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ORIGINAL ARTICLE

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Markedly diminished drug resistance-inducing properties of vinflunine (20′,20′-difluoro-3′,4′-dihydrovinorelbine) relative to vinorelbine, identified in murine and human tumour cells in vivo and in vitro

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Abstract *Purpose*: Vinflunine (VFL) is a novel *Vinca* alkaloid with markedly superior experimental in vivo antitumour activity to its parent molecule, vinorelbine (Navelbine, NVB), against a panel of murine and human tumours. The aim of this study was to establish whether there are differences in the rate and extent of development of resistance, both in vivo and in vitro, to these two newer *Vinca* alkaloids under identical selection conditions. *Methods*: Using P388 leukaemia cells in vivo, it was evident that VFL induced drug resistance far less readily than NVB, as shown by the number of passages required to select for total resistance. Under in vitro conditions, using A549 human lung carcinoma cells, it was also clearly shown by drug sensitivity determinations that VFL was a less-potent inducer of drug resistance than NVB. Resistance resulting from either in vivo or in vitro selection was associated with a classic multidrug resistance profile. Further characterization of the drug-resistance phenotype of the most highly resistant A549 sublines showed that the level of total β-tubulin expression appeared to be modified exclusively in the NVB-resistant cells. *Conclusion*: The clear demonstration that resistance to VFL developed far less readily than resistance to NVB both in vivo and in vitro may have potential clinical implications.

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Introduction

In the field of antineoplastic chemotherapy, tubulin-interacting agents constitute an important class of compounds with broad activity both in solid tumours and in haematological malignancies. Using an original chemical approach employing superacidic chemistry [7], a novel *Vinca* alkaloid derivative vinflunine (20′,20′-difluoro-3′,4′-dihydrovinorelbine, VFL) was synthesized, which demonstrates markedly superior antitumour activity in vivo to navelbine (NVB), its parent molecule, against a series of transplantable murine and human xenografted tumours [10, 16]. Data from in vitro pharmacological studies have provided evidence of its quantitatively distinctive tubulin-binding properties relative to other *Vinca* alkaloids [15, 18, 20]. Murine P388 leukaemia cells selected in vivo for resistance to VFL exhibit a 'classic' multidrug resistance (MDR) phenotype both in vivo and when cultured in vitro [2, 6], including overexpression of a functional P-glycoprotein (Pgp). These findings indicate that VFL belongs to the Pgp-associated MDR group of antitumour agents, like the other *Vinca* alkaloids [5], including NVB [1].

During the course of these initial studies, however, it became apparent that whilst resistance to NVB was readily established, this was not the case for VFL. Therefore, the present study was undertaken to determine whether there really are any identifiable differences in the rate and/or the extent of development of resistance to VFL as opposed to NVB. For these studies, identical selection conditions both in vivo and in vitro were used for each *Vinca* alkaloid. Initial studies were carried out in vivo using P388 murine leukaemia cells and the treatment duration, in terms of the number of

passages, required to select for complete resistance to either VFL or NVB was defined. Resistance to either VFL or NVB was then induced or selected for in vitro in A549 human lung carcinoma cells and the drug resistance levels (RLs) achieved were quantitated. Subsequently, the drug resistance profiles of the various resistant sublines were determined and a preliminary characterization of the associated mechanisms was carried out.

Materials and methods

Compounds

VFL ditartrate was synthesized at the Centre de Recherche Pierre Fabre (Castres, France) [7]. NVB ditartrate and etoposide were provided by Pierre Fabre Médicament (Gaillac, France). MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide), verapamil hydrochloride and doxorubicin hydrochloride were purchased from Sigma Chemical Company (St Louis, Mo.), and DMSO (dimethyl sulfoxide) was purchased from SDS (Peypin, France). Both *Vinca* alkaloids, doxorubicin and verapamil were solubilized in water (1% final concentration) prior to use for in vitro experiments, and DMSO (0.1% final concentration) was used as a solvent for etoposide. The water-soluble compounds (VFL, NVB, doxorubicin) and cisplatin were administered in vivo in 0.9% sodium chloride, whilst the water-insoluble compound camptothecin was administered in 5% Tween 80 in 0.9% sodium chloride (10 ml/kg body weight).

Animals

Viral antibody-free female DBA/2 mice (DBA/2JICO, Iffa Credo, l'Abresle, France) were used for passaging the sensitive and resistant murine P388 leukaemias and experimental chemotherapy was performed using hybrid CDF_1 mice (CD2F1/CrlBR; Charles River, St Aubin-Les-Elbeuf, France). Sterile food and water were given ad libitum. All mice were manipulated in laminar-flow biosafety hoods located in 'specific pathogen organism free' barrier facilities. Their care and housing were as described previously [16].

Cell culture conditions and cell growth inhibition assays in vitro

The parental A549 human tumour cell line and resistant sublines were cultured in MEM (GIBCO, Cergy-Pontoise, France) supplemented with 5% heat-inactivated fetal calf serum, fungizone (1.25 μg/ml), penicillin-streptomycin (100 IU/ml, 100 μg/ml) and 4 m*M* of L-glutamine purchased from GIBCO. Cells were cultivated in a CO_2 incubator (37°C, humidified atmosphere, 5% CO_2 in air). Liquid nitrogen stocks (batches) were made after confirming their negative mycoplasma status by cell culture testing.

