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Antimitotic and Tubulin-Interacting Properties of Vinflunine, a Novel Fluorinated *Vinca* Alkaloid

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ABSTRACT. This study aimed to define the mechanism of action of vinflunine, a novel *Vinca* alkaloid synthesised from vinorelbine using superacidic chemistry and characterised by superior *in vivo* activity to vinorelbine in preclinical tumour models. *In vitro* vinflunine cytotoxicity proved dependent on concentration and exposure duration, with IC_{50} values (72-hr exposures) generally ranging from 60–300 nM. Vinflunine induced G_2 + M arrest, associated with mitotic accumulation and a concentration-dependent reduction of the microtubular network of interphase cells, accompanied by paracrystal formation. These effects, while comparable to those of vincristine, vinblastine or vinorelbine, were achieved with 3- to 17-fold higher vinflunine concentrations. However, vinflunine and the other *Vincas* all inhibited microtubule assembly at micromolar concentrations. Vinflunine, like vinblastine, vincristine and vinorelbine, appeared to interact at the *Vinca* binding domain, as judged by proteolytic cleavage patterns, and induced tubulin structural changes favouring an inhibition of GTP hydrolysis. However, vinflunine did not prevent [^{3}H]vincristine binding to unassembled tubulin at concentrations $\leq$100 μM, and only weakly inhibited binding of [^{3}H]vinblastine or [^{3}H]vinorelbine. Indeed, specific binding of [^{3}H]vinflunine to tubulin was undetectable by centrifugal gel filtration. Thus, the comparative capacities of these *Vincas* to bind to or to interfere with their binding to tubulin could be classified as: vincristine > vinblastine > vinorelbine > vinflunine. By monitoring alkylation of sulfhydryl groups, differential effects on tubulin conformation were identified with vinflunine and vinorelbine acting similarly, yet distinctively from vinblastine and vincristine. Overall, vinflunine appears to function as a definite inhibitor of tubulin assembly, while exhibiting quantitatively different tubulin binding properties to the classic *Vinca* alkaloids. BIOCHEM PHARMACOL 55;5:635–648, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. vinflunine; *Vinca* alkaloids; *in vitro* cytotoxicity; tubulin binding; tubulin assembly; mitotic arrest

The dimeric alkaloids from *Catharanthus roseus* form an important class of antitumour agents, widely used in combination chemotherapy regimens for treating leukaemias and many solid tumours [1]. Vinblastine, the first alkaloid with antiproliferative properties, was discovered in extracts of the leaves of the *Vinca rosea* plant at the University of Western Ontario in 1958 [2] and, independently, at the Lilly Research Laboratories [3]. This discovery was followed rapidly by that of vincristine [4]. Although these two alkaloids differ structurally only in the functional group on the dihydroindole nitrogen, this minor distinction appears responsible for substantial differences in their clinical efficacies and toxicities [5, 6] and provided the impetus to search for new analogues with improved clinical benefits [7]. Amongst the numerous derivatives synthesised, only vindesine and vinorelbine, semisynthetic analogues of vinblastine are in clinical usage [8, 9].

It has been argued that the antiproliferative activity of this class of agents is due largely to their perturbation of mitosis [10, 11], resulting from their alteration of the microtubular dynamics of the mitotic spindle [12]. At the lowest effective *in vitro* concentrations, *Vinca* alkaloids were shown to block microtubule dynamics by reducing the rate of tubulin addition and loss at microtubule ends [13]. Higher drug concentrations resulted in extensive microtubular disassembly and still higher concentrations induced formation of tubulin paracrystals [14]. However, it is becoming clear that the various *Vinca* alkaloids differentially inhibit tubulin function, even if they all bind to the same site or sites on tubulin dimers [15, 16]. In addition, having examined the relationship between inhibition of cell cycle progression and induction of apoptosis by another tubulin-interacting agent, taxol, Lafon *et al.*, [17] have shown that cell cycle arrest was not systematically required for apoptosis. Therefore, the *in vivo* mechanism of action of tubulin-interacting agents might turn out to be much more complex.

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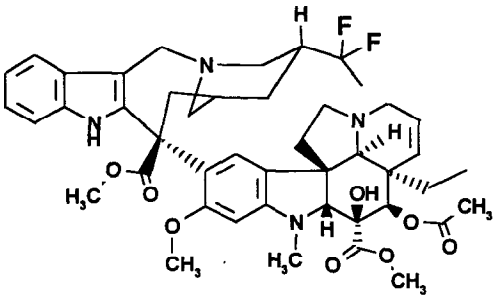
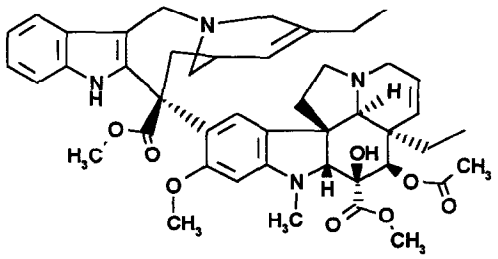


vinflunine



vinorelbine

FIG. 1. Molecular structures of vinflunine and vinorelbine.

Recently, a new *Vinca* alkaloid with substitutions in the little exploited region of the catharanthine moiety was obtained by reaction of an electrophilic agent with vinorelbine in superacid media [18]. Under these unusual conditions, contrasting with the complexity of its molecular structure, such a compound has proved stable and undergoes unexpected chemical transformations leading to 20',20'-difluoro-3',4'-dihydrovinorelbine or vinflunine (Fig. 1). The most impressive modification was the selective introduction of two fluorine atoms at the 20' position, this part of the molecule being previously inaccessible by classical chemistry. The 3',4' double bond was also reduced during the course of the reaction. Of major significance, this new vinorelbine derivative has demonstrated significantly superior antitumour efficacy *in vivo* compared to the other *Vinca* alkaloids against murine P388 leukaemia and B16 melanoma and has shown some definite activity against a number of human tumour xenografts [19]. The present study focuses on *in vitro* studies aimed at investigating the mechanism of action of vinflunine and determining the basis for this superior *in vivo* spectrum of activity. First, the effects of vinflunine on cell proliferation, on cell cycle distribution and on tubulin function within living cells have been studied and compared with those of vinorelbine, vinblastine or vincristine. Second, the action of vinflunine on purified tubulin, its interaction with either the *Vinca* alkaloid or the colchicine binding domains on tubulin, as well as its effects on the conformation of tubulin molecules, have been characterised.

MATERIALS AND METHODS

Materials

Vinblastine and vincristine were extracted, while vinorelbine and vinflunine were synthesised in the Pierre Fabre Laboratories and colchicine was purchased from Sigma. These test compounds were dissolved in deionised water immediately prior to use. RPMI¶ 1640 and BME (Basal Medium Eagle's) media were obtained from PolyLabo, while MEM (minimal essential medium) and foetal calf (FCS) or bovine (FBS) sera or horse serum (HS) were purchased from Life Technologies. Anti-α-tubulin monoclonal antibody and rhodamine-conjugated donkey anti-mouse IgG were obtained from Amersham and Interchim, respectively. Sources of the radiolabelled compounds were as follows: [^{3}H]colchicine (70 Ci/mmol) and [^{33}P]γ-GTP (2 Ci/mol) from DuPont NEN; [^{3}H]vinblastine (13.5 Ci/mmol), [^{3}H]vincristine (6.7 Ci/mmol), [^{3}H]vinflunine (0.25 Ci/mmol) and Iodo[^{14}C]acetamide (60 mCi/mmol) from Amersham; [^{3}H]vinorelbine (0.25 Ci/mmol) from CEA. The radiochemical purity of the *Vinca* alkaloids checked by thin layer chromatography was at least 98% for vinorelbine and vinflunine, but only ca. 90% for vinblastine and vincristine prepared with the higher specific activities. Sephadex G-50, superfine was purchased from Sigma and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was obtained from Sigma Chemical Company.

