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ORIGINAL ARTICLE

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Cell cycle effects of vinflunine, the most recent promising Vinca alkaloid, and its interaction with radiation, in vitro

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Abstract *Purpose*: Vinflunine (VFL) is a novel third generation Vinca alkaloid with superior antitumour activity in preclinical models and an anticipated more favourable toxicity profile compared to the other Vinca alkaloids. *Method*: We investigate the radiosensitising properties of VFL and its cell cycle effects in four human tumour cell lines (ECV304, MCF-7, H292, and CAL-27). The sulforhodamine B test was used to determine cell survival, and cell cycle analysis was performed by flow cytometry. Radiosensitisation (RS) was represented by dose enhancement factors (DEFs). *Results*: Twenty-four hours treatment with VFL before radiation caused dose-dependent RS in all cell lines. This was most pronounced in ECV304 cells with RS already at VFL concentrations that reduced cell survival by 10% (IC10). DEFs ranged from 1.57 to 2.29 in the different cell lines. A concentration-dependent G2/M block was observed (starting at 4 h of incubation). After maximal G2/M blockade cells started cycling again, mainly by mitosis, while a small portion of cells started a polyploid cell cycle. Also drug removal immediately caused recycling of cells and induction of a polyploid cell population. The polyploid cell population was most impressively noticeable after prolonged incubation with VFL (48 h), in particular in CAL-27 and ECV304. This was never observed in a tested normal fibroblast cell line (Fi 360). The fate of these cells is of particular interest, but yet uncertain. *Conclusion*: VFL has radiosensitising potential. The exact role of the cell cycle effects of VFL in its radiosensitising mechanism is still not fully elucidated and requires further study.

Keywords Vinflunine · Chemoradiation · Preclinical · Radiosensitisation · Cell cycle

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Introduction

The Vinca alkaloids form an important class of antitumour agents that are widely used in the treatment of both haematological malignancies and several solid tumours. Vinflunine (VFL; 20′,20′-difluoro-3′,4′-dihydrovinorelbine; Fig. 1) is a novel third generation Vinca alkaloid obtained by hemisynthesis from vinorelbine using superacidic chemistry [8, 9]. In spite of its lower potency in vitro, VFL has markedly superior antitumour activity compared to the other Vinca alkaloids in preclinical models [15, 20, 21].

Key events in the mechanism of action of VFL and the other Vinca alkaloids are the *interaction with tubulin*, the major component of microtubules in the mitotic spindle, and the subsequent *arrest of cells in the G2/M phase* [18, 42]. Contrary to the taxanes, Vinca alkaloids prevent assembly of microtubules without affecting their disassembly. Microtubules are intrinsically dynamic polymers displaying two types of unusual dynamic behaviour, i.e. 'dynamic instability' and 'treadmilling', which appear to be important for progression through mitosis and the cell cycle [42]. Similar to the other Vinca alkaloids, VFL suppresses both these phenomena, but with some differences which, combined with a high

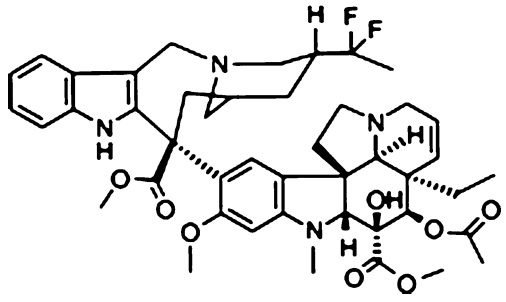

Fig. 1 Chemical structure of vinflunine

intracellular binding, may lead to different effects on cell cycle progression and cell killing [31–33].

The affinity of VFL for the Vinca-alkaloid binding domain on tubulin is much weaker than that of the other Vinca alkaloids [25]. This aspect seems of importance because strong binding to tubulin is not a prerequisite for antitumour efficacy [20], while it does seem to relate to the neurotoxicity observed in the clinic. Therefore, it has been suggested that VFL will likely result in less neurotoxicity than vinorelbine, vinblastine, or vincristine [25].

Another difference which may have clinical implications is the observation that the compound is only a weak substrate for P-glycoprotein and has shown to be a far less potent inducer of resistance than vinorelbine [6, 7].

Finally, VFL induces apoptotic cell death with caspase-3/7 activation, induction of JNK1 (c-Jun N-terminal kinase 1), and involvement of the mitochondria pathway. Differences in Bcl-2 phosphorylation within this process of apoptosis appear to be cell type and concentration-dependent [22, 36].

Preclinical studies indicated that VFL is an attractive candidate to combine with other cytotoxic agents. Synergistic effects were observed when VFL was combined with DNA-damaging agents such as cisplatin and mitomycin C, the DNA-intercalator doxorubicin, the antimetabolite 5-FU, and the topoisomerase I inhibitor camptothecin, in vitro and in vivo [1, 16, 38].

Based on all these favourable characteristics, VFL has been selected for further clinical development. Responses were observed in phase I studies [2], and preliminary phase II results in breast cancer patients indicated activity in that disease and manageable toxicity. At present, phase III trials are ongoing in non-small cell lung cancer (NSCLC), bladder cancer, and advanced breast and ovarian cancer [30].