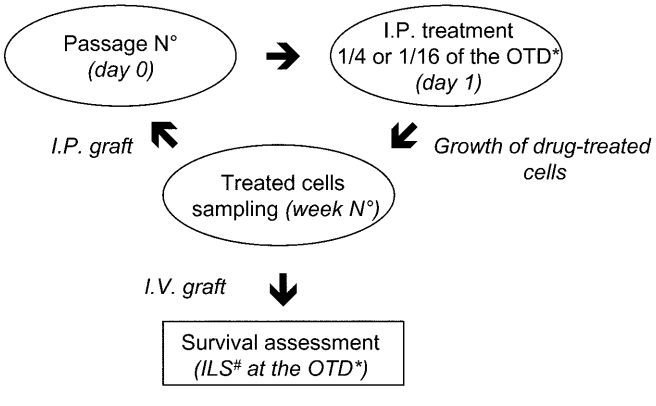
The drug-induced cell growth-inhibiting effects were determined using a colorimetric metabolic dye MTT assay as described previously [6]. Briefly, parental and resistant cells were seeded (2.5×10^3 or 5×10^3 cells/well, respectively) into a series of flat-bottomed 96-well microtitre plates and allowed to reach logarithmic growth (24 h) before adding test compound or solvent at the desired concentration for 72 h (37°C, 5% CO_2 in air). Cell supernatants were then discarded and cells were incubated for 3 h with MTT (0.1 mg/well) prior to centrifugation and the addition of DMSO (0.1 ml) to dissolve the formazan crystals formed. Plates were read using a spectrophotometer MR7000 (Dynatech Laboratories, Guyancourt, France) at 570 nm (test wavelength) and 630 nm (reference wavelength). When used, verapamil (10 μ*M* final concentration) was added simultaneously with the test compound. Irrespective of the cell line used, each drug concentration tested was evaluated at least three times in three independent experiments. IC_{50} values, i.e. the concentration of drug required to reduce growth to 50% of that obtained for control cells, were generated based on pooled data using a custom-made program based on linear interpolation between data points.

Establishment of the VFL- and NVB-resistant P388 sublines in vivo

The P388-sensitive leukaemia cell line, a generous gift from Dr S. Cros, Laboratoire de Pharmacologie et Toxicologie Fondamentales (Centre National de la Recherche Scientifique, CNRS, Toulouse, France) originally obtained from the National Cancer Institute (NCI, Frederick, Md.), were passaged weekly as intraperitoneal (i.p.) implants. All experiments were conducted in compliance with guidelines established in the Centre de Recherche Pierre Fabre (CRPF, Castres, France) based on the United Kingdom Coordinating Commitee on Cancer Research (UKCCCR) guidelines established for the welfare of animals in experimental neoplasia [24].

Murine P388 tumour cells selected for resistance to VFL or NVB were established in vivo as follows. Parental P388 cells were grafted i.p. at 10^6 cells per mouse into a group of five mice on day 0, assigned as one passage, and treated on day 1 after each such passage (Fig. 1) with an i.p. administrations of either VFL or NVB at 25% of their optimal therapeutic dose (OTD), i.e. 10 mg/kg and 2.5 mg/kg, respectively, or fourfold lower doses, i.e. 2.5 mg/kg and 0.63 mg/kg, with the OTD being the dose inducing the greatest increase in lifespan (ILS) with minimal side effects [16]. The antitumour activities of both compounds were then checked at the OTD, at the stage of each passage, as illustrated in Fig. 1, until the acquisition of complete resistance to treatment was documented. Briefly, drug-treated or untreated P388 cells were implanted intravenously (i.v.) into CDF1 mice on day 0 at 10^6 cells/mouse. After randomization, test compounds were administered i.p. on day 1 as a single dose at the OTD, i.e. 40 mg/kg VFL and 10 mg/kg NVB, and mice were checked daily for death and any adverse clinical reaction. ILS values were determined at each time from the relationships: $ILS = T/C - 100$ and $T/C\ (\%) = (\text{median survival cof drug-treated group/median survival of control group}) \times 100$. Once resistance had been established, resistant cells were amplified and stored in liquid nitrogen.

For further antitumour activity trials, P388-resistant cells were used after fewer than 12 passages in vivo with drug selection being maintained on a weekly basis. For evaluations of antitumour activities cells were grafted i.v. and the test compounds were administered i.p. as described above. The OTD (the dose producing the maximum T/C ratio with minimal side effects) was determined for each test compound. According to the NCI standard criteria for the P388 tumour model [23] $120\% \le T/C < 175\%$ is the minimum level for activity (L), and a T/C value ≥175% corresponds to a high



* OTD= optimal therapeutic dosage; # ILS= increase of life span

Fig. 1 Establishment of in vivo resistance

level of antileukaemic activity (H); '0' represents a T/C value $< 120\%$.

Establishment of the VFL- and NVB-resistant A549 sublines in vitro

The A549 human lung cancer cell line was supplied by the American Type Culture Collection (ATCC, Rockville, Md.). VFL- and NVB-resistant A549 sublines were established in our laboratory using comparable selection conditions in vitro, exposing the parental cells to drug as follows. Cells were first treated with six successive 24-h exposures to a concentration of compound approximating eight times the IC_{50} value, i.e. 0.08 μM VFL and 0.008 μM NVB, as defined after 72 h of continuous exposure using the colorimetric-based MTT assay described above. The resistant sublines termed AV6 or AN6 were maintained without further drug selection pressure and proved stable in terms of their levels of resistance for at least 3 months in continuous culture. Aliquots of these cells were then adapted to continuous drug exposure at 25% (about twice the IC_{50} value) or 50% (about four times the IC_{50} value) of the first inducing drug concentration. The cell lines obtained after these more prolonged selection procedures were designated AV2I or AV4I for the VFL-resistant sublines and AN2I or AN4I for the NVB-resistant sublines.

