, Vienna, Austria) and 1% penicillin–streptomycin (Sigma-Aldrich, Munich, Germany). Cells were cultivated in 175 cm^2 plastic flasks in an incubator at 37 °C in 90% H_2O-saturated air and 6% CO_2.

In all experiments a setup was comprised of three identically prepared flasks (25 cm^2 Falcon, Becton Dickinson, East Rutherford, USA) with 5 ml of medium and a defined number of cells ranging in between 100 and 10,000, depending on the setup. Using a Neubauer counting chamber, cell counts were estimated both after trypsinization of stock cells and before seeding for the setups. Each setup of three was repeated three times on different days. Thus every experiment was conducted with nine different samples in total. 2-DG (obtained from Sigma Aldrich, St Louis, USA) was purchased in >97% pure crystalline form and dissolved in the medium of the respective cell line. 2-DG dose response experiments were conducted for CaSki and WiDr with doses ranging in between 0.1 mM and 10 mM prior to deciding on the doses for the combination therapy setups. Two doses for each cell line were chosen from the dose response curves for the combination treatment, one with a high and one with a moderate survival rate. Depending on the combination treatment setup, 2-DG was applied in different doses (0.1 mM/1 mM in CaSki; 0.7 mM/2.5 mM in WiDr) 4 h after cell seeding (when plating on the flask surface was complete) or 24 h after seeding. In the first case, a 2-DG pretreatment was initiated 20 h before irradiation, in the second case it was administered simultaneously with irradiation. The medium was changed 24 h after the application of 2-DG.

For the clonogenic assays, CaSki and WiDr were cultivated for 8 or 9 days respectively, the necessary time required for the formation of colonies and the manifestation of clonogenic cell-death within the two different cell lines. Colonies defined by a content of 50 or more cells were counted after ethanol fixation and optical enhancement with methylene blue under a microscope.

2.2. Irradiation

A laboratory X-ray irradiator designed for biological experiments (X-Rad 320 Precision X-Ray Inc., North Branford) was used for photon irradiation. The doses applied were 2, 4, and 6 Gy at a rate of 1.2 Gy/min with 320 kV and 20 mA. ^{12}C-irradiation was performed using the raster scanning technique at the Heidelberg Ion-Beam Therapy Center. The average penetration depth of the spread-out Bragg peaks occurred at the plating surface. The spread-out Bragg peak consisted of 5 energy layers ranging from 1468 to 1643 MeV. Each layer had 9021 raster points (7.1 mm diameter of maximum energy) with a distance of 2 mm × 2 mm (horizontally and vertically). The average LET was 100 keV/μm (70–170 keV/μm range). ^{12}C irradiation doses applied in this case were 0.125, 0.5, 1 and 2 Gy.

2.3. Data analysis

The data from the clonogenic assays was evaluated with the help of the linear-quadratic model: $-\ln(S) = \alpha D + \beta D^2$ (S being survival and D the applied dose). By applying this mathematical model, α and β values were calculated and fitted linear graphs with data points and corresponding standard deviations were created in SigmaPlot (version 10, Systat Software, Erkrath, Germany). To analyze combination treatments, surviving fractions were normalized to the corresponding values of 2-DG monotherapy by calculating the ratio of the combination treatment's plating efficiency and 2-DG monotherapy. Bonferroni two-way ANOVA post hoc test was employed in SPSS (version 23, IBM, Armonk, USA) to measure statistical significance of differences between the normalized treatment groups and controls. Significant differences in ANOVA tests alone were not considered sufficient to determine superadditivity. For this purpose isobolograms were generated as theoretical control curves. Thus trends towards superadditivity and significant superadditivity could be differentiated [29]. Additivity was assumed for curves in between the control curve and the theoretical control curve. Superadditivity was defined as survival below the theoretical control curve.

3. Results

The LD_{50} (median lethal dose) observed in photon treatment was 1.51 Gy for CaSki and 1.77 Gy for WiDr. In ^{12}C irradiation LD_{50} values were 0.25 Gy for CaSki and 0.19 Gy for WiDr. α/β ratios were 24.34 for CaSki and 13.85 for WiDr in photon irradiation monotherapy (see Table 1). α values were higher and β values lower in the carbon irradiation setting in comparison to photon irradiation ($p < 0.001$). In comparison to photon therapy, the RBE of ^{12}C, which was calculated at 10% survival, was 2.4 for CaSki and 3.5 for WiDr (Fig. 1).