There has been an increasing interest in the concurrent use of chemotherapy and radiation therapy in the clinic for various tumour types, such as NSCLC, head and neck, oesophageal, and cervical cancer [13, 19, 27, 35, 37]. Improved outcomes in patients are most likely a result of improved systemic and local tumour control, and from a direct interaction between cytotoxic agents and radiation. This latter aspect should preferably be investigated first in in vitro studies.

It has long been known that radiosensitivity changes during cell cycle progression. While the S phase is most radioresistant, the G2/M phase is usually considered to be the most radiosensitive [39, 40]. Therefore, synchronisation of cells in G2/M is expected to elicit the maximum response to radiation. Because VFL treatment is known to result in an accumulation of cells in the G2/M phase [20], this is an additional argument to expect that VFL has radiosensitising potential. The current study investigates the interaction between VFL and radiation in vitro, with a special interest into the possible contribution of its cell cycle effects.

Materials and methods

Cell lines

Four different human tumour cell lines have been used: ECV304, an epidermoid bladder cancer cell line; CAL-27, a squamous cell carcinoma cell line of the tongue; MCF-7, a breast cancer cell line; and H292, a muco-epidermoid lung cancer cell line. Also normal cells were used: Fi 360, a human dermal fibroblast cell line. ECV304 cells were grown in Medium 199 (Invitrogen, Merelbeke, Belgium), supplemented with 10% foetal calf serum (Invitrogen). CAL-27 and MCF-7 cells were cultured in DMEM medium (Invitrogen), supplemented with 2 mM glutamine (Invitrogen) and 10% foetal calf serum. H292 and Fi 360 cells were cultured in RPMI-1640 medium (Invitrogen), supplemented with 2 mM glutamine, 1 mM sodium pyruvate (Invitrogen), and 10% foetal calf serum. No antibiotics were added to the media. Cultures were maintained in exponential growth at 37°C in a humidified 5% CO_2 atmosphere.

Vinflunine

Vinflunine was kindly provided by Dr. Breillout (Institut de Recherche Pierre Fabre, Boulogne, France) in its clinical formulation. Each vial consisted of 3 ml containing 90 mg free base in solution (i.e. 30 mg/ml). It was diluted in sterile normal saline (0.9% NaCl) to create a stock solution with a concentration of 30 μM and was stored at 4°C (no longer than 3 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 by trypsinisation, counted, and plated at optimal seeding densities in 48-well plates to assure exponential growth during the experiments. Cell densities were about 70, 150, 200, and 300 cells/well for ECV304, CAL-27, H292, and MCF-7, respectively. After a 24-h recovery, cells were treated with different concentrations of VFL for

24 h, which each time was immediately followed by radiation (Cobalt-60 γ rays, 0–8 Gy, at room temperature). Then, cells were washed with drug free medium and kept at 37°C for 7 or 8 days (about six doubling times). Cell survival was determined by the sulforhodamine B assay, a reliable assay in these circumstances, as described by us previously [34]. Each VFL concentration was tested six times within the same experiment. All experiments were performed at least three times.

Cell cycle experiments

Cells from exponential phase cultures were trypsinised, counted, and plated in 6-well plates. After a 24-h recovery period, two different cell cycle experiments were performed as follows:

- Firstly, the effects of VFL on the cell cycle were investigated. For these experiments, cells were incubated for 24 h with different concentrations of VFL (0–400 nM), and the effects were studied immediately after incubation.
- Secondly, the G2/M block was investigated over time (cell cycle kinetics). For these experiments, not only were different incubation times investigated (4–48 h), but also different time points after a 24-h incubation (3–72 h). The concentrations used for this second set of experiments were those resulting in a clear G2/M block in the first experiments, i.e. 150 nM VFL for ECV304, H292, and MCF-7 cells, and 100 nM VFL for CAL-27 cells.

In order to assure exponential growth during the experiments, seeding densities were about 75,000 cells/well for the first set of experiments, and about 50,000 cells/well for the cell cycle kinetic experiments.

Cell cycle analysis was performed by flow cytometry. DNA was stained according to the Vindelov method, after trypsinisation [41]. In brief, cells were resuspended in 100 µl PBS and incubated with 100 µl solution A (trypsin) for 20 min at room temperature (in the dark). Then, 75 µl solution B (trypsin inhibitor spermine and ribonuclease A) was added and after 10 min incubation at room temperature (in the dark), 75 µl solution C (propidium iodide) was added for at least 30 min at 4°C. 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 OD (optical density) of treated cells/mean OD of untreated cells × 100%. Radiation dose–survival curves were fitted according to the linear-quadratic model: survival = $\exp(-\alpha D - \beta D^2)$, using WinNonlin (Pharsight, Palo Alto, CA, USA). 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). From the dose–survival curves, the following parameters were calculated: the linear component α, the quadratic component β, the ID50, the radiation dose causing 50% growth inhibition; and the mean inactivation dose (MID), which was calculated by numerical integration of the linear-quadratic curve [10]. A two-sample *t*-test was used to investigate significant differences between ID50 values. The results are expressed as means ± standard deviation.