Cell Culture

L1210 murine leukaemia cells and A-10 rat aortic smooth muscle cells, purchased from the National Cancer Institute and the American Type Culture Collection, respectively were grown at 37° in 5% CO_2 in RPMI 1640 or BME media respectively, supplemented with 10% (v/v) HS or FCS respectively and 1% (v/v) penicillin-streptomycin. P388 murine leukaemia cells, a generous gift from Dr S. Cros (Laboratory of Fundamental Toxicology and Pharmacology), obtained originally from the NCI, were grown at 37° in 5% CO_2 in RPMI 1640 medium supplemented with 10% (v/v) HS, 1% (v/v) penicillin-streptomycin and 20 μM beta-mercaptoethanol. Suspension cultures were replaced with new stocks after 20–30 *in vitro* passages. Cultures of the human tumour cell lines (Table 1), grown in 5% CO_2 at 37°, were replaced with new stocks after 40–50 *in vitro* passages. MX-1 cells were purchased from the ATCC, while all other lines were bought from the NCI. The media used were supplemented not only with FCS, but also with fungizone (1.25 μg/mL) and penicillin-streptomycin (100 IU–100 μg/mL), all purchased from GibcoBRL. The nega-

¶ *Abbreviations*: BME, basal medium eagle; FBS, foetal bovine serum; FCS, foetal calf serum; GTP, guanosine triphosphate; HS, horse serum; MAPs, microtubule associated proteins; MEM, minimal essential medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PIPES, piperazine-*N*,*N*'-bis[2-ethanesulfonic acid]; RPMI, Roswell Park Memorial Institute; TCA, trichloracetic acid.

TABLE 1. Calculated IC_{50} values (nM) for each of the test compounds against the panel of cell lines used*

Cell line	Origin/Tumour histology	Vinblastine	Vincristine	Vinorelbine	Vinflunine
Murine tumour cell lines[a]					
L1210	murine leukaemia	16	15	28	80
P388	murine leukaemia	13	6.7	4.4	72
Human tumour cell lines[b]					
A549	lung/non small cell ca.	3.6	23	9.2	81
DLD-1	colon/adenocarcinoma	14	75	120	280
DU145	prostate/metastatic ca.	1.6	47	5.6	71
J82	bladder transitional cell ca.	16000	10000	18000	14000
LoVo	colon/adenocarcinoma	3.3	5.9	7.9	77
MX-1	breast/carcinoma	1.8	0.65	6.7	63
OVCAR-3	ovary/adenocarcinoma	2.0	2.3	5.1	72
SK-OV-3	ovary/adenocarcinoma	1.7	5.9	9.6	11000
T24	bladder transitional cell ca.	4.1	7.6	11	97

*IC_{50} values represent the concentrations required to inhibit cell growth by 50% and were derived from pooled data obtained from at least three independant experiments.

[a] Logarithmically growing cells were exposed to test compounds for 48 hr. Then, for L1210 cells, cell numbers were counted using a Coulter counter, and for P388 cells, proliferation was determined using a standard MTT-based colorimetric assay.

[b] Logarithmically growing cells, maintained in MEM/5% FCS (A-549, DLD-1, LoVo, MX-1, OVCAR-3 and T24) or RPMI/5% FCS (DU145, J82 and SK-OV-3), were exposed to test compounds for 72 hr. Then proliferation was determined using a standard MTT-based colorimetric assay.

tive mycoplasma status of each cell line was confirmed both by PCR testing and by cell culture.

Assays for Cytotoxicity

Effects of test compounds on L1210 cell proliferation were determined using a standard growth inhibition assay. Exponentially growing L1210 cells (1.5×10^5 cells/well) in a 24-well plate were exposed to a range of concentrations of test compounds for 48 hr, prior to determining cell numbers using an electronic particle counter (Coultronics). Effects of test compounds on the *in vitro* proliferation of P388 cells and of all the human tumour cell lines were determined using a standard MTT-based colorimetric assay [20], with minor modifications. Exponentially growing tumour cells were exposed to a range of concentrations of each test compound for a 72 hr incubation period, before MTT (1 mg/mL in RPMI 1640 without phenol red) was added to each well, followed 3 hr later after centrifugation by 0.1 mL DMSO to allow formazan solubilisation. Plates were then shaken before reading at 570 nm, using a microplate reader (MR7000, Dynatech Laboratories). For all tests, the final steps were followed by computerised data acquisition and processing, using in-house custom-made programs. IC_{50} values, i.e. concentration of compound required to reduce absorbance or cell number to 50% of that of control cells, were then determined from replicates of 6 from at least two independent experiments using another in-house program based on linear interpolation between data points.

Flow Cytometry

Flow cytometry analysis of cellular DNA content by propidium iodide staining was performed as described previously [21]. Logarithmically growing P388 cells were incubated with solvent or test compound for 18 hours prior to centrifugation (4°, 250 × g, 10 min), washed twice with cold phosphate-buffered saline (PBS) and slowly fixed with ice-cold 70% ethanol. After at least 30 min at 4°, cells were trypsinised (30 μg/mL, pH 7.6) at room temperature for 10 min, before adding a mixture of trypsin inhibitor (500 μg/mL) and RNase A (100 μg/mL, pH 7.6) for a further 10 min and then staining with a propidium iodide solution (propidium iodide 416 μg/mL and spermine tetrahydrochloride 1160 μg/mL, pH 7.6) for at least 15 min at 4° in the dark. Samples were analysed using an EPICS Elite flow cytometer (Coulter Corporation) and the percentage of cells was calculated in the G_1, S and G_2 + M phases using MultiCycle software (Phoenix Flow Systems).

Mitotic Index Determinations

Exponentially growing L1210 cells were incubated with test compounds for 48 hr, prior to centrifugation at 400 × *g* for 5 min and resuspension of the resultant cell pellet in 1 mL 75 mM KCl at 4°. After 20 min, 1 mL methanol-acetic acid (3:1) as fixative was added slowly under constant mild agitation. Cells were then repelleted and washed twice with 1 mL of fixative, before being resuspended in fixative and dispensed onto glass slides. After drying, samples were stained with Giemsa solution [22]. Four hundred cells/treatment were scored for the presence of mitotic figures by optical microscopy (Nikon, Subra) and the mitotic index was calculated as the proportion of cells with mitotic figures. Results are expressed as means from three independent experiments, associated with the standard error of the estimate of the mean value.

Staining of Microtubule Network by Indirect Immunofluorescence

A-10 cells, grown to near-confluency on glass coverslips, were incubated for 24 hr with test compounds before being fixed with (−20°)-cold methanol for 5 min and then treated with (−20°)-cold acetone for 5 min to increase

their permeability. Cells were then incubated with PBS containing 10% (v/v) FBS to block nonspecific binding sites before incubation at 37° for 60 min with the anti-α-tubulin monoclonal antibody diluted 1/50. Bound primary antibodies were visualised subsequently by a 30 min incubation with rhodamine-conjugated donkey anti-mouse IgG diluted 1/200 [23]. Coverslips were mounted with antifading medium containing 90% (v/v) glycerol and 0.1 mg/mL diazabicyclo-2,2 octane and their fluorescence patterns examined by microscopy (Zeiss) and photographed.

Extraction of Tubulin

Microtubular proteins were prepared from sheep brain by three cycles of assembly and disassembly, as described previously [24]. The final pellet was resuspended in extraction buffer (100 mM PIPES pH 6.6, 0.5 mM $MgCl_2$, 0.1 mM EDTA, 1 μM EGTA) and stored in liquid nitrogen before use or further purification. MAPs (Microtubule associated proteins) were separated from tubulin by ion-exchange chromatography (SP, Pharmacia) in 50 mM PIPES buffer pH 6.6, 0.5 mM EGTA, 0.25 mM $MgCl_2$ and 0.25 mM GTP [25]. Tubulin was eluted in the void volume and MAPs in the fraction eluted by the addition of 0.8 M NaCl to the same buffer. Fractions containing purified tubulin were pooled and stored in liquid nitrogen before use. Protein concentrations were determined by the method of Lowry *et al.* [26], using bovine serum albumin as a standard.

