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ORIGINAL ARTICLE

Further mechanistic unravelling of the influence of the cell cycle effects on the radiosensitising mechanism of vinflunine, in vitro

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Abstract
Purpose Vinflunine is an innovative microtubule inhibitor belonging to the vinca alkaloid class that possesses radiosensitising properties, which could lead to promising activity in chemoradiation studies in the clinic.
Method In the current study, different incubation times with vinflunine, immediately before radiation and different time intervals between vinflunine treatment and radiation were investigated, in vitro, using four different human tumour cell lines differing in cell type and p53 status. Results were correlated with the cell cycle distribution at the moment of radiation, in order to elucidate the role of cell cycle perturbations caused by vinflunine on its radiosensitising effect.
Results Radiosensitisation was observed in all cell lines, and maximal radiosensitisation was both cell line- and schedule-dependent. The cell cycle distributions were cell line-dependent also, and when correlated with the observed radiosensitising effects could explain many (but not all) of the radiosensitising properties of vinflunine.
Conclusion The cell cycle perturbations caused by vinflunine may definitely have an impact on its radiosensitising potential, but other factors must play a role because of some unaccountable differences between cell cycle distribution and the radiosensitising potential.

Keywords Vinflunine · Microtubule inhibitor · Radiosensitisation · Chemoradiation · Cell cycle perturbation

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Introduction

Vinca alkaloids are used as antitumour agents in the treatment of both haematological malignancies and several solid tumours for more than 40 years. Vinflunine (VFL: 20′,20′-difluoro-3′,4′-dihydrovinorelbine) is an innovative microtubule inhibitor belonging to the vinca alkaloid class. It is a semisynthetic vinca alkaloid obtained by hemisynthesis using superacidic chemistry [10].

Mechanism of action of VFL

The antiproliferative activity of the vinca alkaloids in general, and of VFL in particular, arises from their interaction with tubulin, the major component of microtubules in the mitotic spindle. These drugs diminish microtubule dynamics and assembly, resulting in perturbation of mitosis. The affinity of VFL for the vinca alkaloid binding domain on tubulin is much weaker than that of the other vinca alkaloids [23]. However, VFL also suppresses both "dynamic instability" and "treadmilling", but with some differences. Combined with a high intracellular binding, this may lead to different effects on cell cycle progression and cell death [26–28].

VFL has several peculiar drug characteristics. In comparison with the other vinca alkaloids, it exhibits a lower in vitro activity on cell proliferation and mitotic block, shows

a markedly superior in vivo antitumour activity [17, 19], is a far less potent inducer of drug resistance, is less cross-resistant to multidrug resistant tumours [9, 21], shows a higher level of tolerance [3, 20], and finally, might cause less (neuro)toxicity [3, 23].

Preclinical and clinical antitumour activity

VFL exerts in vitro cytotoxic activity against a wide spectrum of tumour cell lines with IC50-values ranging from 10^{-9} to 10^{-7} M, when tested in two murine leukaemias as well as in seven human cell lines derived from lung, colon, prostate, breast, ovarian, and bladder tumours [19]. VFL also proved to be active, both in terms of survival prolongation and tumour growth inhibition, in a panel of transplantable tumour models (both murine and human) with different biological properties and chemosensitivities [3, 17, 20]. Overall, a consistently high level of antitumour activity has been documented in these pre-clinical studies, superior to vinorelbine and the classic vinca alkaloids. Of particular note, this activity was obtained using VFL doses that were not associated with any excessive toxicity. Overall, these data indicated a favourable profile and stimulated further development and initiation of clinical trials with VFL [17].

Early clinical studies demonstrated an interesting level of activity in patients with bladder cancer [6], non-small cell lung cancer (NSCLC) [1] and breast cancer [2], prompting further investigation in phase III trials for each of these tumour types [24].

VFL and radiation

Novel treatment strategies in the field of chemoradiation preferably should be based on adequate pre-clinical data, in order to optimise the clinical application of the combination. Because the enhancement of radiation effects may be influenced by a redistribution of the cell cycle phase [4], we studied the radiosensitising properties of VFL, in vitro, together with its cell cycle effects in an earlier study [32]. 24 h treatment with VFL immediately followed by radiation caused a dose-dependent radiosensitising effect in four human tumour cell lines (ECV304, CAL-27, MCF-7 and H292). Dose enhancement factors (DEF) ranged from 1.57 to 2.29 in the different cell lines. Next to these radiosensitising properties, a concentration-dependent G2/M block was observed after 24 h incubation with VFL. This was of interest because the G2/M phase is usually considered to be the most radiosensitive cell cycle phase [33, 37]. However, the exact role of this G2/M arrest in the radiosensitising effect caused by VFL still needed further investigation. We anticipated—regarding the progress of the G2/M blockade—a clear schedule-dependent radiosensitising effect. In this view, short incubation times (<24 h) with VFL might already show a radiosensitising effect, because a significant G2/M arrest had already been observed after 4 h of incubation, while small intervals between VFL treatment and radiation were expected to result in a decrease of the radiosensitising effect of VFL as a result of rapid recycling of the cells, even from 3 h on after removal of VFL.

Therefore, the current study investigates: (1) different incubation times of VFL immediately before irradiation and (2) different time intervals between VFL treatment and radiation. In addition, these results are correlated with the cell cycle distribution at the moment of radiation. Herewith, we tried to elucidate the role of cell cycle perturbations caused by VFL to its radiosensitising properties.

Materials and methods

Cell lines

Four different human tumour cell lines were used in this study, all obtained from the ATCC Cell Biology Collection: ECV304, an epidermoid bladder cancer cell line (mutant p53); CAL-27, a squamous cell carcinoma cell line of the tongue (mutant p53); MCF-7, a breast cancer cell line (wt p53); and H292, a mucoepidermoid non-small cell lung cancer cell line (wt p53). ECV304 cells were cultured in Medium 199 (Invitrogen, Merelbeke, Belgium); CAL-27 and MCF-7 cells in DMEM medium (Invitrogen) supplemented with 2 mM glutamine (Invitrogen); and H292 cells in RPMI-1640 medium (Invitrogen), supplemented with 2 mM glutamine and 1 mM sodium pyruvate (Invitrogen). All media were completed with 10% foetal calf serum (Invitrogen), no antibiotics were added. Cultures were maintained in exponential growth at 37°C in a humidified 5% CO_2 atmosphere.