Resistance characterization of VFL- or NVB-resistant A549 sublines in vitro

The following studies were conducted using two of the three resistant sublines, i.e. those expressing the lowest (AV6 and AN6) and the highest (AV4I and AN4I) levels of resistance compared with the respective parental lines (A549pv and A549pn).

Flow cytometric analyses of Pgp expression were performed using the anti-Pgp monoclonal antibody MRK16 (Valbiotech, Paris, France). Trypsinized cells were then incubated with mouse IgG2a isotypic control (Immunotech, Marseilles, France) or MRK16 (10 μg for 10^6 cells, 60 min at 4°C). Cells were counterstained with FITC-conjugated goat anti-mouse immunoglobulins (Dako, Glostrup, Denmark; 1/10, 30 min at 4°C in the dark). Propidium iodide (PI) was then added (0.05 mg/ml) to label dead cells. The fluorescence intensity of PI-negative cells (living cells) was determined using a Coulter EPICS XL flow cytometer.

β-Tubulin gene expression was determined using reverse transcription-polymerase chain reaction (RT-PCR). β-Tubulin analyses were performed, as previously described [12], on A549 parental cells and NVB- and VFL-resistant sublines. Briefly, total cellular RNA was isolated, DNase I-treated to remove any contaminating DNA, and reverse transcribed for RT-PCR analysis. To confirm removal of all contaminating DNA, each RNA sample was subjected to a mock reverse transcription by omitting the reverse transcriptase from the reaction mix. Experiments were performed twice with three independent PCR analyses being performed for each gene. PCR products (10 ml) were resolved using 12.5% polyacrylamide gel electrophoresis and then stained with ethidium bromide prior to visualization and imaging on a UV transilluminator. Images were subjected to densitometry and a ratio for the target gene and the β_2-microglobulin PCR product was obtained for each cDNA sample by dividing the densitometric volume of the target gene electrophoretic band by that of the β_2-microglobulin band [12, 21].

Owing to the high degree of amino acid and nucleotide identity between the β-tubulin isotypes [19], consensus primers were designed to amplify total β-tubulin by aligning the various sequences and selecting a region with a very high degree of homology (no more than one base mismatch). Degenerate primer sequences which amplified a 122-bp product were as follows: forward 5′ TTC AAG CGC ATC TCS GAG CA 3′ and reverse 5′ AGG TCR TTC ATG TTG CTC TC 3′. PCR amplification conditions were optimized as previously described for β-tubulin isotypes [12], except that owing to the size similarity between total β-tubulin and the control gene β_2-microglobulin, amplification of each gene was performed in separate tubes.

Results

Development of resistance in P388 cells in vivo

Parental P388 cells were exposed in vivo to either VFL or NVB, as described in Material and methods. At the indicated times (see Fig. 2) drug-treated cells were implanted into mice by the i.v. route and their sensitivities to VFL or NVB administered i.p. at their optimal doses, i.e. 40 mg/kg and 10 mg/kg, respectively, were assessed. As shown in Fig. 2A, the higher drug resistance-inducing dose tested for VFL (10 mg/kg) resulted in the expression of partial resistance over the first 10 weeks, marked resistance from week 12 to week 21, and complete resistance only after 22 weeks (P388/VFL-10). In contrast, with NVB (Fig. 2B) at the higher dose of 2.5 mg/kg, a comparable therapeutic dose level to that of 10 mg/kg VFL (see above), resistance induction was rapid with complete resistance being established after 5 weeks (P388/NVB-2.5). At the lower dose levels studied, with 2.5 mg/kg VFL there was identification of resistance only after 20 weeks and then from week 21 to week 30 partial resistance was expressed, with total resistance being developed after 36 weeks (P388/VFL-2.5). In contrast again, with NVB at 0.63 mg/kg partial

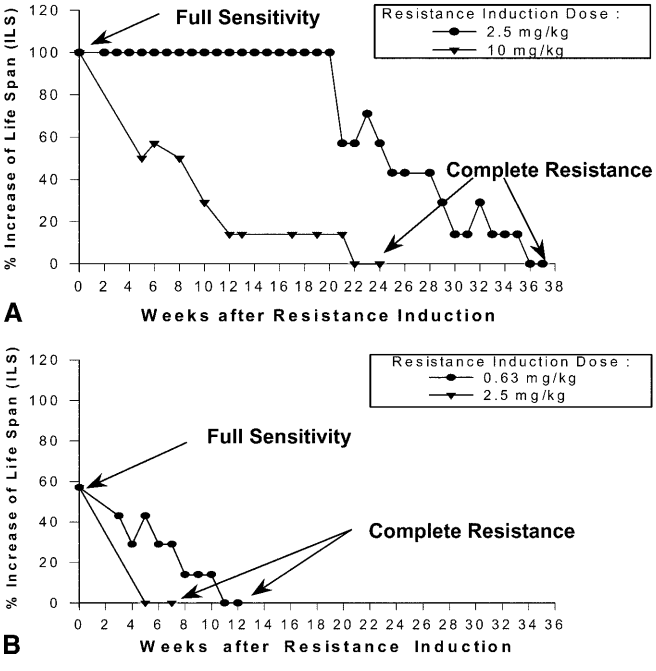


Fig. 2A, B Drug sensitivities of P388-treated cells to VFL or NVB during the course of induction of drug resistance in vivo. Parental P388 cells were exposed to the drugs as described above (see Materials and methods). At the indicated times drug-treated cells were implanted into mice by the i.v. route and their sensitivities to the inducing compound, either VFL (**A**) or NVB (**B**), administered i.p. at the optimal therapeutic dose, i.e. 40 mg/kg and 10 mg/kg, respectively, were assessed. ILS (%) were determined using the formula T/C − 100, with T/C(%) = (median survival of drug-treated group/median survival of control group) × 100