Microtubule Assembly or Disassembly

In vitro microtubule assembly or disassembly was followed turbidimetrically as described by Gaskin and Cantor [27], by quantitating variations in absorbance at 350 nm using a spectrophotometer (DU 640, Beckman) equipped with thermostatically-controlled cuvettes. Turbidity experiments were conducted with 2.3 mg/mL solutions of microtubular proteins in assembly buffer containing 100 mM PIPES pH 6.6, 0.5 mM $MgCl_2$, 0.1 mM EDTA, 1 μM EGTA and 2 mM GTP. Solutions, initially at 0°, were warmed to 37° to initiate assembly, then after 12 min at 37°, the temperature was decreased to 0° again to induce disassembly. When purified tubulin was used, reaction mixtures containing 2 mg/mL of purified tubulin in 700 mM monosodium glutamate pH 6.6, 1 mM $MgCl_2$ and 0.4% DMSO were preincubated at 37° for 15 min without GTP, chilled on ice for 10 min and 0.4 mM GTP then added at the same time as the test compound [28]. Turbidity of the tubulin solution was determined at both 0° and 37°, as described previously. The IC_{50} values for inhibition of assembly or disassembly were those concentrations that reduced assembly or disassembly by 50% as compared to solvent controls, as determined from five independent experiments (or two independent experiments for inactive compounds), using an in-house program.

Tubulin Proteolysis Assay

Assays were performed as described by Sackett and Varma [29]. Briefly, 0.3 mg/mL of purified tubulin were preincubated for 30 min at 30° with or without an excess of test compound and then digested on ice with 5 μg/mL of chymotrypsin or trypsin for 30 min. Enzyme action was halted by the addition of 10 mM PMSF (for chymotrypsin) or 1 mM leupeptin (for trypsin). Then, samples were separated on SDS-polyacrylamide 12% minigels (Bio-Rad). Gels were stained with Coomassie Blue R.

Measurement of GTPase Activity

GTPase activity was determined by measuring the release of ^{33}P from [^{33}P]γ-GTP, as described by Hamel and Lin [30]. Experimental conditions were comparable to those used for following the assembly or disassembly of purified tubulin. Purified tubulin was incubated for 30 min at 37° in the presence of [^{33}P]γ-GTP (2 Ci/mol, 50 μCi/mL). Ten microlitres of the reaction mixture were then mixed with 40 μL of 25% acetic acid before spotting 10 μL of this mixture onto 20 × 20 PEI-cellulose thin-layer chromatography sheets (Macherey-Nagel). The sheets were soaked in methanol for 5 min, air-dried and then soaked in water for 2 min before being subjected to ascending chromatography with 1.0 M KH_2PO_4. Membranes were dried, exposed to a GS525 Molecular Imager system (Bio-Rad) and scanned using a PhosphorImager (Bio-Rad). Patterns of [^{33}P]γ-GTP and free ^{33}P resulting from GTP hydrolysis were visualised and quantified with Molecular Analyst Software (Bio-Rad). Quantification resulting from three independent experiments in duplicate was analysed statistically using the Mann–Whitney test and SigmaStat 2.0 Software (Jandel Corporation).

Centrifugal Gel Filtration Assay

The extent of binding of radiolabelled-*Vinca* alkaloids was followed by a centrifugal gel filtration method described previously [28]. Tubulin obtained from sheep brain by three cycles of assembly/disassembly was incubated at 0.5 mg/mL for 20 min at 22° with [^{3}H]*Vinca* alkaloids (0.1 mCi/mL), in 100 mM PIPES pH 7, 0.5 mM $MgCl_2$, 2% (v/v) DMSO, in the presence or absence of an unlabelled competitor compound at a selected concentration, in a final volume of 0.5 mL. Duplicate or triplicate aliquots (0.15 mL) of this reaction mixture were applied to 1 mL microcolumns of Sephadex G-50 superfine, which were then centrifuged at 900 × g for 4 min at 4°. Protein and radioactivity in the filtrates were determined, using the Lowry method [26] and a β liquid scintillation counter (205 TR, Packard), respectively. The binding of [^{3}H]*Vinca* alkaloids to tubulin was evaluated by calculating the ratio of the amount of bound [^{3}H]compound to tubulin per amount of total microtubular protein for each sample as average values of duplicates or triplicates. The ratio of each sample was expressed as a

percentage of the control ratio, i.e. when the [^{3}H]compound was used in the absence of added competitor. Control experiments showed that no radioactivity passed through the column in the absence of protein. Using Eppendorf plastic tubes (66-317, PolyLabo), relatively weak adsorption (5–10%) occurred during the incubation of [^{3}H]*Vinca* alkaloids or [^{3}H]colchicine at 22° or 37°, respectively.

Tubulin Alkylation

Effects of drugs on the alkylation of tubulin by iodoacetamide were studied as described by Ludueña and Roach [31], with minor modifications. Tubulin (0.7 mg/mL) was incubated for 2 hr at 37° in the reaction buffer (100 mM PIPES pH 6.4, 0.5 mM $MgCl_2$, 0.1 mM EGTA and 1 mM GTP) in the presence of 100 μM of test compound. Iodo[^{14}C]acetamide, diluted with cold iodoacetamide to a final concentration of 1.3 mM and a specific activity of 1.92 Ci/mol, was immediately added to the prepared incubation mixture of tubulin for a second incubation of 0–2 hr at 37°. At each time point, the alkylated tubulin was precipitated by dilution with an equal volume of 10% trichloroacetic acid (TCA) at 4°. Precipitates were collected on GF/C filters (Whatman), washed 3 times with 10% (v/v) TCA and with 95% (v/v) ethanol, before being added to 4 mL scintillation solution (Ultragold XR, Packard) for radioactivity counting. The amount of iodo[^{14}C]acetamide bound to tubulin was evaluated by calculating the ratio of the number of moles of bound iodo[^{14}C]acetamide per mole of tubulin. Mean values were calculated from two separate experiments, performed in triplicate using two independent preparations of tubulin.

RESULTS

Effects of Vinflunine on Cell Proliferation

Data in Fig. 2 illustrate that the cytotoxicity of vinflunine against cultured L1210 murine leukaemia cells is strongly dependent both on concentration and on duration of exposure. Negligible cytotoxicity resulted from a 6-hr exposure, consistent with vinflunine exerting its lethal effects at one or more specific phases of the cell cycle (see below). However, by prolonging the exposure period to 24 or 48 hr, a plateau-shaped dose-response curve was noted, indicating that once a certain concentration is achieved, cell kill becomes more dependent on increased exposure duration than on concentration, an observation consistent with that observed in classic *Vinca* alkaloids [32].