Radiosensitisation (RS) was expressed by the dose enhancement factor (DEF): ID50 of the untreated cells/ID50 of the cells treated with VFL.

Possible synergism was determined by calculation of the combination index (CI) by the Chou and Talalay equation [3], using CalcuSyn (Biosoft, Cambridge, UK), which can be used for chemoradiation combinations [24]. The CI quantifies drug interaction in terms of additive effect (CI = 1), synergism (CI < 1), or antagonism (CI > 1). The CI takes into account both the potency (IC50 or D_m) and the shape of the dose–survival curve (m value, signifying the sigmoidicity of the dose–effect curve). The general equation for the classic isobologram is given by:

$$\mathrm{CI} = (D)_1/(D_x)_1 + (D)_2/(D_x)_2$$

where $(D_x)_1$ and $(D_x)_2$ are the doses (or concentrations) for D_1 (VFL) and D_2 (radiation) alone, required to inhibit cell growth by 50%, and $(D)_1$ and $(D)_2$ are the doses of VFL and radiation in combination that also inhibit cell growth by 50% (i.e. isoeffective as compared with the single treatments).

The $(D_x)_1$ or $(D_x)_2$ (for VFL and radiation) are calculated by the formula:

$$D_x = D_m[f_a/(1 - f_a)]^{1/m}$$

where D_m is the dose required to produce absorbance readings 50% lower than those of non-treated wells (IC50 or ID50), f_a is the fraction affected, and m is the slope of the median-effect plot. The CI values obtained from the classic (mutually exclusive) isobologram calculations were used. In short: $1.10 > \mathrm{CI} > 0.90$, $0.90 > \mathrm{CI} > 0.85$, $0.85 > \mathrm{CI} > 0.70$, and $0.70 > \mathrm{CI} > 0.30$ indicating additivity, slight synergism, moderate synergism, and synergism, respectively.

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 the differences between the percentages of cells in the different cell cycle phases after treatment with VFL versus the untreated cells.

In our experiments, polyploid cell populations appeared after VFL treatment. Therefore, besides the normal cell cycle phases G1, S, and G2/M, also the S2 (second synthesis phase, without previous mitosis) and polyploid G2/M (cells in G2/M after S2), with a double DNA content compared to cells in normal G2/M, were explored.

Results

The IC50-values (concentration of VFL causing 50% growth inhibition) of the four human tumour cell lines used were 34.1 ± 1.3, 47.2 ± 6.0, 56.3 ± 9.8, and 93.6 ± 12.3 nM VFL for CAL-27, MCF-7, H292, and ECV304, respectively. The IC50 value of the dermal fibroblast cell line, Fi 360 was 474.1 ± 63.9 nM VFL.

Chemoradiation experiments

The radiation dose–survival curves of the four tumour cell lines treated with radiation alone or with the combination of VFL and radiation are shown in Fig. 2. The cells were treated with different concentrations of VFL for 24 h, immediately before radiation. The radiation doses ranged from 0 to 8 Gy. The survival data were corrected for the cytotoxic effect of VFL alone; therefore, all dose–survival curves start with 100% at 0 Gy.

In all four cell lines, the highest VFL concentration tested resulted in a clear increase in radiosensitivity (decrease of ID50 values) ($P \leq 0.01$) (data not shown). MID, ID50, DEF, and CI values are summarised in Table 1. As evident from this table, incubation with a higher concentration of VFL resulted in a higher DEF in all cell lines. When using CI calculations, moderate synergism was observed with all tested concentrations in ECV304, with 30 nM of VFL in MCF-7, and with 40 nM of VFL in H292 cells. Clear synergistic effects were observed with 35 and 40 nM of VFL in MCF-7 cells. However, in CAL-27, at concentrations below 30 nM, no significant radiosensitising effect could be observed. Only at a concentration of 30 nM VFL, which caused already 70% cell kill in these chemoradiation experiments, RS was observed.

From these data, it could be concluded that radiosensitising effects were evident with VFL concentrations as low as the IC10 in ECV304, at IC40 concentrations in MCF-7 and H292, and only at a rather toxic concentration (IC70) in CAL-27.