resistance was detectable after 4 weeks, and its extent increased with further passaging, being marked after 8 weeks and complete by week 11 (P388/NVB-0.63). Thus, resistance to VFL was developed in P388 murine leukaemia cells far less readily than to NVB.

Once drug resistance had been established, additional experiments were carried out to define and compare the cross-resistance profiles of these P388 cells selected for resistance either to VFL or to NVB. Definite in vivo antitumour activity was noted when a series of standard antitumour agents were administered as single i.p. doses to mice bearing the i.v.-implanted P388 sensitive cells (see Table 1). A high level of activity was noted with VFL and doxorubicin (T/C values 200% and 186%, respectively), as reported previously [16], and with camptothecin (T/C 214%) and cisplatin (T/C 357%), the two drugs not implicated in the MDR phenotype, whilst lower activity was observed with NVB (T/C 157%). Comparing the responses to the same drugs of mice bearing either of the resistant sublines, it was apparent that the cross-resistance profile for VFL- and NVB-resistant sublines were similar, with full sensitivity to the non-MDR associated agents camptothecin and cisplatin being retained, whilst complete resistance (T/C 100%) to the MDR-associated agents doxorubicin and the other *Vinca* alkaloids was expressed.

Development of resistance in A549 cells in vitro

Resistance to VFL or NVB was established in human lung tumour A549 cells in vitro, as detailed in Materials and methods, and the growth-inhibitory effects of the selecting compound were quantitated in the different resistant sublines and their respective parental line. Comparing the growth-inhibitory effects of VFL (Fig. 3) and NVB (Fig. 4), it was apparent that the cytotoxicity of both *Vinca* alkaloids reached a plateau value that ranged from 20% to 65% of the control value, with this level being markedly higher as the drug RL increased.

Table 1 In vivo antitumour activity of VFL, NVB and a series of standard antitumour agents, administered to mice with i.v.-implanted P388 sublines resistant to either compound. On day 0, 10^6 leukemic cells were inoculated i.v. and then compounds were given as a single dose by the i.p. route on day 1 [T/C = (median survival of drug-treated group/median survival of solvent-treated group) × 100]

Compound	P388 sensitive		P388/VFL-10		P388/VFL-2.5		P388/NVB-2.5		P388/NVB-0.63	
	Optimal T/C (%)	Activity rating[a]	Optimal T/C (%)	Activity rating[a]	Optimal T/C (%)	Activity rating[a]	Optimal T/C (%)	Activity rating[a]	Optimal T/C (%)	Activity rating[a]
VFL	200	H	100	0	100	0	114	0	100	0
NVB	157	L	100	0	100	0	100	0	100	0
Camptothecin	214	H	200	H	200	H	214	H	200	H
Cisplatin	357	H	400	H	328	H	214	H	300	H
Doxorubicin	186	H	100	0	100	0	100	0	100	0

[a]According to the NCI standard criteria [23] for the P388 tumour model, 120% ≤ T/C < 175% is the minimum level for activity (L), and a T/C≥175% corresponds to a high level of antileukemic activity (H); '0' represents a T/C value < 120%

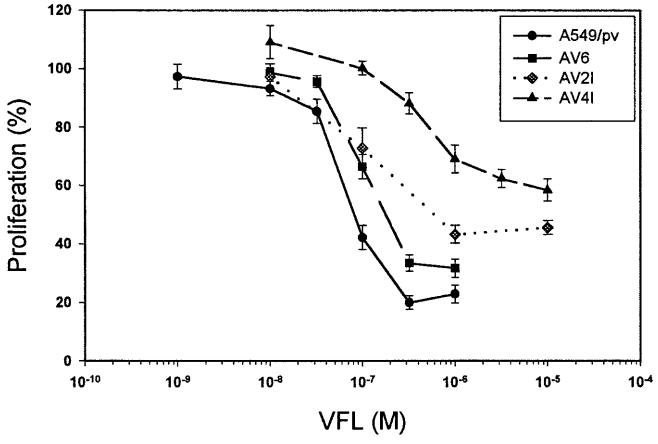


Fig. 3 Drug sensitivities of A549 sensitive and VFL-resistant sublines to VFL in vitro. The various VFL-resistant sublines were established as described above (see Materials and methods). Sensitive and resistant cells were then exposed to various concentrations of test compound for 72 h and cell growth was determined using a tetrazolium dye-based MTT assay. The figure shows dose response curves of VFL against the various cell lines (*bars* SEM obtained from at least three independent experiments)