The study was then extended to include one more murine leukaemia cell line and a panel of nine human tumour cell lines (Table 1), and the results obtained with vinflunine were compared to those obtained with vinorelbine, vinblastine or vincristine under identical experimental conditions. A common feature of all the dose-response curves, irrespective of the cell line or the test compound

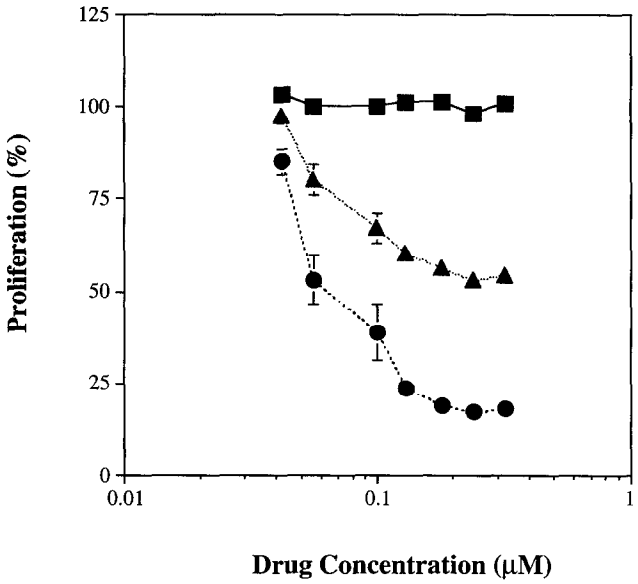


FIG. 2. Time- and concentration-dependent effects of vinflunine on cell proliferation *in vitro*. L1210 cells were incubated with the indicated concentrations of vinflunine for 6 (■), 24 (▲) or 48 (●) hr and then cell numbers were counted and the percent cell proliferation determined. Values reported are the means of 2–3 duplicate cultures obtained from independent experiments. Bars indicate the standard errors.

used, was that cytotoxicity reached a plateau over a two to three log scale of concentrations (data not shown). Comparing the IC_{50} values obtained (see Table 1), vinflunine appeared 3- to 17-fold less cytotoxic than vinorelbine, vinblastine or vincristine against these two murine leukaemias and was also consistently least potent against the panel of nine human tumour cell lines, with higher IC_{50} values by factors of 2- to 44-fold in six of the lines, by 10- to 100-fold against the MX-1 cells, and by up to four orders of magnitude vis-à-vis the SK-OV-3 cell line (see below). In only one of the cell lines selected, namely J82, were similar sensitivities shown for all four test compounds, and it is notable that this particular cell line was markedly unresponsive to all these *Vinca* alkaloids, as reported earlier for the classic *Vinca* alkaloids [33].

Figure 3 provides evidence of the good correlations between log IC_{50} values obtained evaluating cytotoxicity in these nine human tumour cell lines for these three *Vinca* alkaloids relative to vinblastine. A very high correlation coefficient ($r = 0.97$) was identified when comparing log IC_{50} values for vinblastine versus vinorelbine. This correlation was lower with vincristine ($r = 0.85$) and considerably lower with vinflunine ($r = 0.41$). However, this latter poor correlation appears to be due to the high relative 'resistance' of SK-OV-3 cells to vinflunine, since if this cell line is excluded from the analysis, the correlation coefficient is 0.99 (see Fig. 3D). Correlations between vinblastine and vinorelbine or vincristine remained unchanged if the SK-OV-3 cell line data were omitted from the analyses.

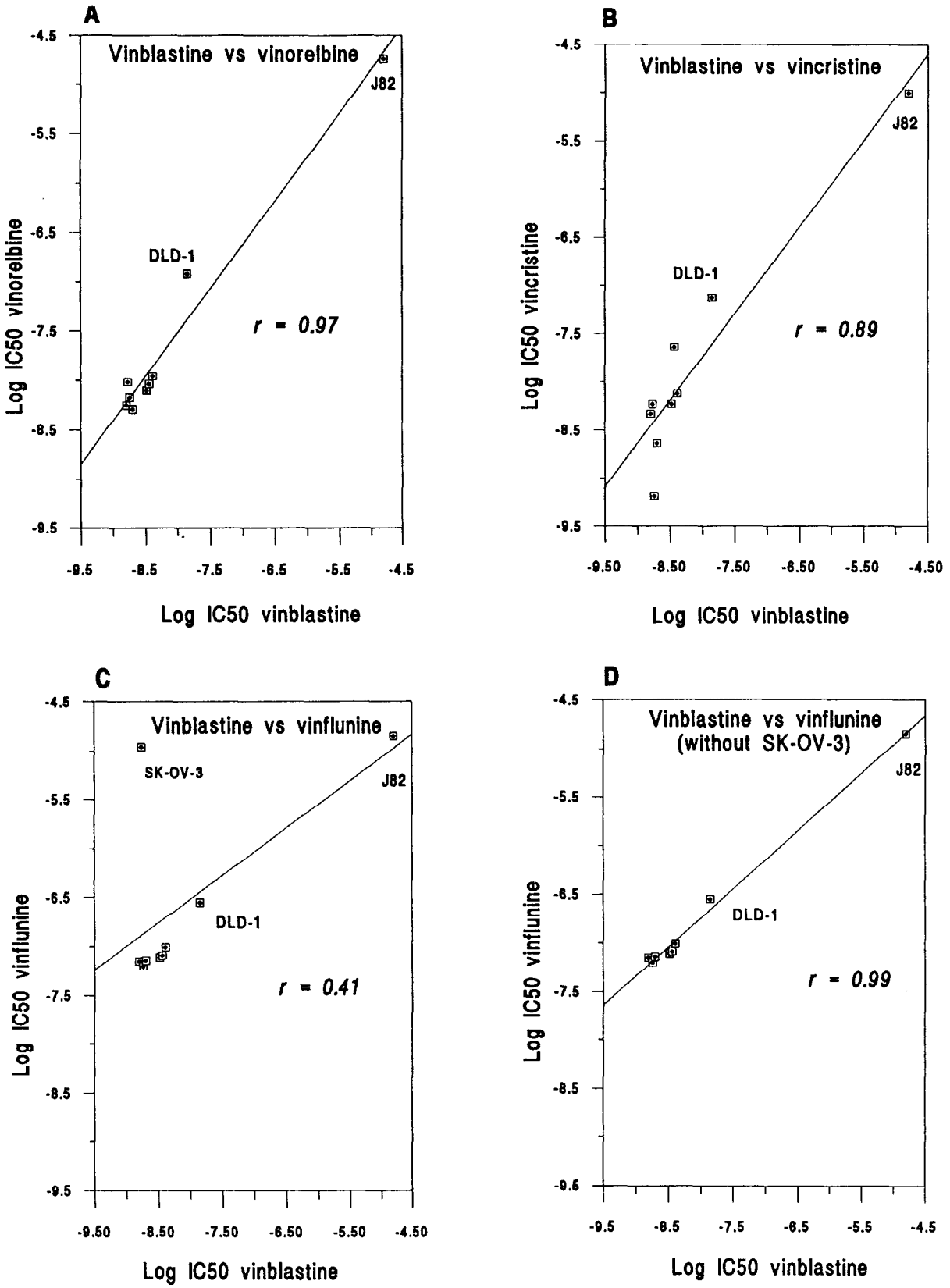


FIG. 3. Correlation between log IC_{50} values for the indicated drugs using vinblastine as the reference compound against a panel of nine (A, B and C) or eight (D) human tumour cell lines. (A) vinblastine versus vinorelbine; (B) vinblastine versus vincristine; (C) vinblastine versus vinflunine; (D) vinblastine versus vinflunine (without the SK-OV-3 cell line data included in the analysis).

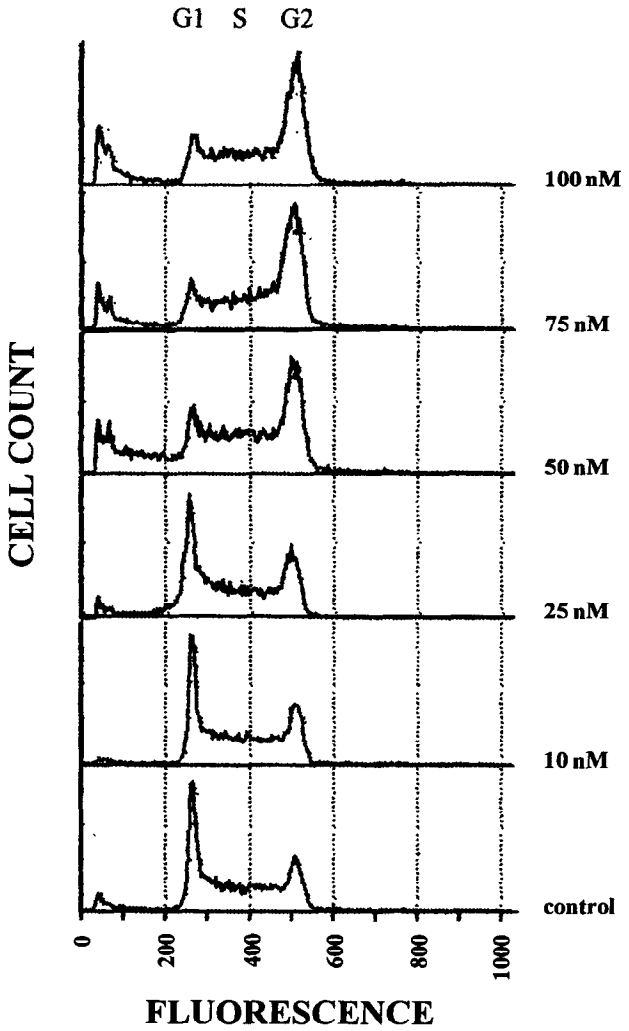


FIG. 4. Effects on the DNA distribution histogrammes of logarithmically growing P388 cells following an 18-hr exposure to increasing concentrations of vinflunine. Samples were prepared and stained with propidium iodide, as described in "Materials and Methods," and analysed using an EPICS Elite flow cytometer. Representative histogrammes from a single experiment using arbitrary fluorescence units and cell counts are depicted.