VFL

VFL was kindly provided by Institut de Recherche Pierre Fabre, Boulogne, France. Each vial consisted of 2 ml containing 50 mg free base in solution (i.e., 25 mg/ml). It was diluted in sterile normal saline (0.9% NaCl) to make a stock solution of 30 μM and was stored at 4°C (no longer than 2 months). Before use, the stock solution was further diluted in 0.9% NaCl to the desired concentration.

Chemoradiation experiments

Cells were harvested from exponential phase cultures (at 50–75% confluence) by trypsinisation, counted and plated at optimal seeding densities in 48-well plates, to assure

exponential growth during the experiments. Cell densities were about 40, 70, 100 and 120 cells/well for ECV304, H292, CAL-27 and MCF-7, respectively. After a 24 h recovery, cells were treated with a fixed concentration of VFL, depending on the cell line, i.e. 50 nM VFL in ECV304, 40 nM VFL in H292, 35 nM VFL in MCF-7 and 20 nM VFL in CAL-27. These concentrations were chosen because they caused a radiosensitising effect in our earlier studies using 24 h incubation immediately followed by radiation [32].

Two treatment schedules were investigated. In the first schedule, different incubation times (8, 24, 32, 48 h) of VFL were studied, immediately followed by radiation (Cobalt-60 γ rays, 0–8 Gy, at room temperature). In the second schedule, different time intervals between VFL treatment and radiation were tested. Therefore, cells were treated for 24 h with VFL and irradiated immediately (24 + 0), 8 h (24 + 8) or 24 h (24 + 24) later. A diagram of these different treatment schedules is presented in Fig. 1.

After radiation (first schedule), or after the 24 h incubation period (second schedule), cells were washed with drug free medium. Cell survival was determined by the sulforhodamine B (SRB) assay, 7 or 8 days (about 6 doubling times) after radiation. It was performed according to the method of Skehan et al. [34] and Papazisis et al. [29], with minor modifications. The SRB is a reliable assay in these circumstances, as described previously [30]. Each VFL concentration was tested six times within the same experiment. All experiments were performed at least three times.

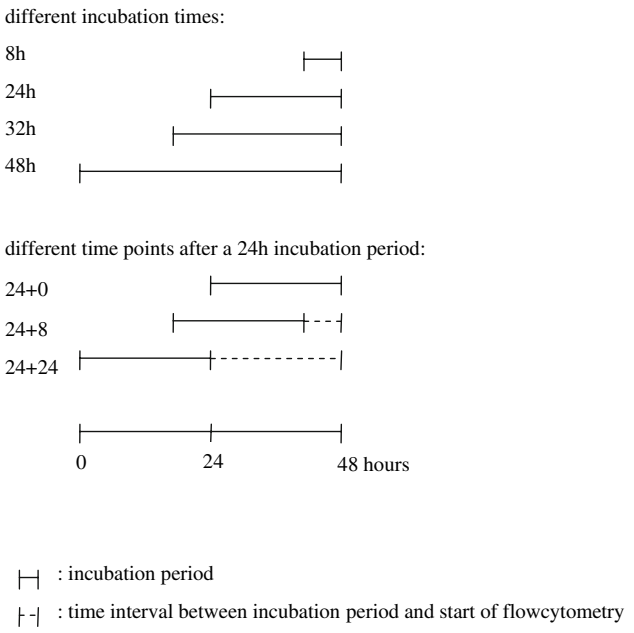

Fig. 1 Different treatment schedules used in the chemoradiation and the cell cycle experiments

Cell cycle experiments

Exponential growing cells (at 50–75% confluence) were trypsinised, counted and plated in 6-well plates. In order to assure exponential growth during the experiments, seeding densities were about 75,000 cells per well. After a 24 h recovery period, cells were treated with the same treatment schedules (see Fig. 1), but without radiation, i.e. 8, 24, 32, 48 h of continuous incubation or different time points after a 24 h incubation period (24 + 0, 24 + 8, 24 + 24), before cell cycle analysis. The concentrations used were those resulting in a clear G2/M block in our previous experiments [32], i.e. 150 nM VFL for ECV304, H292 and MCF-7 cells and 100 nM VFL for CAL-27 cells. To investigate whether VFL-induced cell cycle perturbations might play a key role in the radiosensitising effect, flow cytometry was performed at the time point that cells were irradiated in the above-mentioned treatment schedules. By performing these experiments, a clear picture of the cell cycle distributions caused by VFL at the moment of radiation will be presented.

Cell cycle analysis was performed by flow cytometry after staining of DNA according to the Vindelov method [39], as described earlier [32]. Samples were analysed in a FACScan flow cytometer (Becton-Dickinson, San José, CA, USA).

Data analysis and statistics

Chemoradiation experiments

The survival rates were calculated by: mean optical density (OD) of treated cells/mean OD of untreated cells × 100%. The radiation dose-survival curves were corrected for the cytotoxic effect of VFL alone (the curves were displaced in a vertical direction, so that all dose-survival curves started at 100% survival). These curves were then fitted according to the linear-quadratic model (survival = $\exp(-\alpha D - \beta D^2)$, using WinNonlin (Pharsight, Palo Alto, CA, USA)), in order to calculate the following parameters: the ID50, i.e. the radiation dose causing 50% growth inhibition; the SF2, the surviving fraction at 2 Gy; and the mean inactivation dose (MID), which was calculated by numerical integration of the linear-quadratic curve [11]. A two-sample t test was used to investigate significant differences between ID50 and MID values. Statistical significance was defined at the level of $P < 0.05$. The results are expressed as mean ± standard error. Radiosensitisation was represented by the dose enhancement factor (DEF): ID50 of the untreated cells/ID50 of the cells treated with VFL. In Figs. 2 and 3, the radiation dose-survival curves are presented as the unfitted representative mean of the different independent experiments.