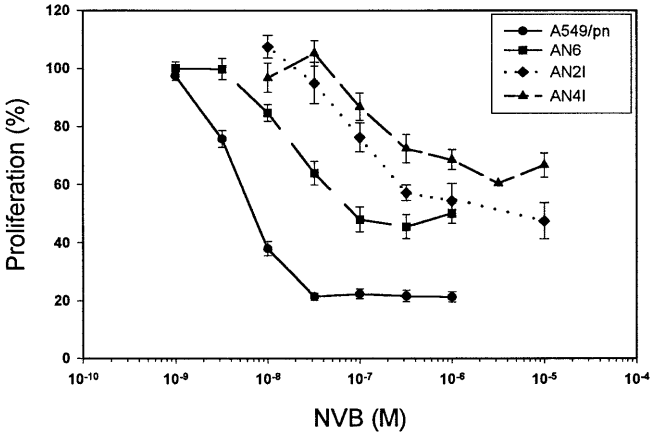


Fig. 4 Drug sensitivities of A549 sensitive and NVB-resistant sublines to NVB in vitro. The various NVB-resistant sublines were established as described above (see Materials and methods). Sensitive and resistant cells were then exposed to various concentrations of test compound for 72 h, and cell growth was determined using a tetrazolium dye-based MTT assay. The figure shows dose response curves of NVB against the various cell lines (*bars* SEM obtained from at least three independent experiments)

IC_{50} values could not be calculated for the more highly resistant sublines (plateau value ≥50%), and thus another parameter, called FCP, representing the first concentration at which the plateau value was reached, was determined for these sublines, and compared with the FCP value of their sensitive counterpart. For each resistant subline, RL represented by either IC_{50} ratios (IC_{50} resistant cells/IC_{50} sensitive cells) or FCP ratios (FCP resistant cells/FCP sensitive cells) were determined, and in each case the duration of the selection procedure was defined (Tables 2 and 3).

Considering the sublines obtained after six successive 24-h pulsed drug exposures, at eight times the IC_{50} values, a significant level of resistance to NVB was obtained, as revealed by an RL of 14 in the AN6 subline, although only a low level of resistance (twofold) to VFL resulted in the AV6 subline. Under conditions of continuous exposure to NVB ($2\times IC_{50}$ values), resistance was readily obtained (within 2 months) in subline AN2I. In contrast, a prolonged period of selection (8 months) was needed to adapt cells to survive continuous exposure to $2\times IC_{50}$ values of VFL, eventually generating subline AV2I. On the other hand, increasing the drug concentration used for selection to $4\times IC_{50}$ values of either test compound resulted in resistant sublines being obtained within 2 months using either NVB (AN4I) or VFL (AV4I). However, in general the variously derived sublines expressing resistance to VFL expressed markedly lower levels of resistance ($2 \le RL \le 10$) compared with the corresponding NVB-resistant subline ($14 \le RL \le 100$). These results strongly indicate that resistance to VFL was considerably more difficult to induce or select for than resistance to NVB using these A549 cells in vitro. These in vitro findings are clearly consistent with those obtained when selecting for resistance to either NVB or VBL in vivo using the P388 leukaemia model.

Table 2 Characteristics of the VFL-resistant sublines in terms of drug sensitivities (i.e. IC_{50} and FCP values determined from Fig. 3) and selection of drug resistance (*FCP* first concentration reaching the plateau value, *RL* resistance level, *n.c.* not calculable, *n.d.* not determined)

Cell line	IC_{50}	RL	Plateau value (%)	FCP	RL	Selection period (months)
A549pv	8.2×10^{-8}		20	3.2×10^{-7}		
AV6	1.8×10^{-7}	2	30	n.d.		2/3
AV2I	n.c.		50	1.0×10^{-6}	3	8
AV4I	n.c.		60	3.2×10^{-6}	10	2

Table 3 Characteristics of the NVB-resistant sublines in terms of drug sensitivities (i.e., IC_{50} and FCP values determined from Fig. 4) and selection of drug resistance (*FCP* first concentration reaching the plateau value, *RL* resistance level, *n.c.* not calculable, *n.d.* not determined)

Cell line	IC_{50}	RL	Plateau value (%)	FCP	RL	Selection period (months)
A549pn	7.0×10^{-9}		20	3.2×10^{-8}		
AN6	1.0×10^{-7}	14	45	n.d.		2/3
AN2I	n.c.		55	1.0×10^{-6}	30	2
AN4I	n.c.		65	3.2×10^{-6}	100	2

Characterization of the A549 sensitive and VFL- or NVB-resistant A549 sublines selected in vitro

Studies were conducted with certain of these resistant A549 sublines, i.e. those expressing the lowest (AV6, AN6) and the highest (AV4I, AN4I) levels of resistance, and were aimed at comparing their growth characteristics and their drug sensitivity profiles. The doubling time of the least-resistant AV6 cell line was almost identical to that of the parental line (25 h), while the growth of lines AN6, AV4I and AN4I were significantly decreased, with doubling times of 29, 34 and 33 h, respectively (data not shown). The cell cycle distributions of logarithmically growing cultures of the various resistant and sensitive cell lines were essentially comparable (data not shown).