Effects of Vinflunine on Cell Cycle Distribution

The results of flow cytometric analyses of cellular DNA content of P388 leukaemic cells using propidium iodide staining (Fig. 4) indicate that an 18-hr incubation (approximately one population doubling time) with vinflunine induced a dose-dependent accumulation of P388 cells in the G_2 + M phases of the cell cycle. Control cells and those incubated with 10 nM vinflunine had 12 ± 1.2 and 13 ± 1.3% of the population, respectively distributed in the G_2 + M phases of the cycle, whereas following exposure to higher concentrations of 50 or 100 nM, 28 ± 4.1 and 51 ± 4.8% of the population, respectively accumulated in G_2 + M. In no case were polyploid cells observed. In contrast, the peak corresponding to amounts of DNA lower than in the G1-phase was most likely due to cell fragmentation.

Effects of Vinflunine on Cell Division

Since vinflunine induced cell proliferation arrest in the G_2 + M phases of the cycle, its specific effects on mitosis were next evaluated. Vinflunine was shown to induce accumulation of L1210 cells in mitosis, the extent of which increased with increasing drug concentration (data not shown). At a vinflunine concentration 5-fold the IC_{50} value, i.e. 1 μM, the mitotic index estimation was 23 ± 3%. These effects on L1210 cells were similar to those observed with vinblastine, vincristine or vinorelbine, also using concentrations approximating to 5-fold the IC_{50} values, when mitotic index values of 29 ± 2, 15 ± 1 and 25 ± 2%, respectively were recorded.

Effects of Vinflunine on the Interphase Microtubular Cytoskeleton

The interaction of vinflunine with tubulin within cells was investigated by fluorescence microscopy and compared to that of vinorelbine and vinblastine, using A-10 rat smooth muscle cells because of their extensive networks of microtubules. These cells grow as thinly spread, but tightly adherent monolayers, hence their cytoskeleton is easily visualised and photographed. Equitoxic concentrations of each test compound, namely those resulting in 30–60% inhibition of A-10 cell proliferation, were used. Untreated cells showed a rich and intact cytoplasmic microtubular cytoskeleton (Figs. 5A and 5B), whereas vinflunine induced a concentration-dependent reduction of their microtubular network (Figs. 5C–5F). After exposure to 0.5 μM vinflunine, wide cytoplasmic areas were apparent with a low microtubule content, while the remaining microtubules localised around the nucleus (Figs. 5C and 5D). At 1 μM, vinflunine induced a more marked effect on the microtubular network of these A-10 cells, and only a few short microtubules remained around the nuclei (Figs. 5E and 5F), with some cells appearing to have completely lost their microtubules. Under these conditions, vinorelbine and vinblastine induced similar effects, except that approximately 5-fold and 20-fold lower concentrations, respectively, were sufficient to cause complete microtubular network disruption (data not shown). In addition, no microtubular bundles were observed in A-10 cells treated with vinflunine, suggesting that this new derivative did not stabilise microtubules. It was also notable that at concentrations that induced a marked reduction of the microtubular cytoskeleton, neither vinflunine, vinorelbine, nor vinblastine modified the actin network, another major constituent of the cellular cytoskeleton (data not shown). At high concentrations, *Vinca* alkaloids can aggregate microtubules, resulting in the formation of paracrystals. Following exposure of A-10 cells for 24 hr to 50 μM vinflunine, paracrystals of different sizes, but with a majority of large structures, were observed within all cells (Figs. 5G and 5H). Similar effects were seen with vinorelbine and vinblastine, but again at 3-fold and 17-fold lower doses,

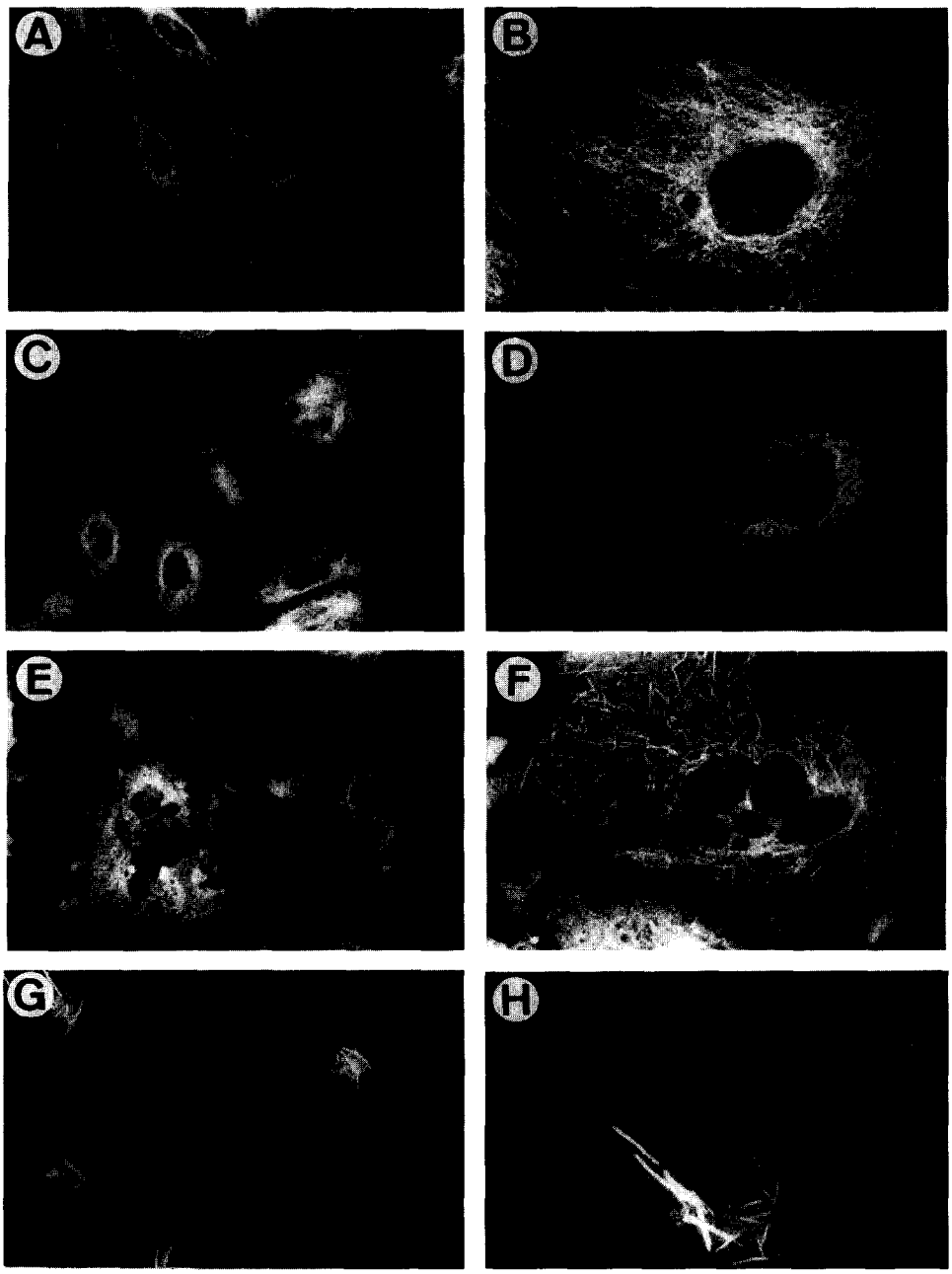


FIG. 5. Effects of vinflunine on the interphase microtubular cytoskeleton of A-10 cells. Cells were grown on coverslips and treated with sterile demineralised water (A and B), 0.5 μM vinflunine (C and D), 1 μM vinflunine (E and F) or 50 μM vinflunine (G and H) for 24 hr. The cells were then stained for microtubules using an anti-α monoclonal antibody. The fluorescence photographs were taken using either a ×40 (A, C, E and G) or a ×100 (B, D, F and H) objective.