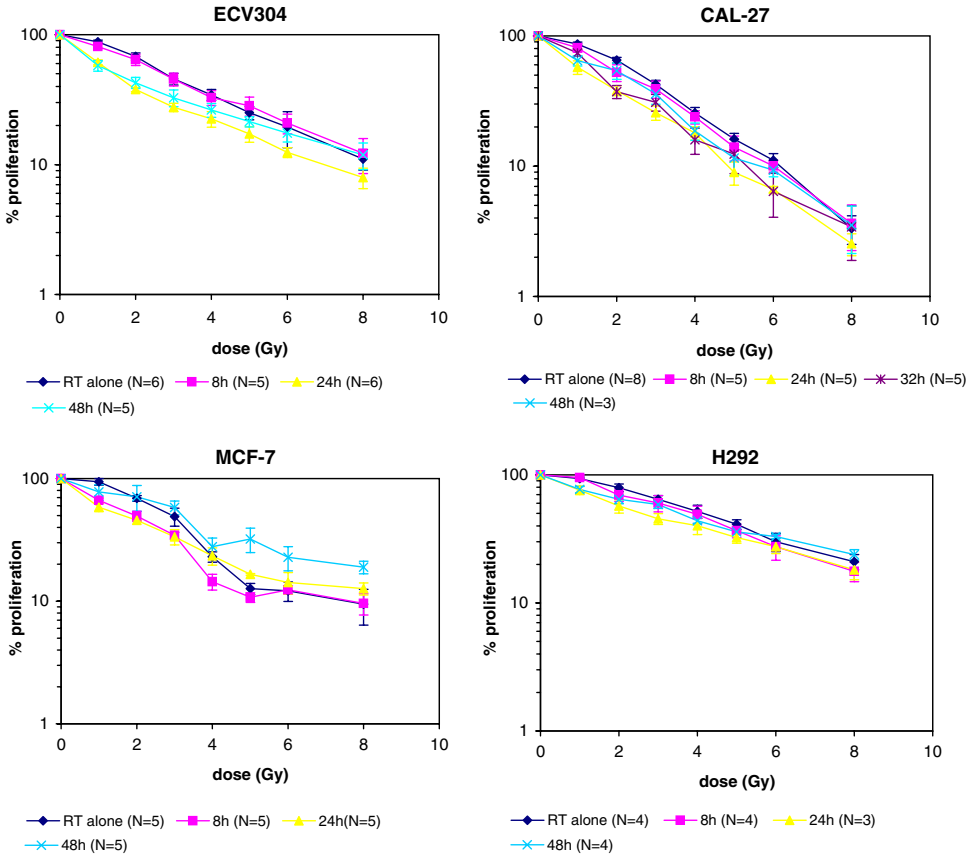

Fig. 2 Unfitted radiation dose-survival curves of four human tumour cell lines when treated with radiation alone or the combination of vinflunine and radiation using different incubation times (mean ± standard error) *RT* radiation, *VFL* vinflunine, *Gy* gray, *N* number of independent experiments

Cell cycle experiments

Flow cytometric data were analysed using Cell Quest (Becton Dickinson). A two sample *t* test was used to investigate the significance of differences between the percentages of cells in different cell cycle phases after treatment versus the untreated cells and versus the 24 h incubation with VFL. Statistical significance was defined at the level of $P < 0.05$.

In our experiments, polyploid cell populations appeared after VFL treatment. Therefore, besides the normal cell cycle phases G1, S and G2/M; S_2 (2nd synthesis phase, without previous mitosis) and polyploid G2/M [$(G2/M)_2$, cells in G2/M after S_2], with a double DNA content compared to cells in normal G2/M, were explored.

Results

Influence of the incubation time on the radiosensitising potential of VFL

Figure 2 shows the unfitted radiation dose-survival curves (mean of the different independent experiments) of the four human tumour cell lines treated with radiation alone or with the combination of VFL and radiation. The cells were treated with a fixed concentration of VFL using different incubation times (8, 24, 32, 48 h), immediately before radiation. The radiation doses ranged from 0 to 8 Gy. Since the survival data were corrected for the cytotoxic effect of VFL alone, all the dose-survival curves start at 100% survival at 0 Gy. The radiation parameters are summarised in Table 1. As evident from Fig. 2 and Table 1, cell line-related differences were observed regarding the influence of the incubation time on the radiosensitising potential of VFL. In ECV304, CAL-27 and H292 a similar course was seen. In these cell lines, the short incubation period of 8 h did not result in a sensitising effect (DEF = 1.09, 1.22 and 1.16, respectively). The previously investigated schedule of 24 h incubation with VFL immediately prior to radiation caused a maximal radiosensitising effect (DEF = 2.06, 2.04 and 1.61, respectively), which declined towards the 32 and 48 h continuous incubation with the same VFL concentration (DEF = 1.84, 1.58 and 1.29, respectively). In MCF-7, however, both the 8 h (98% survival) and 24 h (50% survival) schedule resulted in an equal radiosensitising potential of VFL (DEF = 1.67), which in turn decreased towards the 48 h incubation period (DEF = 1.31).