2-DG monotherapy achieved survival rates of 82.4 ± 1.2% and 39.2 ± 4.5% in CaSki at concentrations of 0.1 mM and 1 mM respectively at an LD_{50} of 0.78 mM. In WiDr similar survival rates were reached at higher doses (73.8 ± 3.1% at 0.7 mM and 51.0 ± 4.8% at 2.5 mM, LD_{50} 2.57 mM). The survival rates given here were observed when 2-DG was applied 4 h after seeding with medium change after 24 h. The actual monotherapy controls were run simultaneously with each combination experiment. The effects of 2-DG on clonogenic survival are shown in Fig. 2.

All of the following percentages were calculated in relation to survival of 2-DG monotherapy. The percentages thereby represent surviving fractions normalized to the corresponding values of 2-DG monotherapy. This facilitates the evaluation of additivity versus superadditivity, as the percentages can be directly compared to the surviving fractions of irradiation monotherapy.

Table 1
Overview of α and β values of the setups.

Cell line		pre						sync					
		CaSki			WiDr			CaSki			WiDr		
2-DG (mM)		0	0.1	1	0	0.7	2.5	0	0.1	1	0	0.7	2.5
Photon	$\alpha\ (Gy^{-1})$	0.39	0.43	0.47	0.33	0.33	0.35	0.39	0.36	0.46	0.33	0.34	0.51
	$\beta\ (Gy^{-2})$	0.02	0.001	0.003	0.02	0.03	0.03	0.02	0.03	0.02	0.02	0.04	0.02
	$\alpha/\beta\ (Gy)$	24.34	359.94	173.21	13.85	12.08	11.56	24.34	12.68	26.63	13.85	8.47	24.94
^{12}C	$\alpha\ (Gy^{-1})$	1.91	1.98	2.08	2.2	2.22	2.3	1.91	2.1	2.2	2.2	2.44	2.54
	$\beta\ (Gy^{-2})$	0	0	0	0	0	0	0	0	0	0	0	0
	$\alpha/\beta\ (Gy)$	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a