respectively (data not shown). In all cases, the size and incidence of these crystals increased with test compound concentration (data not shown).

Effects of Vinflunine on Tubulin Assembly or Disassembly

The capacity of vinflunine to inhibit tubulin assembly *in vitro* was determined by monitoring turbidity changes with time. Figure 6 shows the concentration-dependent inhibition of microtubule assembly by vinflunine, using 2.3 mg/mL of microtubular proteins. At concentrations higher than 10 μM, vinflunine appeared to cause paracrystal formation, characterised by an increase in turbidity at 350 nm (Fig. 6). Concentrations of vinflunine necessary to inhibit the assembly of microtubule proteins or purified tubulin by 50% were comparable (Table 2), consistent with the molecular target of this new derivative being tubulin rather than MAPs. These effects, recorded with vinflunine, were similar to those observed with the three other *Vinca* alkaloids tested (Table 2). In addition, neither vinflunine nor any of the

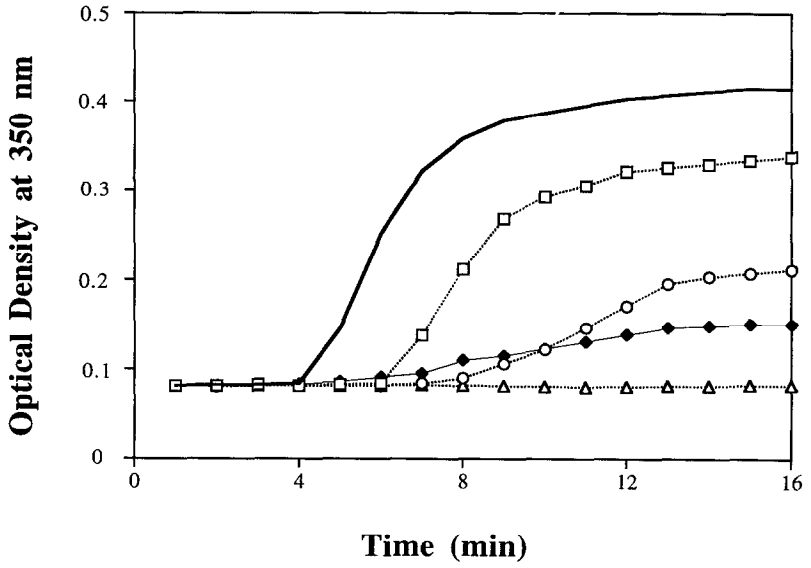


FIG. 6. Effects of vinflunine on assembly of microtubular proteins. *In vitro* microtubule assembly was followed turbidimetrically, as described in "Materials and Methods." Microtubular proteins were incubated in the presence of vinflunine at concentrations of 1 μM (□), 3.2 μM (○), 5.6 μM (△) and 100 μM (◆) or in the presence of water only used as control (▪).

other *Vinca* alkaloids exhibited a stabilising effect on assembled microtubules (data not shown).

Effects of Vinflunine on the Drug Binding Domains of Tubulin

Two major tubulin binding sites have been identified, termed the colchicine and the *Vinca* alkaloid sites [34]. Interactions of vinflunine with these tubulin domains were assessed. Initially, a proteolysis assay was used, as described by Sackett [35], based on the fact that the binding of tubulin-interacting agents to tubulin modifies the proteolysis of purified tubulin by chymotrypsin or trypsin, with the proteolytic pattern observed dependent on the binding site of the test compound and on any structural modifications induced by this binding. Native tubulin can be cleaved by trypsin and by chymotrypsin. In each case, two major characteristic fragments are produced, but any binding of colchicine or *Vinca* alkaloids to tubulin differentially alters these cleavage patterns. Chymotryptic cleavage of colchicine-tubulin complexes produced a new band ("β-col"), while tryptic cleavage was enhanced. In contrast, chymotryptic cleavage of vinblastine- or vincristine-tubulin complexes was enhanced, whereas tryptic cleavage was inhibited [35]. Experimental conditions were optimised to exclude paracrystal formation and tubulin assembly [14, 27] and thus, to reduce structural modifications as an exclusive effect of the binding. The results of our study show that vinflunine and vinorelbine induced similar patterns of chymotryptic and tryptic cleavage and that these were identical to those identified with vinblastine and vincristine (Figs. 7A and 7B), indicating that vinflunine, as expected, interacts with the *Vinca* alkaloid binding domain of tubulin. However, this technique has not permitted identification of any definite differences between these various *Vinca* alkaloid derivatives relating to their efficiencies in either stimulating chymotryptic activity or inhibiting tryptic cleavage.

TABLE 2. Effects of vinflunine on tubulin assembly—comparison with the effects of vinblastine, vincristine or vinorelbine

Compound	Inhibition of tubulin assembly IC_{50} (μM)*	
	microtubular proteins	purified tubulin
Vinflunine	3.1	2.5
Vinblastine	2.2	2.7
Vincristine	1.7	0.5
Vinorelbine	1.7	0.9

The inhibition of tubulin assembly was determined using a turbidity assay, with either a solution of microtubular proteins (three-cycle-purified microtubular proteins) or purified tubulin (three-cycle-purified microtubular proteins further purified by ion-exchange chromatography). *IC_{50} is the concentration that inhibits tubulin assembly by 50%.

GTP Hydrolysis by Tubulin

Vinflunine bound to tubulin induced structural changes which favoured an inhibition of GTP hydrolysis. This inhibition was concentration-dependent with 39 ± 11% inhibition observed at 10 μM vinflunine and complete inhibition being registered at 100 μM (data not shown). Similar results were obtained with the other *Vinca* alkaloids tested and these, as expected, were opposite to the stimulatory effects observed with colchicine, with GTP hydrolysis being elevated to 220 ± 20% in the presence of 10 μM colchicine.