In ECV304 cells, also different concentrations of VFL (250–1,500 nM VFL) were tested in the 8 h incubation schedule, to identify whether more toxic concentrations (80–60% survival) for a short incubation period would lead to a radiosensitising effect. However, concentrations resulting

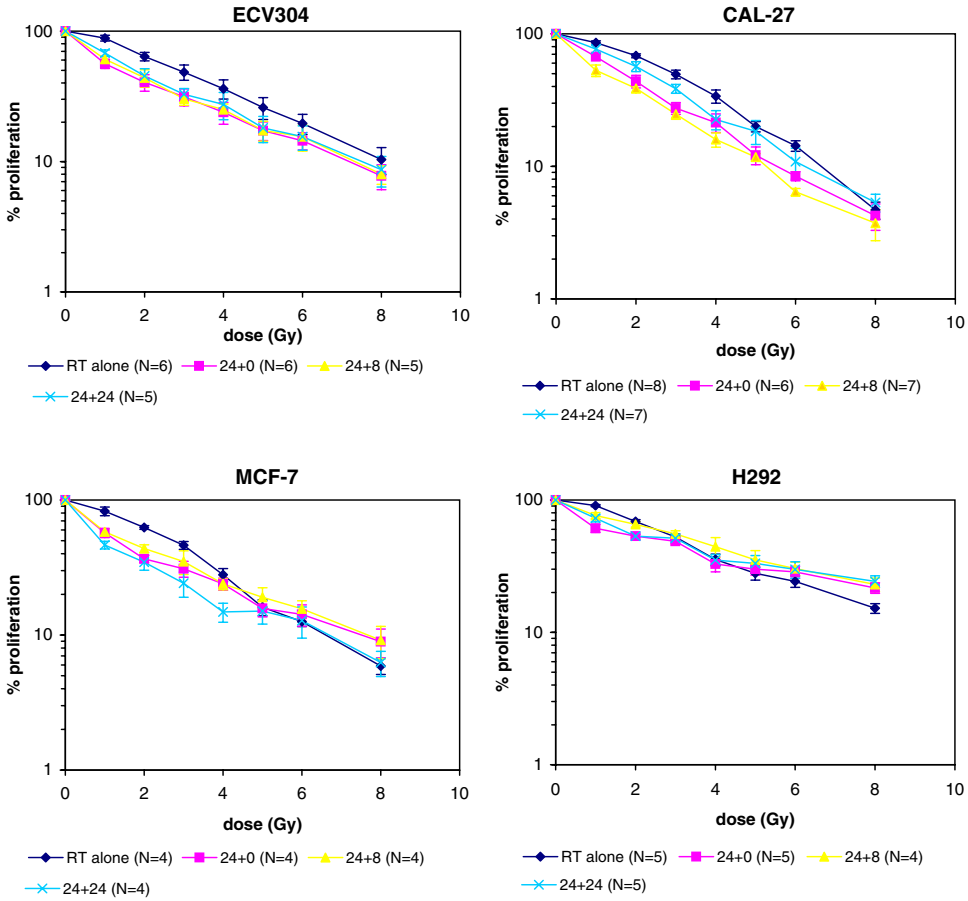

Fig. 3 Unfitted radiation dose-survival curves of four human tumour cell lines when treated with radiation alone or the combination of vinflunine and radiation using different time intervals between 24 h vinflunine treatment and radiation (mean ± standard error) *RT* radiation, *VFL* vinflunine, *Gy* gray, *N* number of independent experiments

in 60% cell survival still did not cause an increase of cell kill in combination with radiation (DEF = 1.13) (results not shown). To eliminate a possible contribution of the starting point of treatment on the radiosensitising effect in CAL-27 cells, also a 32 h continuous incubation with VFL was investigated, next to the 24 + 8 schedule.

So, cell line-related differences were observed with different incubation times of VFL prior to radiation. Generally, 8 h incubation caused no radiosensitisation, 24 h incubation caused a maximal radiosensitising effect, which declined towards a 48 h incubation period. Only in MCF-7 cells, both 8 and 24 h incubation with VFL showed a similar radiosensitising potential.

Influence of a time interval between 24 h VFL treatment and radiation on the radiosensitising potential of VFL

Figure 3 shows the unfitted radiation dose-survival curves (mean of the different independent experiments) of the four human tumour cell lines using different time intervals between 24 h VFL treatment and radiation. The cells were treated with a fixed concentration of VFL during a 24 h incubation period followed by radiation after 0, 8 or 24 h. The radiation parameters are also depicted in Table 1.

Regarding the influence of a time interval between 24 h VFL treatment and radiation on the radiosensitising potential of VFL, clear cell line-dependent differences were observed. In ECV304 and H292 cells, a maximal radiosensitising effect (DEF = 2.01 and 1.46, respectively) was seen when the 24 h VFL treatment was immediately followed by radiation (24 + 0 schedule). In ECV304, the radiosensitising effect gradually decreased with an increasing time interval between VFL treatment and radiation. An 8 h time interval before radiation resulted in a DEF of 1.71, a 24 h interval in a DEF of 1.57. However, in H292, the radiosensitising effect of VFL disappeared in the 24 + 8 schedule (DEF = 0.99), while in the 24 + 24 schedule it was still present but decreased (DEF = 1.26), compared to 24 + 0. In CAL-27, maximal radiosensitisation was observed in the 24 + 8 schedule. The DEF was 2.36 versus 1.79 immediately after the 24 h incubation period (24 + 0). A 24 h interval between VFL treatment and radiation (24 + 24) resulted in a manifest decrease of radiosensitisation, the DEF was 1.36. In MCF-7, however, maximal radiosensitisation was seen after a 24 h interval between VFL treatment and radiation (24 + 24 schedule: DEF = 2.44). The effect was lower in the 24 + 0 schedule (DEF = 1.87) and even less in 24 + 8 (DEF = 1.66).

Table 1 Percentage survival, number of experiments (*N*), mean SF2, MID, ID50 and DEF of the four human tumour cell lines using different treatment schedules with VFL; mean values ± standard error