The sensitivity profiles of these resistant sublines in terms of their responses to VFL, NVB and two other drugs implicated in 'classic' MDR phenotype (doxorubicin and etoposide) were next defined so as to determine whether resistance was related to the Pgp-MDR phenotype. RL values, as defined above, were determined and are shown in Table 4. VFL-resistant AV6 cells proved weakly cross-resistant or non-cross-resistant ($RL \le 3$) to these drugs, while the corresponding NVB-resistant subline AN6 expressed significant levels of cross-resistance to all these agents ($3 < RL < 17$). However, when evaluating the more highly resistant sublines AV4I and AN4I, adapted to growth in the continuous presence of $4\times IC_{50}$ values of VFL or NVB, respectively, comparable high RL levels ($10 < RL < 105$) were noted

Table 4 In vitro cross-resistance profiles of VFL- and NVB-resistant A549 sublines and their modulation by 10 μ*M* verapamil (*n.d.* not determined)

Compound	Resistance levels[a]			
	VFL-resistant sublines		NVB-resistant sublines	
	AV6	AV4I	AN6	AN4I
VFL	2	10[b]	6	30[b]
VFL + verapamil	n.d.	1[b]	2	3[b]
NVB	3	100[b]	14	100[b]
NVB + verapamil	n.d.	3[b]	3	3[b]
Doxorubicin	3	33	3	28
Doxorubicin + verapamil	n.d.	9	n.d.	7
Etoposide	1	100	17	105
Etoposide + verapamil	n.d.	118	24	140

[a]Resistance level = IC_{50} (or FCP) resistant cell line/IC_{50} (or FCP) sensitive cell line
[b]Resistance level calculated using FCP value (first concentration reaching the plateau value)

in both sublines to each of this group of compounds, clearly suggesting a Pgp-mediated MDR mechanism.

Modulation by verapamil of the resistance of the various A549 sublines to VFL or NVB in vitro was then evaluated in terms of the RL values obtained in the presence or absence of 10 μ*M* verapamil. This verapamil concentration was selected for its nonsignificant cytotoxicity (i.e. permitting ≥80% proliferation) against the various sublines studied (data not shown). As illustrated in Table 4, the addition of verapamil to cells expressing definite levels of drug resistance (RL≥3), relative to their selecting compound, i.e. AV4I, AN6, AN4I, resulted in marked decreases in their levels of resistance, as shown by the lower RL values obtained to VFL, as well as to NVB and to doxorubicin, although this was not the case with etoposide. Overall, these results again strongly indicate that these VFL- and NVB-resistant A549 sublines all express a Pgp-mediated MDR phenotype. Indeed, this observation was further confirmed by flow cytometry, using MRK16 as a specific antibody against human Pgp (Fig. 5), which revealed strong overexpression of Pgp by the AN4I and AV4I sublines, with an approximate 40-fold increase in fluorescence relative to the parental cells. Moreover, in recent studies in which the uptake of tritiated VFL by AN4I and AV4I was measured, similar minimal uptake was noted in these resistant cells relative to the parental cells (data not shown), such a reduced accumulation having been demonstrated in most Pgp-overexpressing cell lines.

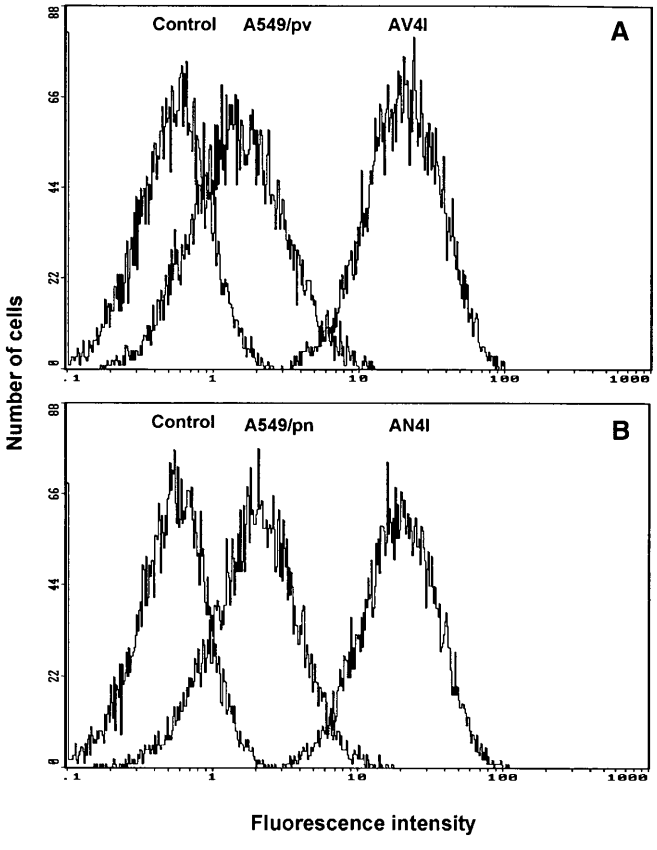


Fig. 5A, B Flow cytometric analysis of Pgp expression in A549 sensitive and VFL- and NVB-resistant sublines. Permeabilized cells were labelled with MRK16 or an isotype-matched control antibody (see Materials and methods). **A** A549 parental and VFL-resistant sublines; **B** A549 parental and NVB-resistant sublines

Complementary characterization of the MDR phenotype of the resistant A549 sublines