Influence of Vinflunine on the Tubulin Binding of Radiolabelled-Vincristine, -Vinblastine or -Vinorelbine, and Binding of Radiolabelled-Vinflunine to Tubulin

The centrifugal gel filtration technique used to evaluate the ability of vinflunine to compete with the tubulin binding of

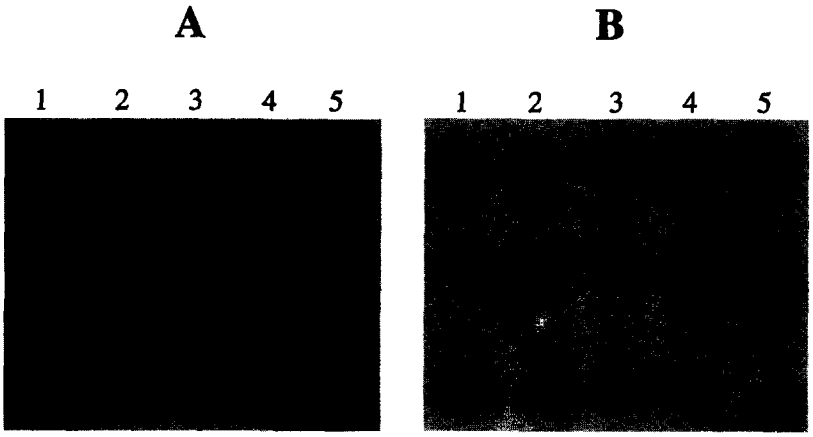


FIG. 7. Effects of vinflunine on the proteolysis of purified tubulin in comparison with those of colchicine or three classical *Vinca* alkaloids. After a preincubation without (lane 1) or with 100 μM colchicine (lane 2), 100 μM vinflunine (lane 3) or 100 μM vinorelbine (lane 4) for 30 min at 30°, the tubulin was digested at 4° with either chymotrypsin (A) or trypsin (B) for 30 min. At the end of the incubation, samples were separated on SDS-PAGE 12% minigels and stained with coomassie Blue R. Lane 5 shows tubulin before proteolysis. *New band "β-col" produced by chymotryptic cleavage of colchicine-tubulin complexes.

[^{3}H]*Vinca* alkaloids did not reveal, as indicated in Table 3, any marked inhibition by vinflunine of the binding of labelled vinorelbine, vinblastine or vincristine to tubulin at concentrations ≤50 μM. Even when the vinflunine concentration was increased to 100 μM, there was still no apparent effect on the binding of [^{3}H]vincristine to tubulin, although some slight inhibition of the other two *Vinca* alkaloids became detectable, with residual bound [^{3}H]vinblastine = 62.8% and residual bound [^{3}H]vinorelbine = 60.7%. In contrast, as shown by data in Table 3, the addition of 50 μM of vincristine, vinblastine or vinorelbine to the reaction mixture markedly reduced the binding of the corresponding labelled *Vinca* alkaloids by 57.4% to 98.4%. At these same concentrations, neither vinflunine nor any of the other *Vinca* alkaloids were shown to interfere with the binding of [^{3}H]colchicine to tubulin (data not shown), consistent with the results of the proteolysis assays.

The extent of binding of [^{3}H]vinflunine to tubulin under these experimental conditions was next determined (Fig. 8A). The results showed that under these experimental conditions, specific binding of radioactive vinflunine to tubulin was undetectable, with a comparable extent of binding being observed to tubulin and serum albumin. In contrast, [^{3}H]vinorelbine, [^{3}H]vinblastine or [^{3}H]vincristine all showed saturable and specific binding, under the same experimental conditions (Fig. 8B–D).

TABLE 3. Effect of vinflunine on the binding of [^{3}H]vincristine, [^{3}H]vinblastine, [^{3}H]vinorelbine to tubulin*—comparison with the effects of vinorelbine, vinblastine or vincristine

Added competitor (50 μM)	Residual bound [^{3}H]*Vinca* alkaloids (% of control)		
	[^{3}H]vincristine	[^{3}H]vinblastine	[^{3}H]vinorelbine
Vinflunine	96 ± 4	90 ± 4	83 ± 15
Vinorelbine	43 ± 0.4	20 ± 2	18 ± 3
Vinblastine	32 ± 7	28 ± 3	4 ± 0.1
Vincristine	15 ± 2	6 ± 1	2 ± 0.5

*Binding of [^{3}H]*Vinca* alkaloids to tubulin was determined by centrifugal gel filtration. Reaction solutions containing 0.5 mg/mL tubulin, 3 μM [^{3}H]*Vinca* (0.1 Ci/mmol) and 50 μM of unlabelled competitor compound were incubated at 22° for 20 min. At the end of this incubation, the amount of [^{3}H]*Vinca* remaining bound to tubulin was determined as described in "Materials and Methods" and expressed as a percentage of control. Mean values ± SEM were calculated from triplicates or duplicates.

Data reported in Table 3 show that vincristine proved most effective at competing for the binding to tubulin of both [^{3}H]vinblastine and [^{3}H]vinorelbine, with vinorelbine appearing a less effective competitor, while vinblastine expressed intermediate competitive properties. Results illustrated in Fig. 8 also indicate the differing extents of the binding of the three *Vinca* alkaloids to tubulin, with vincristine apparently being bound most strongly and vinorelbine least strongly. Overall therefore, in terms of their capacities to interfere with the binding of [^{3}H]*Vinca* alkaloids to tubulin or to bind to tubulin under these experimental conditions, the various compounds tested could be classified as follows: vincristine > vinblastine > vinorelbine > vinflunine.

Effects of Vinflunine on the Alkylation of Tubulin by Iodo[^{14}C]acetamide and a Comparison with the Effects of Vinorelbine, Vincristine or Vinblastine

Sulfhydryl groups on the tubulin molecules have emerged as potential probes for characterising drug binding sites, based on their reactivity with the alkylating agent iodo[^{14}C]acetamide [31]. Specific alkylation of the sulfhydryl groups of tubulin was monitored in the presence of vinflunine to investigate the drug's effects on tubulin conformation. When tubulin was incubated with either vinflunine, vinorelbine, vinblastine or vincristine at 100 μM prior to alkylation, the quantity of iodo[^{14}C]acetamide bound to tubulin after 60 min was limited to a similar extent (Fig. 9). However, after the incubation was prolonged to 120 min, whereas vinblastine and vincristine still inhibited the alkylation, this reaction was no longer affected by the presence of either vinflunine or vinorelbine (Fig. 9). This observation could indicate that the vinflunine-tubulin and vinorelbine-tubulin complexes dissociate more rapidly than those formed with vinblastine or vincristine. Alternatively, the structural modifications of the tubulin molecule induced by vinflunine and vinorelbine might actually be different from those induced by