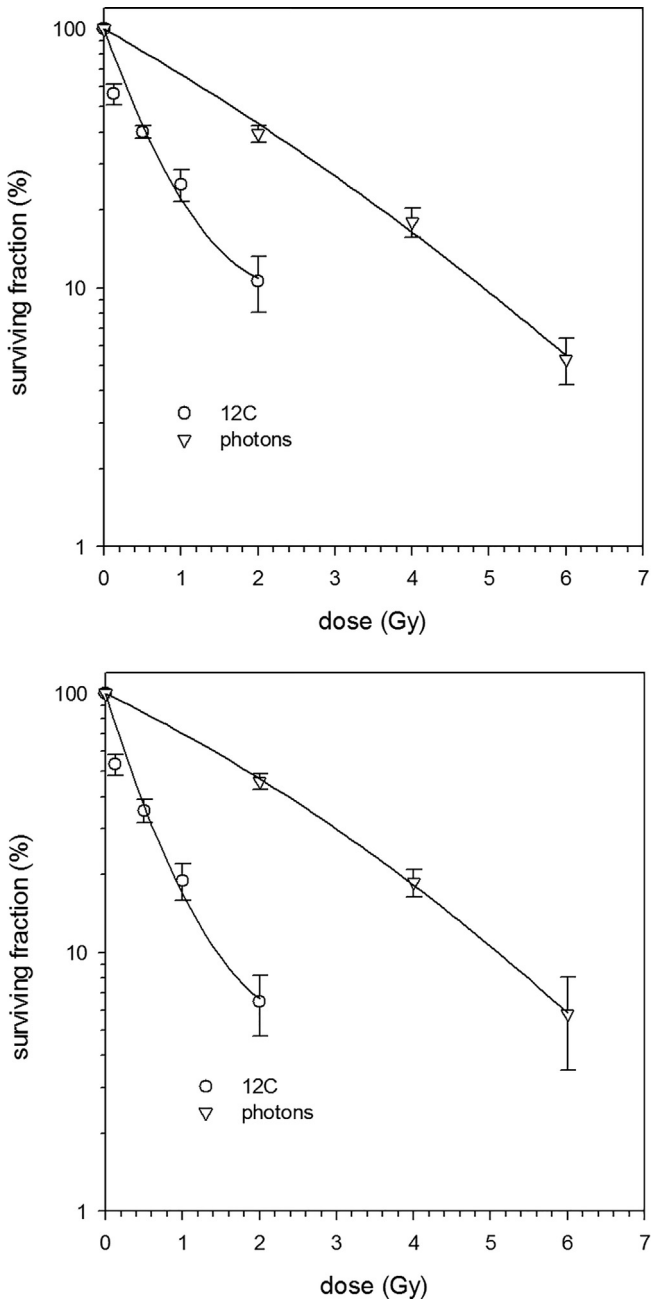

Fig. 1. Clonogenic survival after irradiation with ^{12}C or photons. CaSki left and WiDr right. Symbols represent experimental data with their standard deviations, lines are results of the fit described in the text.

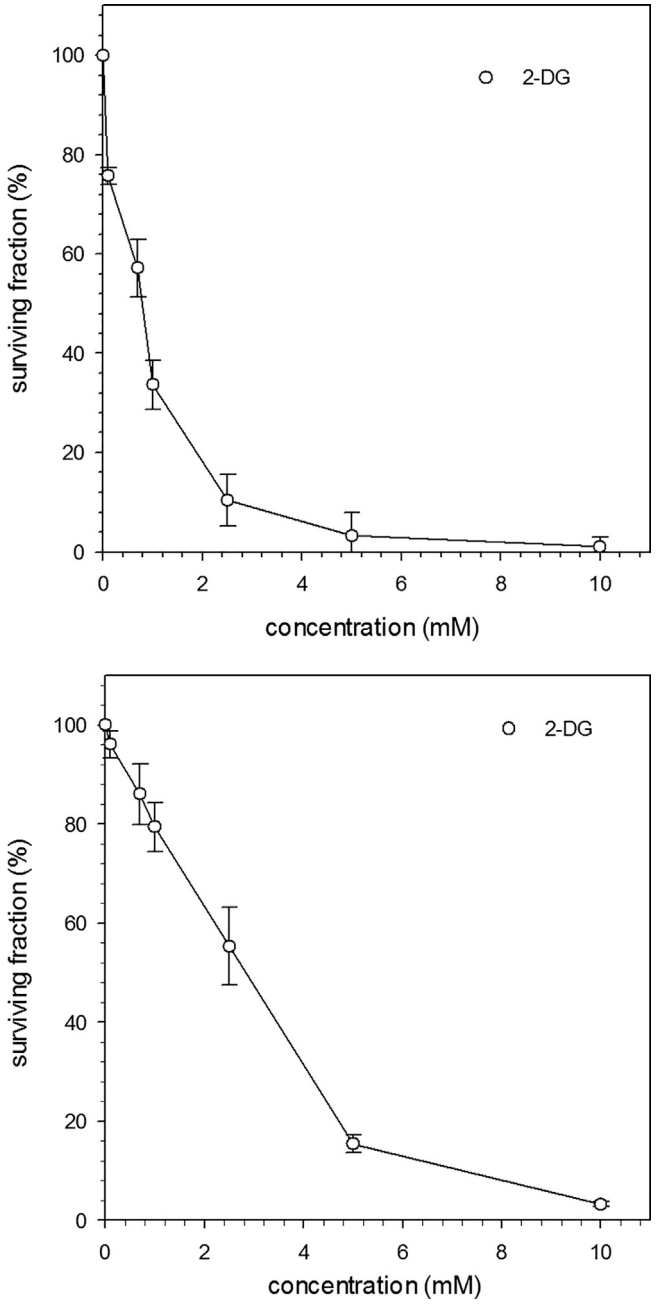

Fig. 2. Clonogenic survival after 2-DG monotherapy. CaSki left and WiDr right. Symbols represent experimental data with their standard deviations, the lines are visual guides.