Cell line (conc. VFL)	Treatment schedule	Survival (%)	*N*	Mean SF2	Mean MID	Mean ID50	Mean DEF
Different incubation times with VFL immediately followed by radiation							
ECV304	Control	100	6	66.05 ± 3.64	3.58 ± 0.27	3.06 ± 0.27	
	8 h	96 ± 5	4	61.97 ± 5.59	3.77 ± 0.55	2.90 ± 0.38	1.09 ± 0.05
	24 h	73 ± 9	6	36.16 ± 2.55[a]	1.99 ± 0.14[a]	1.49 ± 0.10[a]	2.06 ± 0.16
	48 h	36 ± 4	3	36.92 ± 7.86[a]	2.11 ± 0.51[a]	1.67 ± 0.33[a]	1.84 ± 0.17
CAL-27	Control	100	8	64.17 ± 2.58	3.01 ± 0.12	2.17 ± 0.14	
	8 h	83 ± 11	4	55.80 ± 4.42	2.73 ± 0.19	2.31 ± 0.22	1.22 ± 0.12
	24 h	55 ± 14	7	35.00 ± 4.33[a]	1.88 ± 0.18[a]	1.42 ± 0.17[a]	2.04 ± 0.20
	32 h	39 ± 17	4	44.48 ± 3.98[a]	2.30 ± 0.28[a]	1.80 ± 0.17[a]	1.45 ± 0.08
	48 h	32 ± 14	4	54.94 ± 1.78[a]	2.54 ± 0.11[a]	1.96 ± 0.26	1.58 ± 0.13
MCF-7	Control	100	8	65.28 ± 2.33	3.36 ± 0.19	2.93 ± 0.16	
	8 h	98 ± 15	4	44.24 ± 5.56[a]	2.45 ± 0.31[a]	1.82 ± 0.23[a]	1.67 ± 0.18
	24 h	50 ± 14	7	41.30 ± 3.50[a]	2.33 ± 0.23[a]	1.80 ± 0.18[a]	1.67 ± 0.08
	48 h	17 ± 9	3	55.47 ± 11.52	2.88 ± 0.58	2.82 ± 0.73	1.31 ± 0.46
H292	Control	100	10	78.04 ± 2.18	4.86 ± 0.20	4.28 ± 0.22	
	8 h	99 ± 8	5	73.73 ± 6.08	4.53 ± 0.42	3.87 ± 0.54	1.16 ± 0.11
	24 h	81 ± 10	9	56.73 ± 3.79	3.76 ± 0.45	2.77 ± 0.29	1.61 ± 0.08
	48 h	57 ± 22	4	63.57 ± 4.45[a]	4.25 ± 0.49	3.29 ± 0.35	1.29 ± 0.09
Different time intervals between 24 h VFL treatment and radiation							
ECV304	Control	100	6	65.80 ± 5.16	3.66 ± 0.38	3.09 ± 0.35	
	24 + 0	72 ± 12	5	36.97 ± 6.35[a]	2.13 ± 0.34[a]	1.59 ± 0.25[a]	2.01 ± 0.13
	24 + 8	67 ± 12	4	41.19 ± 5.97[a]	2.36 ± 0.36[a]	1.67 ± 0.23[a]	1.71 ± 0.07
	24 + 24	71 ± 11	3	43.64 ± 6.90[a]	2.53 ± 0.48	1.91 ± 0.35[a]	1.57 ± 0.09
CAL-27	Control	100	6	67.47 ± 2.67	3.31 ± 0.18	3.00 ± 0.16	
	24 + 0	55 ± 11	6	43.29 ± 4.18[a]	2.33 ± 0.21[a]	1.73 ± 0.18[a]	1.79 ± 0.14
	24 + 8	43 ± 11	3	32.95 ± 6.50[a]	1.83 ± 0.29[a]	1.34 ± 0.21[a]	2.36 ± 0.38
	24 + 24	32 ± 13	3	54.66 ± 5.77[a]	2.72 ± 0.21[a]	2.29 ± 0.31[a]	1.36 ± 0.12
MCF-7	Control	100	9	62.75 ± 2.11	3.08 ± 0.09	2.69 ± 0.11	
	24 + 0	57 ± 4	8	35.06 ± 2.18[a]	1.93 ± 0.11[a]	1.46 ± 0.08[a]	1.87 ± 0.11
	24 + 8	46 ± 13	5	39.65 ± 3.04[a]	2.19 ± 0.18[a]	1.66 ± 0.15[a]	1.66 ± 0.16
	24 + 24	41 ± 14	4	26.91 ± 3.95[a]	1.54 ± 0.17[a]	1.17 ± 0.13[a]	2.44 ± 0.42
H292	Control	100	9	68.44 ± 1.36	3.95 ± 0.17	3.29 ± 0.13	
	24 + 0	49 ± 5	8	48.65 ± 1.24[a]	2.79 ± 0.10[a]	2.26 ± 0.10[a]	1.46 ± 0.04
	24 + 8	36 ± 4	5	63.73 ± 3.87	4.39 ± 0.55	3.39 ± 0.39	0.99 ± 0.10
	24 + 24	39 ± 7	3	53.75 ± 2.59[a]	3.25 ± 0.24[a]	2.64 ± 0.24[a]	1.26 ± 0.07

SF2 survival fraction at 2 Gy, *MID* mean inactivation dose, *ID50* radiation dose causing 50% growth inhibition, *DEF* dose enhancement factor

[a] $P < 0.05$ compared to control

Cell cycle distribution at the moment of radiation, using different incubation times with VFL

The distribution of the cells in the different cell cycle phases after different incubation times with VFL are summarised in Table 2. Incubation times of 8, 24, 32 and 48 h (same schedules as used in the chemoradiation experiments) were examined to obtain a clear picture of the cell cycle distribution at the moment of radiation. The experiments were performed in the four cell lines, with fixed concentrations resulting in a clear G2/M block after 24 h incubation in previous cell cycle experiments [32].