To determine whether a component of the resistance phenotype expressed by VFL- and NVB-resistant A549 cells was associated with altered expression of the cellular target of the selecting drugs, the expression of specific β-tubulin isotypes was examined [12]. Gene expression levels of Hβ4, which encodes for the class III β-tubulin protein, were decreased in AV6, AV4I, AN6 and AN4I cells, by 16%, 29%, 25% and 22%, respectively, compared to parental A549 cells (Fig. 6). The constitutively expressed class I β-tubulin isotype gene, HM40, did not differ significantly in the VFL- and NVB-resistant cells compared to parental A549 cells. There was, however, a small decrease in HM40 expression ($P < 0.05$) in the AV4I cells compared to the A549 cells. Class II β-tubulin, Hβ9, was expressed at low levels in the VFL and NVB cell lines and a significantly decreased expression was observed only in the AN4I cell line ($P < 0.05$). The β-tubulin genes H5β (class IVa), Hβ2 (class IVb), and the haematopoietic-specific Hβ1 (class VI) were expressed at very low, but unaltered levels in all the cell populations studied (data not shown). To establish whether the decreased expression of specific β-tubulin isotypes was due to changes in the levels of total β-tubulin, RT-PCR was performed on samples using primers designed to a consensus region of β-tubulin. Total β-tubulin expression in the resistant cells did not differ from that in the parental A549 cells (Fig. 6), except in the AN4I cells which showed a 20% decrease in gene expression ($P < 0.05$).

Discussion

VFL, a recently synthesized novel *Vinca* alkaloid, has demonstrated an overall in vivo antitumour activity superior to NVB, its parent molecule, against transplantable murine and human xenografted tumours [10, 16]. Resistance of tumour cells to multiple cytotoxic drugs, termed MDR, is a major limitation to successful chemotherapy. The widely reported mechanism of resistance to tubulin-binding agents is the so-called 'classic' MDR phenotype mediated by the Pgp efflux pump, a 170-kDa membrane protein belonging to the ABC cassette transporters and encoded by the *mdr1* gene. As shown previously, the murine P388 leukaemia cells selected in vivo for resistance to VFL exhibit such a 'classic' MDR phenotype [2, 6], as described previously for NVB, its parent molecule [1]. The aim of this study, was to establish whether there were any differences in the

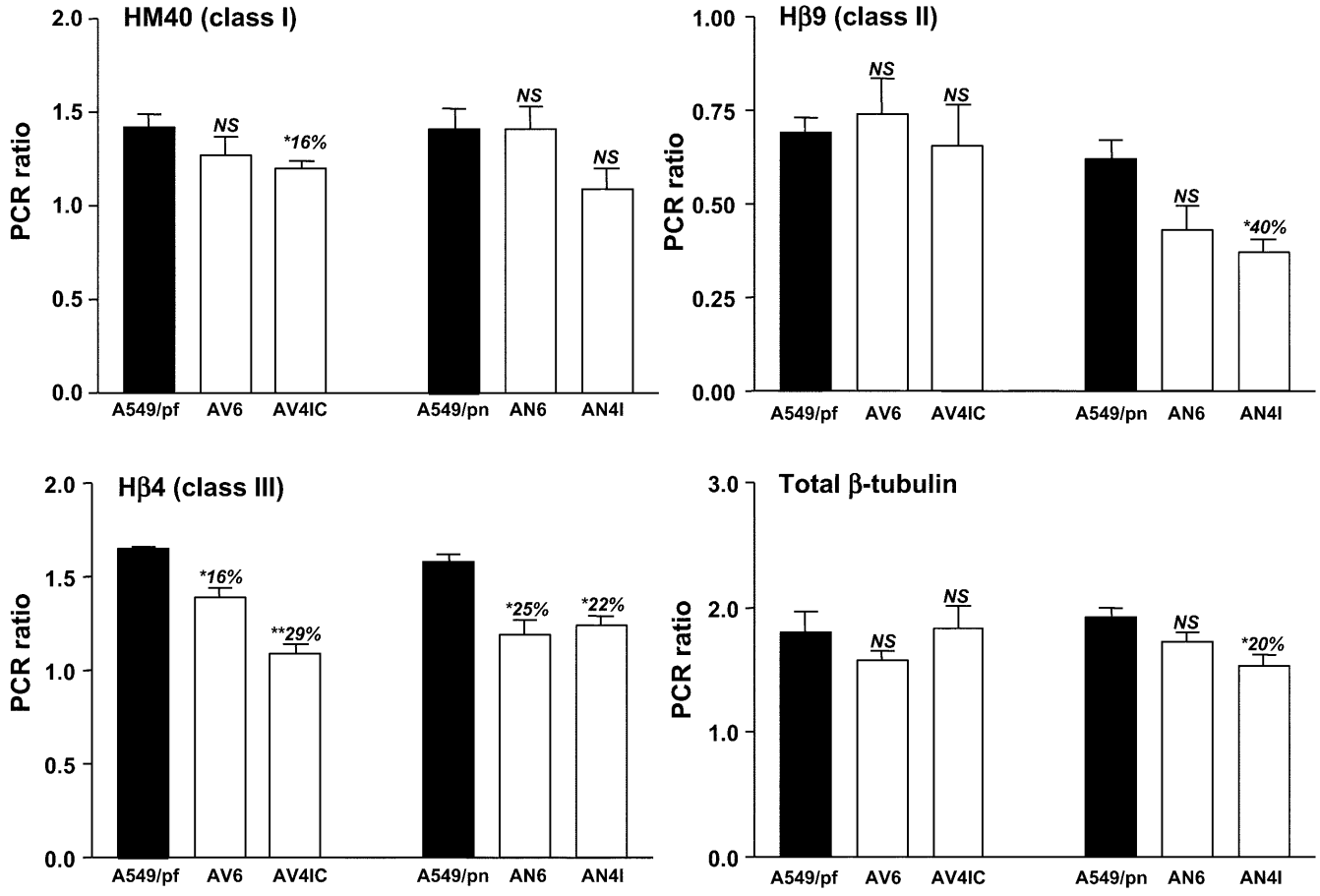


Fig. 6 Expression of β-tubulin isotypes in parental A549 sensitive and VFL- and NVB-resistant sublines. Following competitive RT-PCR, analysis of HM40, Hβ9, Hβ4 and single primer analysis of total β-tubulin, PCR ratios between the target and control β_2-microglobulin gene products were determined by densitometry for each sample. Between three and five independent PCR reactions were performed on each sample. The columns indicate mean values (*bars* SE) and the numbers at the top of the columns indicate the percentage decrease compared to parental cells ($^*P < 0.05$, $^{**}P < 0.005$; *NS* not significant)