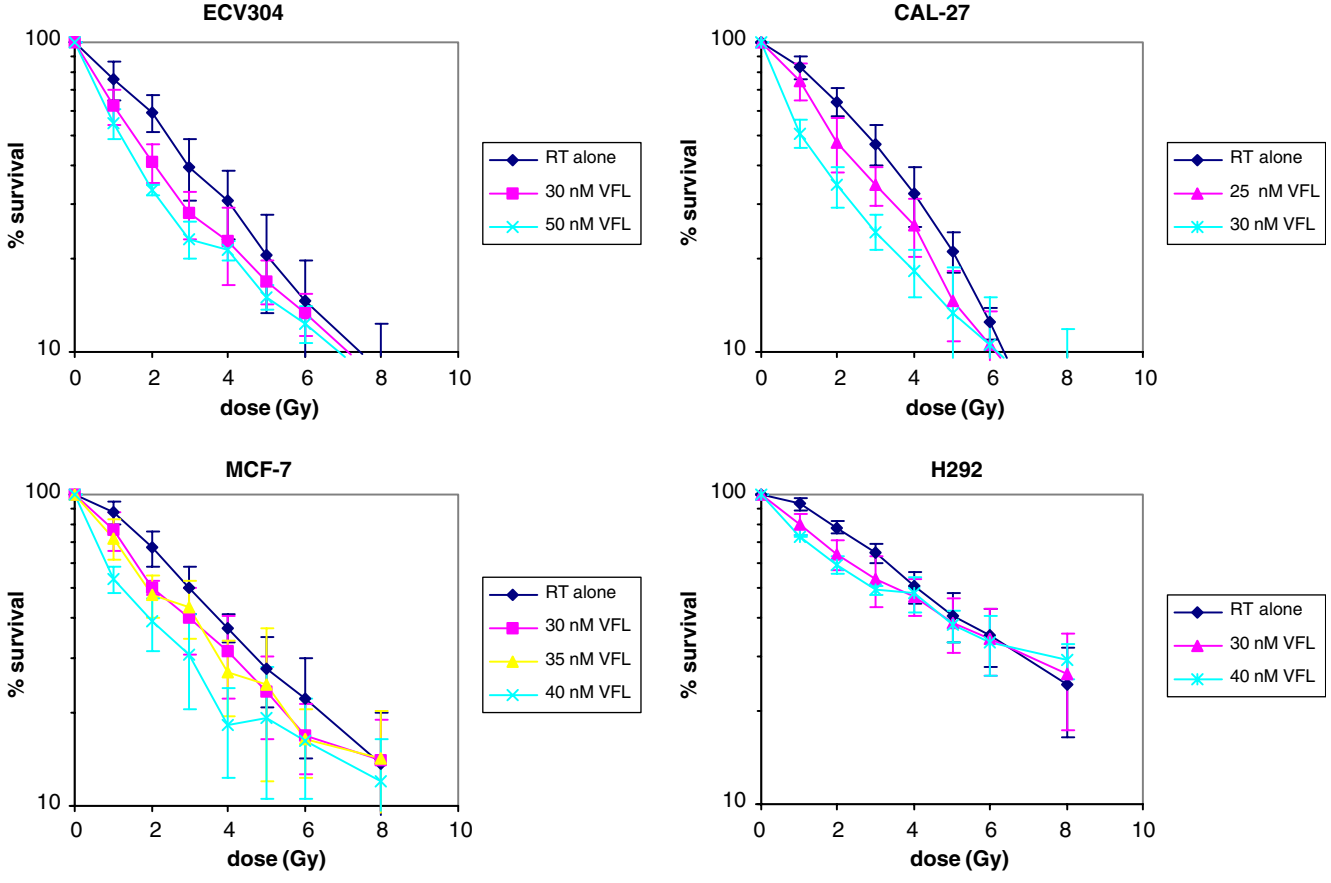

Fig. 2 Radiation dose–survival curves of the four human tumour cell lines when treated with radiation alone versus those of radiation in combination with different dosages of vinflunine for 24 h immediately prior to radiation. *RT* radiotherapy, *VFL* vinflunine, *Gy* gray

Table 1 Percentage survival, MID, ID50, DEF, and CI values of the four human tumour cell lines, mean values ± standard deviation

cell line	conc. VFL (nM)	survival (%)	mean MID	mean ID50	mean DEF	mean CI	
ECV304	0	100	3.18 ± 0.56	2.46 ± 0.62			
	30	88 ± 7	2.12 ± 0.58[b]	1.56 ± 0.36[b]	1.57 ± 0.25	0.84 ± 0.03	*moderate synergism*
	50	74 ± 11	1.66 ± 0.17[a]	1.26 ± 0.12[a]	1.93 ± 0.31	0.71 ± 0.05	*moderate synergism*
CAL-27	0	100	3.24 ± 0.29	2.81 ± 0.38			
	25	59 ± 21	2.48 ± 0.45[b]	2.01 ± 0.51[b]	1.41 ± 0.17	0.98 ± 0.13	*additivity*
	30	29 ± 9	1.63 ± 0.12[a]	1.22 ± 0.09[a]	2.29 ± 0.45	0.57 ± 0.06	*synergism*
MCF-7	0	100	4.00 ± 0.67	3.18 ± 0.52			
	30	59 ± 9	3.07 ± 0.75	2.29 ± 0.58	1.42 ± 0.26	0.77 ± 0.04	*moderate synergism*
	35	49 ± 7	2.84 ± 0.22[b]	2.16 ± 0.26[b]	1.57 ± 0.10	0.69 ± 0.05	*synergism*
	40	41 ± 20	1.93 ± 0.31[a]	1.48 ± 0.27[a]	2.24 ± 0.15	0.54 ± 0.04	*synergism*
H292	0	100	5.16 ± 0.72	4.42 ± 0.62			
	30	87 ± 12	4.55 ± 1.28	3.55 ± 0.91	1.29 ± 0.27	1.27 ± 0.48	*additivity*
	40	62 ± 13	3.60 ± 0.37[b]	3.03 ± 0.34[a]	1.53 ± 0.04	0.77 ± 0.08	*moderate synergism*