Cell line-related differences in the cell cycle distribution at the moment of radiation after the different incubation times were observed. After 8 h incubation with VFL, a significant amount of cells was arrested in the G2/M phase, but an almost equal amount of cells was blocked in the S phase at that time point, in all tested cell lines. Twenty-four

Table 2 Distribution of the cells in the different cell cycle phases after different incubation times with VFL, mean values ± standard error (*N* = number of independent experiments)

Cell line	Treatment schedule	*N*	Cell cycle phases				
			G1 (%)	*S* (%)	G2/M (%)	S2 (%)	(G2/M)2 (%)
ECV304	Control	6	40.6 ± 1.2	36.5 ± 1.0	18.2 ± 0.6	4.2 ± 1.0	0.7 ± 0.0
	8 h	4	14.3 ± 2.2[a]	52.1 ± 0.8[ab]	30.4 ± 2.7[ab]	4.7 ± 0.3	1.0 ± 0.1[a]
	24 h	6	19.2 ± 3.3[a]	25.1 ± 2.1[a]	47.5 ± 4.9[a]	6.1 ± 0.9	1.5 ± 0.2[a]
	48 h	3	5.1 ± 1.2[ab]	14.7 ± 2.0[ab]	42.4 ± 3.5[a]	22.4 ± 0.8[ab]	15.6 ± 0.8[ab]
CAL-27	Control	8	61.3 ± 1.3	21.3 ± 1.1	14.8 ± 0.7	2.2 ± 0.3	0.5 ± 0.1
	8 h	4	21.3 ± 1.9[ab]	38.1 ± 1.5[ab]	33.3 ± 3.3[ab]	6.1 ± 2.4	1.1 ± 0.2[ab]
	24 h	7	10.1 ± 1.8[a]	16.1 ± 0.8[a]	57.7 ± 2.9[a]	10.8 ± 1.4[a]	5.2 ± 0.6[a]
	32 h	4	10.9 ± 3.2[a]	12.9 ± 2.0[a]	34.8 ± 2.8[ab]	23.8 ± 2.8[ab]	17.4 ± 2.5[ab]
	48 h	4	4.5 ± 1.4[ab]	9.6 ± 1.8[ab]	21.3 ± 2.9[b]	21.8 ± 1.4[ab]	41.8 ± 4.9[ab]
MCF-7	Control	8	53.7 ± 1.3	25.6 ± 1.1	18.5 ± 0.8	2.0 ± 0.2	0.5 ± 0.1
	8 h	4	18.6 ± 2.4[a]	34.9 ± 2.0[a]	42.3 ± 2.7[a]	3.1 ± 0.6	1.5 ± 0.2[a]
	24 h	7	13.3 ± 1.3[a]	33.0 ± 1.8[a]	48.9 ± 2.4[a]	3.5 ± 0.3[a]	1.6 ± 0.3[a]
	48 h	3	10.7 ± 0.6[a]	27.3 ± 1.0[b]	49.3 ± 2.9[a]	8.8 ± 1.6[a]	4.4 ± 0.8[a]
H292	Control	10	56.9 ± 1.8	23.5 ± 1.6	17.2 ± 0.6	2.2 ± 0.4	0.4 ± 0.1
	8 h	5	14.0 ± 2.0[a]	39.7 ± 2.4[ab]	43.0 ± 3.3[ab]	2.3 ± 0.6[b]	1.2 ± 0.4
	24 h	9	8.8 ± 1.1[a]	22.0 ± 2.4	61.8 ± 3.1[a]	5.5 ± 0.8[a]	2.0 ± 0.3[a]
	48 h	4	4.2 ± 0.4[ab]	16.5 ± 2.4[a]	65.7 ± 1.8[a]	7.1 ± 0.9[a]	7.0 ± 0.9[ab]

[a] $P < 0.05$ compared to control

[b] $P < 0.05$ compared to 24 h

hour incubation with VFL resulted in a maximal G2/M block in ECV304, CAL-27 and H292. In MCF-7, however, the cell cycle distribution after both 8 and 24 h incubation was equal, so still a significant amount of cells was arrested in S after 24 h incubation in this cell line. The difference with the 32 and 48 h incubation period compared to 24 h, was the impressive appearance of a polyploid population in CAL-27 and ECV304 cells. In MCF-7 and H292, a significant amount of polyploid cells was also observed, but to a lesser extent than in CAL-27 and ECV304, and still accompanied by a maximal arrest of cells in G2/M.

Cell cycle distribution at the moment of radiation using different time intervals after 24 h VFL treatment

Table 3 summarises the distribution of the cells over the different cell cycle phases, different time points after 24 h VFL treatment. In these experiments, the cell cycle effects were determined 0, 8 and 24 h after VFL removal. Again, the same schedules as with the chemoradiation experiments were used, to obtain a clear picture of the cell cycle distribution at the moment of radiation.

Regarding the cell cycle distribution at the moment of radiation, different time points after 24 h VFL treatment, cell line-dependent differences were also seen in these experiments. The 24 + 0 schedule caused a maximal G2/M block in all tested cell lines; however, in MCF-7 this coincided with a similar sized arrest of cells in S, as observed in the experiments of the different incubation times (24 h incubation). The differences that became apparent in the 24 + 8 schedule compared to 24 + 0 were the increased G1 population in ECV304 and MCF-7 cells and the increase of both G1 and the polyploid population in CAL-27 and H292 cells. This caused a decrease of the amount of G2/M arrested cells, in all tested cell lines. The 24 + 24 schedule resulted, in ECV304 cells, in an increased polyploid population at the expense of the G2/M blocked cells, compared to 24 + 0. In the other cell lines, 24 + 24 showed an almost equal distribution over the different phases of the cell cycle compared to 24 + 8.

Discussion

In this study, cell line-related differences in the radiosensitising effect of VFL, both after different incubation times and different time intervals between VFL treatment and radiation, are presented.

Some of these radiosensitising effects can be related to the cell cycle perturbations caused by VFL at the moment of radiation (Figs. 4, 5).