In the pretreatment setting, low dose 2-DG (0.1 mM for CaSki and 0.7 mM for WiDr) in combination with photon irradiation in the standard fraction dose of 2 Gy yielded a survival of 36.4 ± 0.7% of 2-DG monotherapy survival in CaSki and 44.5 ± 2.9% in WiDr. In the synchronous setting, in which all other parameters were identical to those above with the exception of the moment of treatment application, survival rates of 36.0 ± 2.0% and 35.1 ± 4.6% were noted respectively for CaSki and WiDr. No statistically significant difference was seen in CaSki between the pretreatment and synchronous treatment, whereas a significant difference was determined in WiDr with $p < 0.05$. LD_{90} for WiDr in the pretreatment setup was 4.93/4.65 Gy for low/high 2-DG dose and 4.33/3.83 Gy for low/high 2-DG dose in the synchronous treatment setting, respectively. For a comparison of pretreatment to synchronous treatment in the photon setup see Fig. 3. The low survival of WiDr in synchronous treatment of 2-DG and photons was prevalent in all tested doses of 2-DG and irradiation and the greatest synergistic effect was seen in these setups. The superadditive reduction of the survival rate, determined by calculating the difference between additive survival rates of the monotherapies and the survival rate of combination treatment, ranged between 1.6 ± 0.73% (6 Gy, 2.5 mM 2-DG) and 7.5 ± 2.7% (2 Gy, 2.5 mM 2-DG) for WiDr in the synchronous treatment setup. When this difference is set in relation to the additive survival rates of the monotherapies, the potential of combination therapy at already low survival rates is more suitably represented. This relative reduction of the survival rate ranged between 15.9 ± 11.3% (2 Gy, 0.7 mM 2-DG) and 54.8 ± 22.5% (6 Gy, 2.5 mM 2-DG). The highest relative reduction of the survival rate was observed at the highest radiation dose. The survival curves of the combination therapy for both low and high 2-DG doses in the WiDr synchronous treatment setup were below the isobolograms, which were calculated for the respective 2-DG doses (Fig. 4). This was the only setup in which the respective survival curves or their error bars did not cross the isobolograms. Statistical analysis for all data points determined a statistically significant difference between normalized treatment group and control group as well in this setting ($p < 0.05$).

When irradiation was performed with carbon ions in the pretreatment setting with the aforementioned doses of 2-DG and irradiation, survival rates of 8.1 ± 1.5% for CaSki and 4.7 ± 1.4% for WiDr were seen. In the carbon ion setting, synchronous treatment yielded a clonogenic survival of 8.5 ± 1.0% for CaSki and 4.8 ± 1.8% for WiDr. The standard deviations of the combination treatment curves crossed the isobologams in all ^{12}C setups. Although the combination with 2-DG relatively benefited photon therapy, carbon ion combination therapy was still more effective at equal irradiation dose (Fig. 5) with a high statistical significance ($p < 0.001$ for all data points). RBE values at 10% survival were 2.75 for CaSki