MID mean inactivation dose, *ID50* radiation dose causing 50% growth inhibition, *DEF* dose enhancement factor, *CI* combination index
[a] $P \leq 0.01$ compared to control (0nM VFL)
[b] $P < 0.05$ compared to control (0nM VFL)

Cell cycle experiments

Cell cycle effects after 24 h treatment with vinflunine

Figure 3 shows representative histograms of the four human tumour cell lines and the normal fibroblast cell line treated with different concentrations of VFL (i.e. 100 nM in CAL-27, and 150 nM in the other cell lines) during 24 and 48 h. Table 2 summarises the percentages of cells in the different phases of the cell cycle for all the tested cell lines.

Immediately after treatment, a concentration-dependent G2/M block was observed. In ECV304, a significant G2/M block was apparent with VFL concentrations of 150 nM and higher, while cells in G1 and S phase decreased. In CAL-27, MCF-7, and H292, a significant G2/M block was already observed when cells were incubated for 24 h with 60 nM VFL, and the percentage of cells in G2/M increased with increasing concentrations. In CAL-27, an expected decrease in G1 and S coincided with the G2/M block. In MCF-7 and H292 however, the percentage of cells in S phase was unchanged, but the percentage of G1 phase cells decreased.

Cell cycle kinetics

Table 3 summarises the percentages of cells in the G2/M phase of the cell cycle after different incubation times with VFL, and Table 4 those of different hours after drug removal.

Table 2 Percentages of cells in the G2/M, S, and G1 phase of the cell cycle: after 24 h incubation with different concentrations of vinflunine, mean ± standard deviation

Cell line (%)	Control (%)	60 nM (%)	150 nM
	% of cells in G2/M		
ECV304	21.5 ± 3.4	19.2 ± 1.8	50.2 ± 2.9[a]
CAL-27	15.9 ± 2.5	30.4 ± 6.8[b]	55.4 ± 5.8[a]
MCF-7	21.2 ± 3.4	47.2 ± 10.6[b]	52.5 ± 8.2[a]
H292	16.4 ± 2.2	28.6 ± 2.3[a]	53.2 ± 11.7[b]
	% of cells in S		
ECV304	41.3 ± 6.3	44.1 ± 5.0	26.4 ± 5.5[b]
CAL-27	25.3 ± 1.2	33.3 ± 4.2	14.9 ± 3.3[b]
MCF-7	27.0 ± 4.9	26.4 ± 2.5	29.7 ± 3.8
H292	21.8 ± 2.2	23.5 ± 1.7	23.8 ± 3.9
	% of cells in G1		
ECV304	36.7 ± 2.5	36.3 ± 3.8	15.0 ± 5.9[a]
CAL-27	56.5 ± 4.2	19.0 ± 1.7[a]	9.2 ± 1.9[a]
MCF-7	49.5 ± 7.6	19.1 ± 3.9[a]	11.3 ± 5.3[a]
H292	59.6 ± 4.8	39.3 ± 4.1[a]	15.0 ± 5.2[a]

[a] $P \leq 0.01$ compared to control
[b] $P < 0.05$ compared to control

Table 3 Percentages of cells in the G2/M phase of the cell cycle: after different incubation times with vinflunine, mean ± standard deviation

Cell line	Control (%)	4 h incubation (%)	Max. of cells (%)	(After)	24 h incubation (%)	48 h incubation (%)
	% of cells in G2/M					
ECV304	20.6 ± 4.3	32.7 ± 6.5[b]	64.1 ± 9.7[a]	(16 h)	57.3 ± 7.9[a]	42.4 ± 6.1[a]
CAL-27	17.1 ± 3.9	33.7 ± 4.1[a]	63.0 ± 11.3[b]	(20 h)	59.7 ± 4.7[a]	21.3 ± 5.8
MCF-7	18.7 ± 2.4	25.5 ± 3.1[b]	59.7 ± 1.7[a]	(16 h)	53.3 ± 10.1[b]	49.3 ± 5.1[a]
H292	18.7 ± 3.4	37.8 ± 5.6[b]	69.4 ± 5.9[a]	(24 h)	69.4 ± 5.9[a]	65.7 ± 3.6[a]

[a] $P \leq 0.01$ compared to control
[b] $P < 0.05$ compared to control

Table 4 Percentages of cells in the G2/M phase of the cell cycle: different hours after drug removal after 24 h incubation with vinflunine, mean ± standard deviation