development of resistance to VFL and NVB under strictly comparable selection conditions using either in vivo-treated murine leukaemia cells (P388) or in vitro-treated human lung carcinoma cells (A549). The drug resistance levels and the cross-resistance profile were then established, either in vivo or in vitro, for the various drug-resistant cell lines. Finally, MDR mechanisms associated with the A549 drug-resistant subline were characterized and compared.

First it was noted that resistance to VFL and NVB developed gradually over time in P388 cells, as shown by monitoring the sensitivities of P388-treated cells to the inducing compound during the course of resistance induction. Clearly, VFL-induced drug resistance developed far less readily than NVB, as shown in terms of the number of passages required to select for complete resistance. Regarding in vivo drug sensitivity profiles of the VFL- and NVB-resistant P388 sublines, all proved cross-resistant to the other *Vinca* alkaloids, doxorubicin and etoposide, but showed no such resistance to drugs such as cisplatin and camptothecin not implicated in the MDR phenotype, thus confirming data obtained with the P388/VFL-resistant subline reported previously [6].

A similar approach was next used in vitro to establish VFL- and NVB-resistant sublines of A549 human lung carcinoma cells. Resistance selected for in vitro by either VFL or NVB developed gradually over time in these cells. However, drug resistance to VFL was more difficult to induce than to NVB, as shown by drug sensitivity estimations. Indeed, a significant level of resistance to NVB but only a low level of resistance to VFL was noted after the first step of drug resistance induction, and resistance to continuous exposure to an intermediate concentration (twice the IC_{50} level) of NVB was readily obtained (2 months) but a significantly longer period (8 months) was needed for A549 cells to survive equivalent dosages of VFL. Moreover, sublines resistant to VFL generally expressed lower RL values compared with the corresponding NVB-resistant sublines. In terms of chemosensitivity profiles, the A549 sublines resistant to either compound proved cross-resistant to drugs implicated in the 'classic' MDR phenotype (other *Vinca* alkaloids, etoposide, doxorubicin), and sensitive to the reversal effects of verapamil, indicating that these resistant cells overexpress a functional MDR protein, namely Pgp, as previously described for VFL- and NVB-resis-

tant P388 cells [6] and generally for other *Vinca* alkaloid-resistant sublines [9].

Multidrug resistance in the clinical setting appears to be multifactorial and probably involves mechanisms besides Pgp overexpression. Alterations in microtubule structure and/or function can represent an important mechanism of resistance to tubulin-interacting compounds [4, 5]. Thus, we examined the expression of the specific β-tubulin isotypes in VFL- and NVB-resistant A549 sublines using RT-PCR analysis [12]. A significant decrease in expression of the Hβ4 gene, which encodes for the class III β-tubulin isotype, was quantitated in both VFL- and NVB-selected cells. These decreases ranged from 16% to 29% and although not large, are of interest as this isotype has previously been associated with resistance to paclitaxel [11, 12, 13, 22]. In contrast to the microtubule-stabilizing drug paclitaxel, where resistance is associated with increased expression of the Hβ4 β-tubulin gene, resistance to the microtubule-depolymerizing agents, VFL and NVB, was associated with decreased expression of this gene. This is consistent with our recent finding that Hβ4 (class III) β-tubulin is decreased in *Vinca* alkaloid-resistant paediatric tumour cell lines [14].

One of the NVB-selected cell lines, AN4I, displayed a 46% decrease in expression of the Hβ9 gene (class II β-tubulin isotype). This isotype has been found to be increased in a highly paclitaxel-resistant murine J744.2 cell line [8] and in paclitaxel-resistant A549 cells [12]. Consistent with other cell lines selected for resistance to antimitotic agents, expression of the HM40 gene (class I β-tubulin isotype) did not significantly change in the resistant cells. In order to identify whether the decreases observed in specific β-tubulin isotypes were due to decreases in the total levels of β-tubulin, consensus PCR primers were designed to amplify β-tubulin. A small reduction in expression of total β-tubulin in the AN4I cells (20%) was observed. Previous studies of cells selected for resistance to low levels of antimitotic agents have not found any change in overall tubulin expression [3, 17]. The observed decreases in specific β-tubulin isotypes are relatively small and are likely to have occurred as an early event during the drug selection process, possibly giving the cells an initial survival advantage prior to another mechanism such as Pgp-MDR being activated.

Overall, these results complement and extend the results of our previous detailed in vivo and in vitro studies in providing an interesting profile of preclinical activity for VFL. Indeed, evidence that VFL is a less-potent inducer of drug resistance than NVB adds weight to its potential value as a candidate for further clinical development. Phase I clinical studies of VFL in Europe have now been completed and phase II trials are scheduled for late 2000.

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