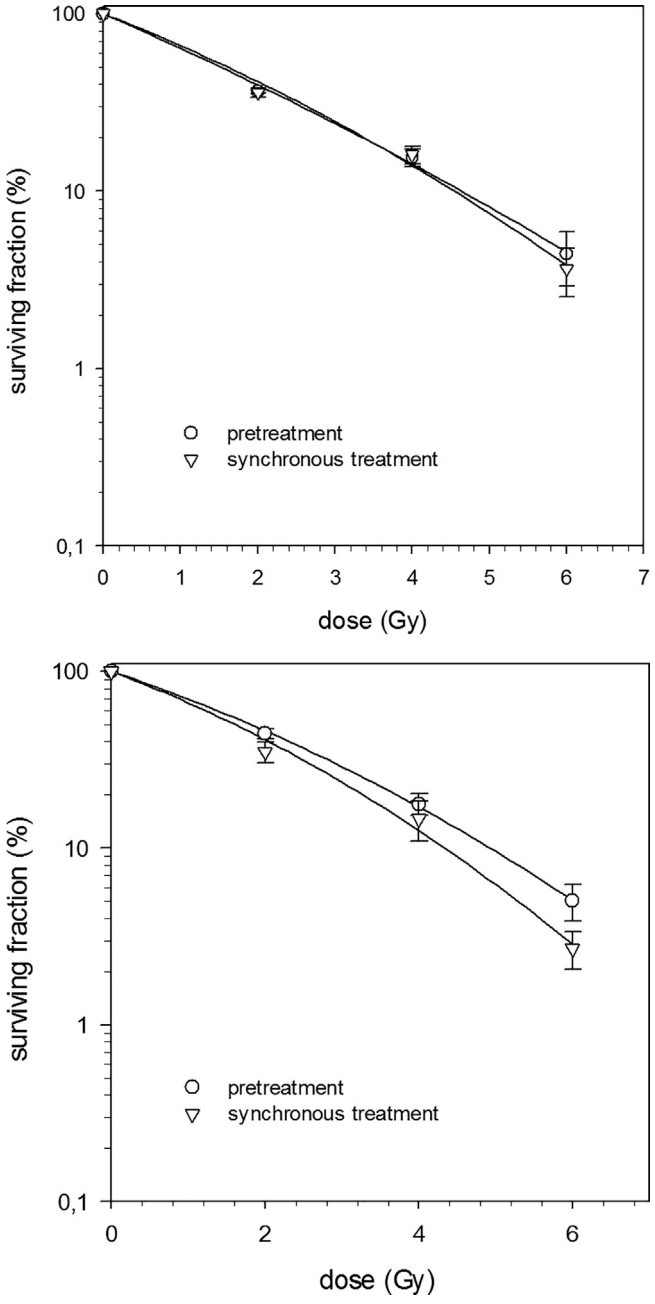

Fig. 3. Comparison of clonogenic survival between low dose 2-DG as a pretreatment to photon therapy and synchronous combination therapy. Results for CaSki (left) and WiDr (right). Doses were 0.1 mM and 0.7 mM, respectively. Symbols represent experimental data with their standard deviations, the lines are results of the fit described in the text.

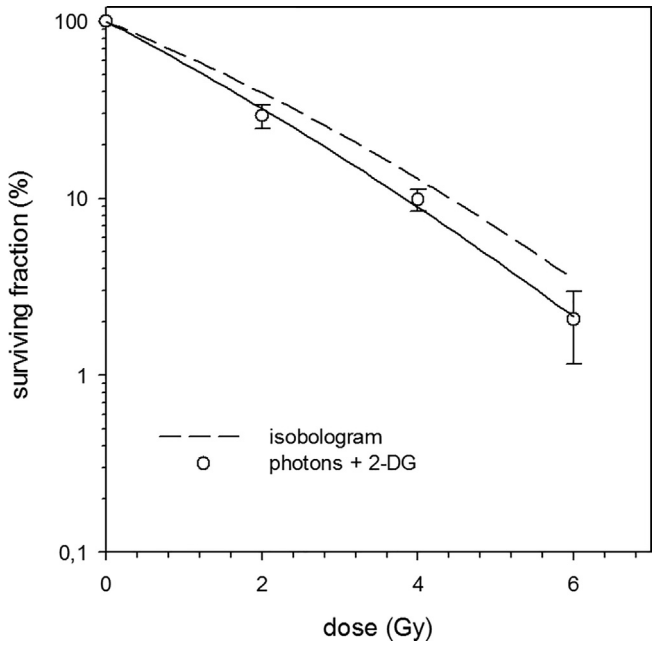

Fig. 4. Comparison of clonogenic survival between synchronous combination therapy of high dose 2-DG with photon irradiation in WiDr and the matching isobologram. Symbols represent experimental data with their standard deviations, the lines are results of the fit described in the text.