Cell line	24 + 0 h (%)	24 + 3 h (%)	24 + 5 h (%)	24 + 24 h (%)	24 + 48 h (%)	24 + 72 h (%)
	% of cells in G2/M					
ECV304	57.3 ± 7.9	29.1 ± 5.5[a]	29.6 ± 6.3[a]	33.4 ± 1.4[b]	28.6 ± 1.7[b]	23.4 ± 3.0[a]
CAL-27	59.7 ± 4.7	33.2 ± 7.9[a]	29.6 ± 7.0[a]	25.0 ± 4.7[a]	24.9 ± 1.7[a]	27.6 ± 1.7[a]
MCF-7	53.3 ± 10.1	36.2 ± 7.5	33.5 ± 9.4[b]	27.8 ± 6.7[b]	27.3 ± 6.9[b]	29.8 ± 6.6[b]
H292	69.4 ± 5.9	39.5 ± 1.7[a]	42.7 ± 2.0[a]	41.3 ± 4.0[a]	41.0 ± 4.3[a]	26.9 ± 6.8[a]

[a] $P \leq 0.01$ compared to 24 h incubation
[b] $P < 0.05$ compared to 24 h incubation

Table 5 Percentages of cells in S2 and polyploidy after different incubation times with vinflunine and after drug removal, mean ± standard deviation

Cell line	Control (%)	24 h incubation (%)	48 h incubation (%)	24 + 24 (%)	24 + 48 (%)	24 + 72 (%)
	% of cells in S2					
ECV304	3.6 ± 2.3	7.4 ± 1.8[a]	22.4 ± 1.4[a]	12.7 ± 2.5[d]	16.0 ± 1.2[c]	17.1 ± 0.5[c]
CAL-27	2.4 ± 1.1	5.5 ± 3.2	21.8 ± 2.7[a]	11.2 ± 0.9	21.7 ± 2.0[c]	20.2 ± 3.2[c]
MCF-7	2.1 ± 0.8	2.8 ± 1.7	8.8 ± 2.8[b]	5.2 ± 0.8	10.6 ± 2.5[c]	9.4 ± 2.7[c]
H292	2.2 ± 1.5	2.9 ± 0.8[b]	7.1 ± 1.7[a]	3.6 ± 2.0	4.5 ± 0.9	5.8 ± 2.6
	% of cells in Polyploidy					
ECV304	0.6 ± 0.2	1.1 ± 0.8	15.6 ± 1.3[a]	9.2 ± 2.6[d]	10.2 ± 1.2[c]	8.4 ± 0.7[c]
CAL-27	0.6 ± 0.2	2.8 ± 1.4[a]	41.8 ± 9.8[a]	5.8 ± 0.5[d]	7.7 ± 1.1[c]	8.3 ± 2.2[d]
MCF-7	0.5 ± 0.2	1.3 ± 0.3[b]	4.4 ± 1.4[b]	2.0 ± 0.9	4.4 ± 1.0[c]	4.4 ± 1.8[d]
H292	0.4 ± 0.2	1.2 ± 0.3[b]	7.0 ± 1.8[a]	1.9 ± 0.7	2.4 ± 0.5[d]	1.5 ± 0.3

[a] $P \leq 0.01$ compared to control
[b] $P < 0.05$ compared to control
[c] $P \leq 0.01$ compared to 24 h incubation
[d] $P < 0.05$ compared to 24 h incubation

In all cell lines, a significant increase in the percentage of cells in the G2/M phase was already visible after 4 h incubation. A maximal G2/M block was reached after 16 h in ECV304 and MCF-7, after 20 h in CAL-27, and after 24 h in H292.

In all but MCF-7 cell lines, the percentage of cells in G2/M decreased significantly from 3 h after removal of VFL (after the 24-h incubation period). In MCF-7, at least 5 h were needed for a significant release of cells from the G2/M block. This release of cells from G2/M coincided with an increase in the number of cells in the G1 phase (data not shown), and suggested that the accumulated cells in G2/M had re-entered the cell cycle.

Polyploidy

Table 5 summarises the percentages of cells in S2 and in polyploidy after 24 and 48 h of incubation with VFL, and after drug removal.

After 48 h of continuous incubation with VFL, a polyploid cell population was clearly observed in CAL-27 and ECV304 cells, and to a lesser degree also in MCF-7 and H292 cells. This is depicted in Fig. 3. In our experiments, polyploidy was never observed in the normal fibroblast cell line Fi 360 (Fig. 3).