Generally, 8 h incubation caused no radiosensitisation, while 24 h incubation induced a maximal radiosensitising effect, which declined using an 48 h incubation period. The absence of radiosensitisation in the 8 h incubation schedule can be explained by an equal or even more pronounced increase in S phase cells (the most radioresistant phase of the cell cycle [35]) together with a simultaneous increase in G2/M phase cells. The maximal G2/M block observed with 24 h incubation can explain the maximal radiosensitising

Table 3 Distribution of the cells in the different cell cycle phases, different time points after 24 h VFL treatment, mean value ± standard error (N = number of independent experiments)

Cell line	Treatment schedule	N	Cell cycle phases				
			G1 (%)	*S* (%)	G2/M (%)	S2 (%)	(G2/M)2 (%)
ECV304	Control	6	39.3 ± 1.5	41.3 ± 1.8	17.4 ± 0.6	1.9 ± 0.4	0.4 ± 0.1
	24 + 0	5	17.5 ± 3.6[a]	24.8 ± 1.9[a]	48.4 ± 4.6[a]	6.4 ± 1.1[a]	1.1 ± 0.4
	24 + 8	4	53.6 ± 2.0[ab]	18.8 ± 1.4[ab]	19.4 ± 0.7[b]	6.4 ± 0.3[a]	1.4 ± 0.2[a]
	24 + 24	3	20.5 ± 1.6[a]	23.7 ± 2.2[a]	33.4 ± 0.8[ab]	12.7 ± 1.4[ab]	9.2 ± 1.5[ab]
CAL-27	Control	6	64.3 ± 2.2	19.3 ± 1.5	14.4 ± 0.7	1.8 ± 0.4	0.4 ± 0.1
	24 + 0	6	16.3 ± 3.3[a]	17.8 ± 0.8	53.9 ± 3.1[a]	8.2 ± 1.8[a]	4.0 ± 1.0[a]
	24 + 8	3	32.4 ± 0.9[ab]	20.9 ± 0.8[b]	21.9 ± 1.4[ab]	15.2 ± 1.0[ab]	8.4 ± 1.5[a]
	24 + 24	3	37.0 ± 1.8[ab]	21.4 ± 0.9[b]	25.0 ± 2.7[ab]	11.2 ± 0.5[a]	5.8 ± 0.3[a]
MCF-7	Control	9	54.4 ± 1.2	25.6 ± 1.0	17.3 ± 0.8	2.2 ± 0.2	0.6 ± 0.1
	24 + 0	8	12.8 ± 1.0[a]	32.7 ± 1.6[a]	49.9 ± 2.4[a]	3.3 ± 0.4[a]	1.6 ± 0.2[a]
	24 + 8	5	42.6 ± 5.6	24.1 ± 3.4	29.0 ± 2.9[ab]	3.5 ± 0.5	1.1 ± 0.1[ab]
	24 + 24	4	42.5 ± 1.8[a]	22.3 ± 1.9[b]	27.8 ± 3.4[ab]	5.2 ± 0.4[ab]	2.0 ± 0.5[a]
H292	Control	9	59.7 ± 2.7	23.0 ± 1.9	15.3 ± 0.8	1.6 ± 0.3	0.3 ± 0.1
	24 + 0	8	10.5 ± 1.5[a]	23.1 ± 2.2	59.5 ± 2.5[a]	5.1 ± 1.0[a]	1.9 ± 0.4[a]
	24 + 8	5	24.5 ± 3.5[ab]	25.8 ± 1.7	36.3 ± 4.3[ab]	10.3 ± 0.4[ab]	3.0 ± 0.4[a]
	24 + 24	3	36.5 ± 1.5[ab]	16.5 ± 0.5[ab]	41.3 ± 2.3[ab]	3.6 ± 1.2	1.9 ± 0.4

[a] $P < 0.05$ compared to control

[b] $P < 0.05$ compared to 24 h

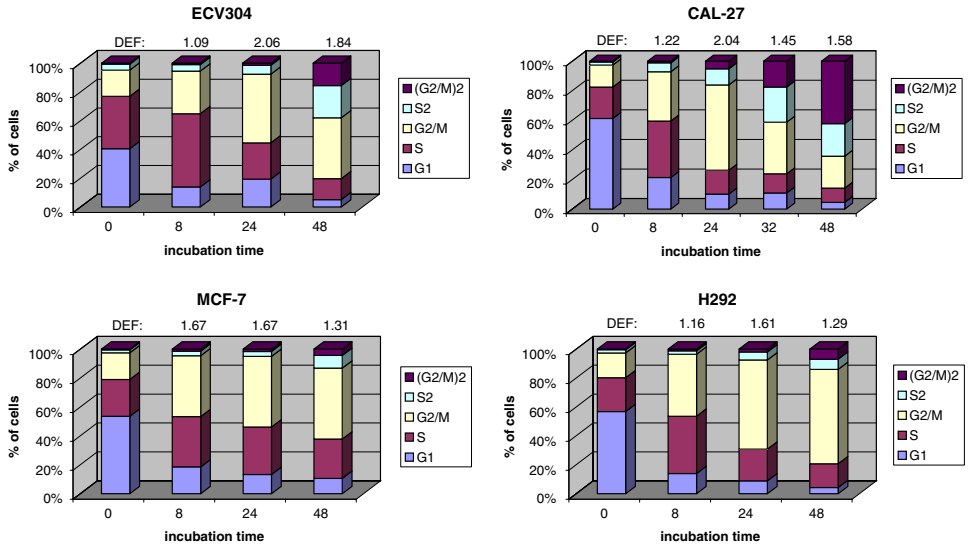

Fig. 4 Dose enhancement factors (*DEFs*) and cell cycle distributions after different incubation times with vinflunine immediately followed by radiation

effect at that time point. Only in MCF-7 cells, both 8 and 24 h incubation with VFL showed a comparable radiosensitising potential, which coincided with an equal cell cycle distribution.

Using different time intervals, the differences between the cell lines were more pronounced. Maximal radiosensitisation was observed in ECV304 and H292 in the 24 + 0 schedule, which coincided again with a maximal G2/M arrest, in CAL-27 in the 24 + 8 schedule and in MCF-7 cells in the 24 + 24 schedule.