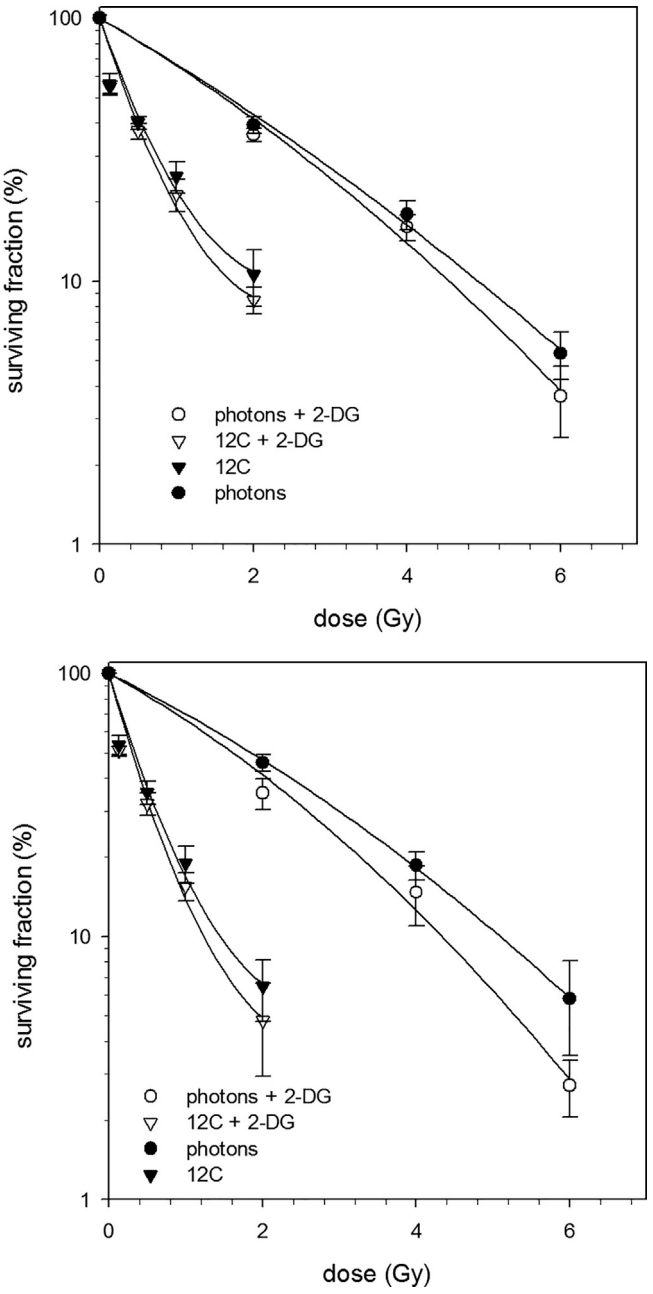

Fig. 5. Comparison of clonogenic survival between synchronous combination therapy of low dose 2-DG with photon and carbon ion irradiation. CaSki left and WiDr right. Symbols represent experimental data with their standard deviations, the lines are results of the fit described in the text.

and 3.71 for WiDr after pretreatment and 2.68 for CaSki and 3.51 for WiDr after synchronous treatment with low dose 2-DG.

4. Discussion

The high α values found in ^{12}C irradiation may be explained by the high LET of heavy ions. Low α/β ratio in photon monotherapy has been associated with high RBE [30]. WiDr, which was more resistant to photon irradiation in monotherapy than CaSki, had the lower α/β ratio in photon monotherapy and a higher RBE of ^{12}C. The efficacy of ^{12}C irradiation was therefore higher for WiDr than for CaSki. Photon irradiation α/β ratios were comparable to those of studies done in vivo, e.g. α/β of 26 Gy for cervical and 11.1 Gy for colorectal carcinoma [31,32]. The RBE of ^{12}C found in this study is similar to the results reported in previous studies on different cell lines [33,34]. WiDr was also affected more by the combination of photon irradiation with 2-DG. Both therapies were able to overcome WiDr's radioresistance.

A trend towards an increase of α values, which is associated with higher radiation sensitivity was observed in 2-DG combination treatment. Significant elevation of α values was demonstrated for ^{12}C irradiation. An increase of α values reflects higher radiation sensitivity [33].

The 2-DG dose needed for radiosensitization was relatively low. Effects of 2-DG have often been studied at higher doses up to 20 mM [28]. These studies often had a medium change following application of 2-DG in <24 h [35,36,37]. Perumal et al. changed the medium as early as 3 h after application [38]. In other cases the high dose was used to achieve a measurable effect in as early as 24 h [39] and not 8–9 days as presented here. These differences explain the higher doses compared to our study. The results of the simultaneous treatment group suggest that the duration of treatment with 2-DG can also be shortened.