From 5 h up to 24 h after removal of VFL, the percentage of ECV304 and CAL-27 cells in G1 phase decreased significantly (results not shown), however,

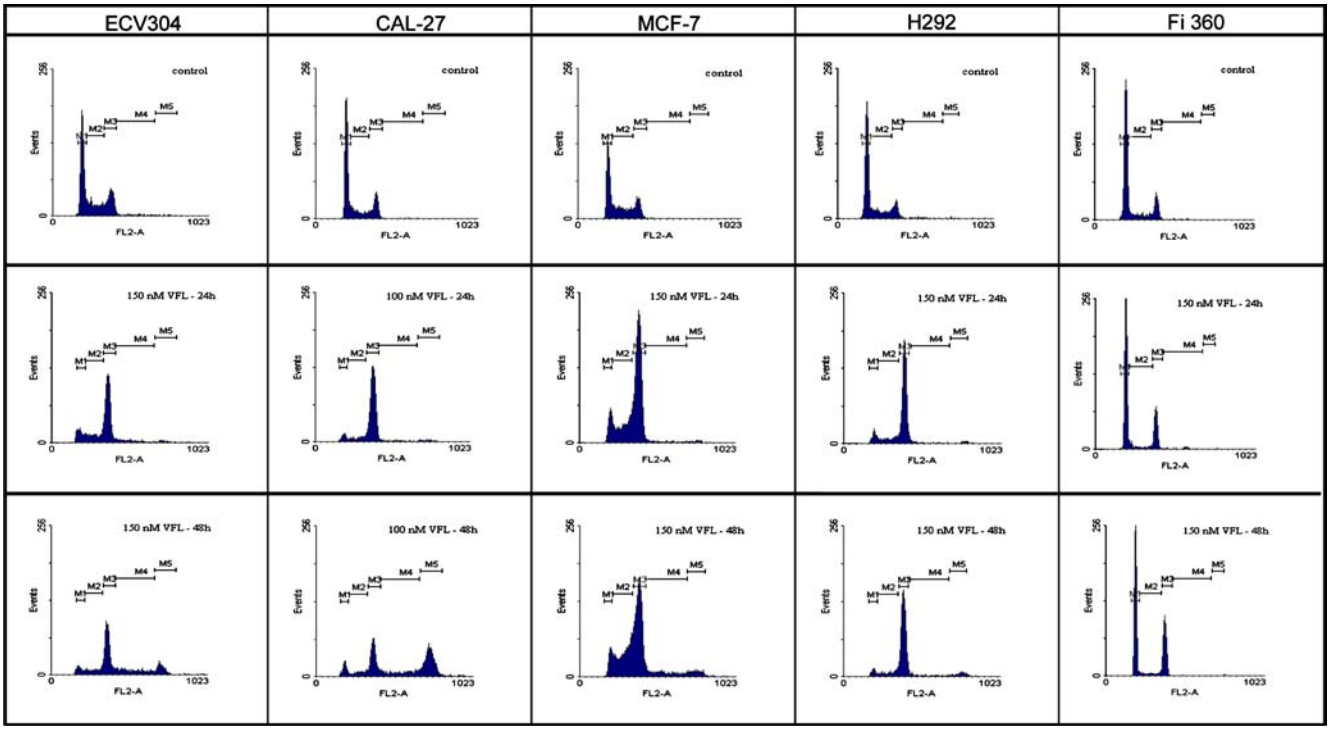

Fig. 3 Histograms of the four human tumour cell lines and the normal fibroblast cell line (Fi 360), treated with different concentrations of vinflunine during 24 and 48 h. FL-2A = DNA content, Events = number of fluorescent nuclei, M1 = G1, M2 = S, M3 = G2/M, M4 = S2, M5 = polyploidy

without any significant change in the percentage of cells in S and G2M (Table 4). On the other hand, a significant increase of cell number was observed in S2 and polyploidy (Table 5), meaning that VFL induces a polyploid cell population. So, the decrease in G1 phase cells can be explained by the fact that a number of G2 phase cells will continue in a (polyploid) cell cycle, without preceding mitosis.

In MCF-7 and H292, the amount of cells in S2 and polyploidy only reached a significant level 48 h after drug removal, and this was again less than what was observed with ECV304 and CAL-27.

Discussion

In the current in vitro study, we investigated the interaction between VFL and radiation together with the effects of VFL on the cell cycle. Twenty-four hours incubation with VFL, immediately followed by radiation, caused RS in the four tumour cell lines tested. Our cell cycle experiments showed a concentration-dependent G2/M block after 24 h of incubation with VFL. A rapid accumulation of cells in G2/M was observed, but the number of accumulated G2/M phase cells also rapidly decreased after drug removal. A prolonged continuous exposure to VFL (48 h) resulted in a polyploid cell population, especially in CAL-27 and ECV304 cells.

Until now, the interaction between VFL and radiation had not been investigated. Since VFL treatment was known to result in an accumulation of cells in the G2/M phase, this new third generation Vinca alkaloid was expected to have radiosensitising properties. This turned out to be the case in all four human tumour cell lines that we investigated in this study. In ECV304, RS was already observed at concentrations around IC10 (DEF = 1.57). For MCF-7 and H292, concentrations around IC40 were needed to cause RS (DEF = 1.42 and 1.53, respectively). In CAL-27 cells, only rather toxic concentrations, around IC70, resulted in a DEF significantly higher than 1 (DEF = 2.29).