Besides a G2/M and/or S phase blockade, the formation of polyploid cells can also have an influence on radiosensitisation. The small percentage of polyploid cells in H292 and MCF-7, compared to ECV304 and CAL-27, can possibly be explained on the basis of their p53 status. In H292 and MCF-7, both wild type p53, an increase in ploidy after prolonged mitotic arrest is countered by p53 [16]. Since ECV304 and CAL-27 cells both have a mutant p53 status, a more pronounced polyploid fraction is not unexpected. It has been described that alterations in p53 abolish the G2/M checkpoint and allow reduplication leading to a polyploid cell population in the presence of tubulin binding agents [8, 14, 16]. This explains why in these two cell lines the observed polyploid population is more pronounced. We assume that the polyploid cell population is less radiosensitive than G2/M and G1 cells, in order to explain the observed effects, such as the trend towards a decreased radiosensitising potential in the 32 and 48 h incubation

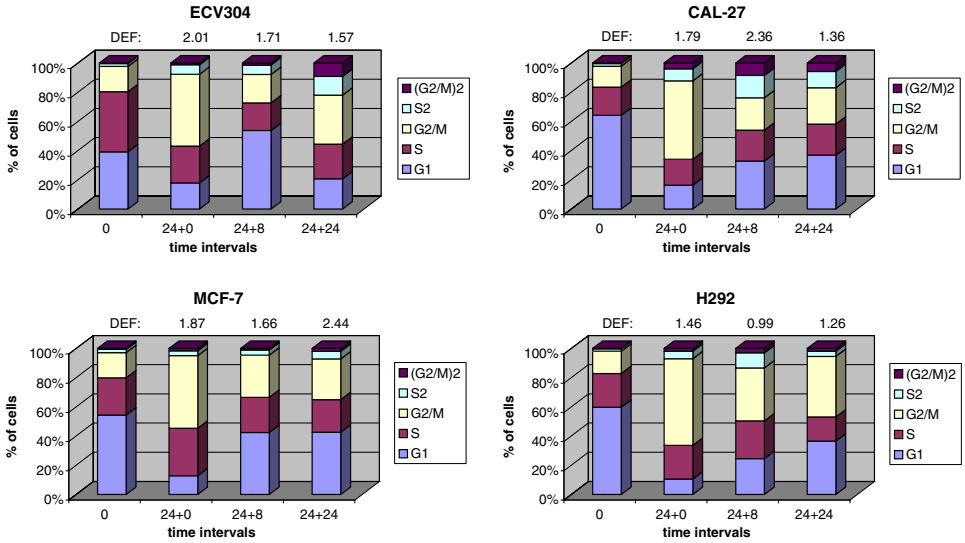

Fig. 5 Dose enhancement factors (*DEFs*) and cell cycle distributions after different time intervals between 24 h vinflunine treatment and radiation

schedule, and in the 24 + 24 schedule in ECV304 cells. To our knowledge, no unequivocal sensitivity of these polyploid cells towards radiation has been described. Recently, Hau et al. [16] conveyed the possibility that the elevated DNA content in polyploid cells could contribute for the higher sensitivity to DNA damaging agents (such as ionising radiation). This would make this type of cells even more sensitive to irradiation than cells in G2/M. On the other hand, while polyploid cells have always been regarded as being reproductively dead, Illidge et al. [18] discovered that polyploid giant cells provide a survival mechanism for p53 mutant cells after DNA damage. If polyploid cells do provide a survival advantage after genotoxic insult, then presumably they must be able to repair DNA. Somatic cell division of polyploid cells, including pairing of homologs similar to that in meiosis, may therefore represent an effective means of both DNA repair and return to diploidy and the mitotic cycle [8]. This mechanism would result in a reduced sensitivity to radiation-induced cell kill, and could support our observations/hypothesis regarding the polyploid cells.

The radiosensitising effect of vinorelbine another semi-synthetic vinca alkaloid was also described to be correlated with the cell cycle perturbations that it caused. The maximal radiosensitising effect was observed after G2/M accumulation alone or in combination with continuous polyploidisation and enhanced apoptosis [7, 13]. In the chemoradiation experiments performed with paclitaxel, another microtubule binding agent, Choy et al. [5] also found radiosensitisation caused by paclitaxel correlated with a G2/M block.

On the other hand, the correlation between the radiosensitising effect and the observed cell cycle effects of vinflunine was unclear for other treatment schedules (Figs. 4, 5). For example, the radiosensitising effect of 32 and 48 h incubation in CAL-27 cells is about equal although the cell cycle distribution is different, and the trend towards a decreased radiosensitising effect using the 48 h incubation period in H292 and MCF-7 cells is also difficult to correlate to the cell cycle observations. In CAL-27 and MCF-7 cells, maximal G2/M arrest was reached in the 24 + 0 schedule, while the maximal radiosensitising effect was not yet reached at that time point; and an equal cell cycle distribution was observed for the 24 + 8 and the 24 + 24 schedule, while the radiosensitising effect was significantly different in these treatment schedules.

So, regarding the chemoradiation and cell cycle results presented in this study, the cell cycle can definitely have an impact on the radiosensitising potential of VFL, but other influencing factors must exist also. The same holds for vinorelbine, different causative factors for radiosensitisation, besides or without G2/M accumulation, were reported: enhanced apoptosis [40] or impairment of DNA repair following radiation induced DNA damage [12]. In the case of paclitaxel, Steren et al. [36] described a radiosensitising effect without a G2/M block. Gorodetsky et al. [15] observed a G2/M block but no radiosensitisation, only an additive effect between paclitaxel and radiation. Liebmann et al. [22] considered the development of a G2/M block a necessary, but insufficient condition for paclitaxel radiosensitisation. Paclitaxel may enhance the sensitivity of tumour cells to radiation, but also additive or subadditive effects have been reported [25]. The outcome of the taxane-radiation interaction, in vitro, was thought to depend on many factors, including cell type [25], proliferation state of cells [25, 36], drug concentration [25], exposure duration [38], and timing of radiation delivery in relation to drug administration [25, 31, 36]. So, also with paclitaxel, cell cycle distributions could not explain the radiosensitising effect all by itself.

In conclusion, cell line-related differences were observed in the radiosensitising potential of VFL using different treatment schedules. Also, the cell cycle distributions caused by VFL at the moment of radiation were cell line-dependent in all the different schedules. Some, but not all of the increased or decreased radiosensitising effects could be explained by the cell cycle perturbations caused by VFL at the moment of radiation. Taken together, the cell cycle perturbations can definitely have an impact on the radiosensitising potential of VFL, but other influencing factors must exist because of some unaccountable differences between cell cycle distribution and the radiosensitising potential.

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