The combination of photon irradiation and 2-DG led to an additive effect on colony formation in CaSki with a trend towards superadditivity under almost all experimental conditions. A trend towards superadditivity was seen for WiDr when 2-DG was added 20 h before irradiation and a statistically significant superadditive effect was seen after simultaneous application of irradiation and 2-DG. Simultaneous treatment was more effective for WiDr. The superadditive potential of simultaneous treatment had already been shown for 2-DG in an early murine experiment [40]. Nevertheless, many studies were conducted under pretreatment conditions hereafter [14,15]. In the present study, the synergistic potential of 2-DG was lost within the 20 h of incubation before irradiation in the pretreatment setup. Apparently, this period was sufficient for a metabolic adaptation in the surviving cells to these doses that diminished a later synergistic effect.

The difference between the effect of 2-DG on the two cell lines becomes more discernable when observing the survival data in monotherapy. The decrease of survival in WiDr can clearly be divided into two linear phases changing at 4 mM, whereas CaSki's decrease of survival is exponential. CaSki's colony formation was also more sensitive to lower doses of 2-DG. Large differences between cell lines regarding 2-DG therapy response have been reported before [41]. In the case of CaSki the high sensitivity to 2-DG has been linked to a high glucose metabolism [42].

To the best of our knowledge, there is no other study on the combination therapy of 2-DG and carbon ion irradiation. The combination led to an additive effect with a trend towards superadditivity. Accordingly, RBE values at 10% survival were only insignificantly higher for combination treatment in both cell lines in comparison to monotherapeutic irradiation. Survival was significantly lower compared to equal doses in the photon setting ($p < 0.001$). As a result, much lower doses could be used in order to achieve the same clonogenic cell death rates. This is in accordance with findings from other studies [43,44]. The effect of radiosensitization in WiDr in simultaneous treatment could not be reproduced in the heavy ion setting, only a trend towards a synergistic effect was observed. Similar observations have been made before with different cell lines and different substances with high potential for radiosensitization in photon irradiation [45]. Nonetheless, multiple studies have shown that radiosensitization occurs in different cell lines in heavy ion combination therapy as well [46,47,48]. In the case of the present study, an antimetabolite was used that restricts DNA repair mechanisms. While photon irradiation primarily causes non-DSB clustered DNA lesions, the characteristic property of high LET radiation is the induction of DSB [49]. DSB are repaired with less success than other DNA damage

because of a high error rate [50,51]. Sublethal radiation damage as it often occurs with photon irradiation can become fatal due to missing repair mechanisms and lead to a synergistic effect of photon irradiation and antimetabolite treatment. The lack of measurability of a synergistic effect in the ^{12}C setup can in turn be explained by a lesser importance of these repair mechanisms. This is supported by the high alpha values in the ^{12}C setups which have been linked to irreparable cell damage [52].

At high radiation doses the relative reduction of the survival rate by combination therapy was highest, which shows that especially the highly resistant tumor cells can be targeted by combination therapy for maximum reduction of tumor load.

The advantages of carbon ion irradiation cannot be fully comprehended in an in vitro setting. Our department has experienced success in sparing structures at risk during carbon irradiation [53,54], which is another benefit that can be expected during the clinical trials following this study.

In terms of feasibility, 2-DG is a valid option for cancer therapy with wide applicability, as it is regarded to be a safe substance [55,56]. Mohanti et al. successfully tested the substance in doses of up to 200 mg/kg on glioma patients as early as 1996 with no relevant toxicity [16]. Thus the effective doses applied and considered safe in vivo where considerably higher than those of the present study.

5. Conclusions

In the present study we investigated the radiosensitizing potential of the antimetabolite 2-deoxyglucose in combination with photon and carbon ion irradiation in vitro in two cell lines of pelvic tumors commonly found in females.

2-DG was able to radiosensitize cells successfully when applied simultaneously with irradiation. Whether the moment of the application of 2-DG has relevant effects in vivo is yet to be explored. High RBE carbon ion irradiation can be complemented with 2-DG for an additive effect and maximum reduction of cell survival.

Both the combination of photon therapy with 2-DG as well as the combination of irradiation with carbon ions and 2-DG are effective measures to counter radioresistant cancer cells like WiDr. The effectiveness of these therapies could also be used to lower the radiation dose.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

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