It should be kept in mind that radiosensitising effects do not by definition lead to an improved therapeutic index. The selectivity of the interaction between the cytotoxic agents and radiotherapy is a key issue for improved clinical outcome. Non-tumour cells with 'normal' checkpoint proteins might tolerate the relatively less potent inhibitory effects of VFL on microtubule dynamics better than, e.g. the more potent inhibitory effects of vinblastine, irrespective of their antitumour activity. Furthermore, since checkpoint mechanisms in tumour cells are frequently faulty, cancer cells may be more susceptible than normal cells to VFL [31]. Nevertheless, in vivo studies should always be used to verify an improved therapeutic index.

For vinorelbine, a second generation Vinca alkaloid, the radiosensitising effect has already been investigated both in vitro and in phase I trials. The outcomes of in vitro studies have been variable. In some studies in which tumour cells were exposed to vinorelbine and

then irradiated, the drug enhanced the radiation effects in a dose-dependent manner, which was thought to be linked up with a block in the G2/M phase of the cell cycle [4] as well as with continuous polyploidisation and induction of apoptosis [11]. In other studies, only an additive effect was observed [5] or the association between RS and G2/M blockade was not found [12]. A phase I study in 14 patients with locally advanced NSCLC indicated that thoracic radiotherapy and daily (4 mg/m^2) vinorelbine (used as a radiosensitiser) was feasible, and resulted in partial and complete responses [14].

In all cell lines we tested, a concentration-dependent G2/M block was observed after 24 h of incubation with VFL. This is in agreement with the observations by Kruczynski et al. [20]. They described a dose-dependent accumulation of P388 leukaemic cells in the G2/M phase of the cell cycle after 18 h incubation (approximately one population doubling time). Although maximal G2/M blockade in our study occurred only after 16–24 h of incubation with VFL, the number of cells in G2/M was already increased significantly after 4 h of incubation. This should be seen in relation to the fact that VFL, like vinorelbine and vinblastine, enters the cells gradually, reaching maximum levels within 4 h [32]. We noticed that the number of cells in G2/M rapidly decreased after drug removal (from 3 h after treatment), which seemed to be mainly caused by mitosis and re-entry in G1 phase, but also by a small portion of cells that started a polyploid cell cycle. Interestingly, Jean Decoster et al. [17] showed that the separation of centrosome units induced by VFL was rapidly reversed after drug 'wash-out', while this process was slower in cells treated with the three other Vinca alkaloids. VFL, with the weakest overall affinity for tubulin, appears to have the most readily reversible interaction with tubulin. Important in view of our observation, Lobert et al. [26] also stated that the smaller spiral polymers induced by VFL have a more rapid relaxation time, and thus a potential for faster clearance from the cells.

After 48 h continuous incubation with VFL, we observed a polyploid cell population in CAL-27 and ECV304, and to a much smaller degree also in MCF-7 and H292 cells. However, in the normal fibroblast cell line (Fi 360) these polyploid cells could not be found. While Kruczynski et al. [20] did not observe polyploid cells in P388 cells after incubation with VFL, other studies have shown polyploid cell populations after incubation with other members of the Vinca alkaloid family, like vinblastine, vincristine, and vinorelbine [11, 28, 29]. Also with other microtubule binding agents, such as taxanes, polyploid cell formation has been observed [23]. A possible explanation for this phenomenon is that altered expression or inactivation of cell cycle regulatory molecules (JNK, p21Cip1/Waf1, cdk1, etc.) is involved in the deregulation of cell cycle checkpoint control, which could lead to the induction of polyploidisation [11].

Using Facs analysis, we were unable to assess the fate of the polyploid cells as yet. We are uncertain whether these cells ultimately undergo apoptosis, or persist as aneuploid cells with possible increased resistance to chemotherapeutic agents. Further studies to investigate this phenomenon are ongoing.

The radiosensitising effect of VFL apparently can be correlated to the G2/M block, since concentrations causing RS (25–50 nM VFL) are in the same nanomolar range as the concentrations resulting in a significant G2/M block (from 60 to 150 nM VFL). When the cell cycle effects of VFL would indeed play a key role in its radiosensitising properties, a clear schedule-dependent radiosensitising effect would be expected. In this view, short incubation times are expected to result already in a radiosensitising effect, whereas small intervals between VFL treatment and radiation would already result in a clear decrease in the radiosensitising effect. Taking into account that similar observations related to G2/M blockade and polyploidisation have been observed earlier with vinorelbine [11], more research on the schedule dependency of the radiosensitising effects of VFL is needed to further elucidate the role of the cell cycle effects in its radiosensitising mechanism.

In conclusion, VFL causes RS in all four human tumour cell lines tested, most pronounced in ECV304 cells, with radiosensitising effects at non-toxic concentrations. In this study, we report these radiosensitising properties in relation to the cell cycle effects of VFL. A concentration-dependent G2/M block was observed after 24 h incubation with VFL. Rapid accumulation of cells in G2/M was seen, whereas cells rapidly re-entered the cell cycle after drug removal. Treatment with VFL, especially with longer incubation times (48 h), resulted in the appearance of a polyploid cell population, caused by further DNA synthesis after G2 phase without mitosis. This was observed in tumour cells only, and not in normal cells.

Given the importance of the observed RS caused by VFL, the exact role of the cell cycle effects warrants further investigation.

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