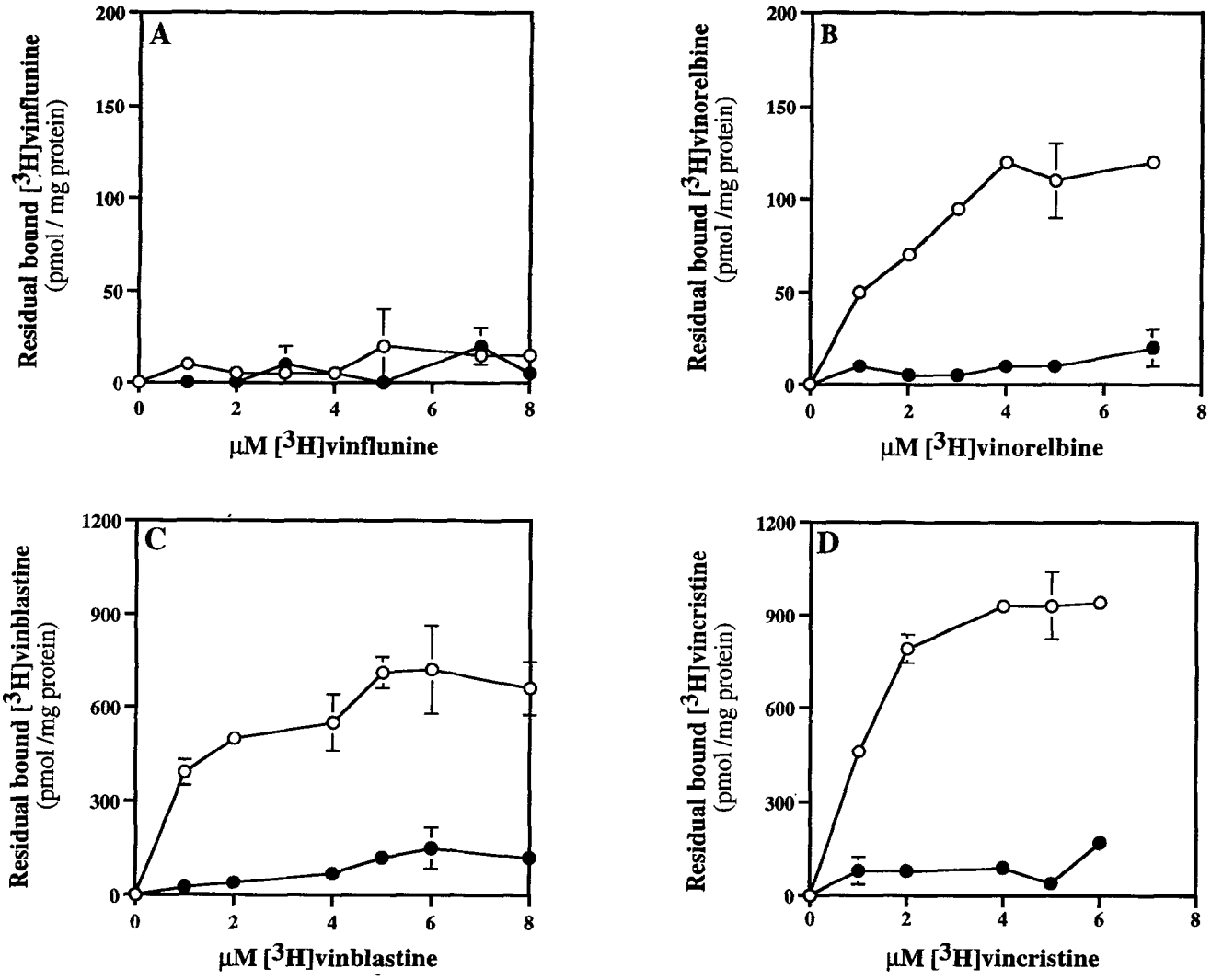


FIG. 8. Binding of [^{3}H]vinflunine to tubulin. Comparison with vincristine, vinblastine or vinorelbine. The binding of [^{3}H]vinflunine was followed using a centrifugal gel filtration assay. Reaction solutions containing 0.5 mg/mL tubulin and a variable concentration of [^{3}H]vinflunine (A), [^{3}H]vinorelbine (B), [^{3}H]vinblastine or [^{3}H]vincristine (D) (0.1 Ci/mmol) were incubated at 22° for 20 min. At the end of this incubation, the amount of [^{3}H]*Vinca* alkaloid remaining bound to tubulin was determined, as described in "Materials and Methods." Results are expressed as means from triplicates or duplicates, with the associated standard error of the estimate of the mean value.

vinblastine and vincristine. Overall, therefore, these results suggest that these four *Vinca* alkaloids may exert differential effects on the conformation of the tubulin molecule, with vinflunine acting in a similar manner to vinorelbine and quite different from that of vinblastine and vincristine.

DISCUSSION

Vinflunine, a novel fluorinated synthetic analogue of vinorelbine, has shown marked *in vivo* antitumour activity, with good overall tolerance in preclinical models, one which was generally superior to that of the other *Vinca* alkaloids, including vinorelbine, tested concurrently [19]. In attempting to define the basis for this enhanced spectrum of antitumour activity, the effects of vinflunine within cells and on tubulin function *in vitro* have been analysed. The first series of studies provide clear evidence that vinflunine exhibited several characteristics in common with the other *Vinca* alkaloids, but differences were identified in certain tubulin-interacting properties of this compound and these were examined in more detail in the second series of investigations.

Vinflunine induced a concentration-dependent inhibition of L1210 cell growth, featuring a steep dose-response curve indicative of a narrow dose range of cytotoxicity commonly observed with *Vinca* alkaloids. Vinflunine cytotoxicity also increased with exposure duration. Survival curves for two murine leukaemia and nine human solid tumour cell lines were all exponential-plateau type, characteristic of Class II agents in the Kinetic Classification of Antitumour Agents [36, 37]. The implication of this classification is that once the plateau-level concentration

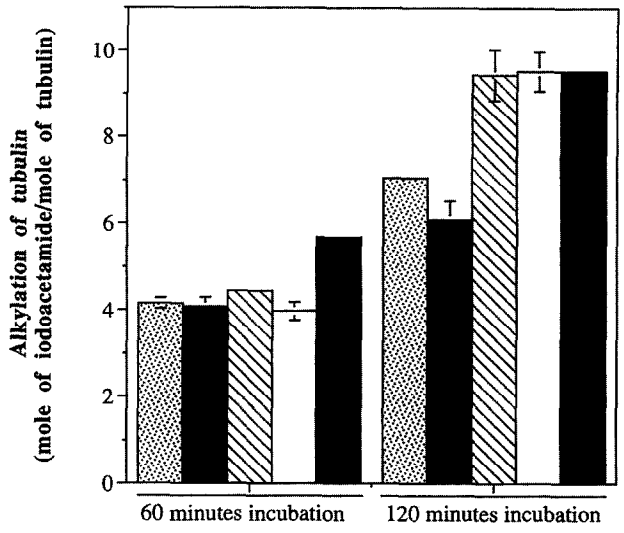


FIG. 9. Effects of vinflunine on the alkylation of purified tubulin by iodo[^{14}C]acetamide in comparison with those of colchicine or the classical *Vinca* alkaloids. Purified tubulin was incubated in the presence of vinflunine (white bars), vinorelbine (hatched bars), vinblastine (grey bars), vincristine (dotted bars) or water (black bars) used as control. The iodo[^{14}C]acetamide bound to tubulin was quantified as described in "Materials and Methods." Results are expressed as means from duplicates, with the associated standard error.

has been achieved, drug-induced cytotoxicity becomes dependent on increasing the duration of exposure, rather than the concentration. The high order of correlations between log IC_{50} values, obtained when evaluating cytotoxicities against a panel of nine unselected human tumour cell lines, for vinflunine, vinorelbine or vincristine relative to vinblastine are consistent with all these compounds having a similar intracellular target. Vinflunine resulted in similar overall biological effects to those of other *Vinca* alkaloids [38] on mammalian tumour cells *in vitro*, causing cell cycle arrest at G_2 + M, associated with an accumulation of cells in mitosis and producing a concentration-dependent reduction of the microtubule network, together with the formation of paracrystalline structures at high concentrations [39] in interphase cells. However, these effects, which all proved to be concentration-dependent, generally required 3- to 17-fold higher concentrations of vinflunine relative to those of the other *Vincas* tested. This finding appears consistent with the relatively higher levels of vinflunine needed to inhibit tumour cell proliferation *in vitro* and *in vivo*, as well as to increase the survival of tumour-bearing animals [19]. *In vitro* studies have established that vinflunine prevents assembly of microtubules without affecting their disassembly, with an IC_{50} value similar to those noted with vincristine, vinblastine or vinorelbine. In addition, vinflunine appears to interact at the *Vinca* alkaloid binding site on tubulin. This was shown first using a test based on the differential proteolysis of purified tubulin by chymotrypsin or trypsin [35] and confirmed by the fact that vinflunine, when permitted to interact with tubulin, induced structural changes which favoured an inhibition of GTP hydrolysis [25]. In this respect, therefore, vinflunine appears to function as a specific inhibitor of tubulin assembly, like the classic *Vinca* alkaloids.

However, vinflunine also expressed some distinctive features in terms of its binding to free tubulin and its ability to compete for the binding with the other [^{3}H]*Vinca* alkaloids. Under the conditions used, vinflunine did not appear to compete at all with [^{3}H]vincristine, and only slightly inhibited the binding of [^{3}H]vinblastine and [^{3}H]vinorelbine when used at the highest concentration of 100 μM. This quantitative difference might be due to a higher dissociation constant for vinflunine than for the other *Vinca* alkaloids, thereby permitting the equilibrium between bound and free vinflunine to be easily displaced in favour of free drug during the course of an experiment based on the centrifugal gel filtration technique. Support for this hypothesis also comes from evaluating the effects of vinflunine, relative to the other *Vinca* alkaloids, on tubulin conformation. Results of monitoring alkylation of the sulfhydryl groups of tubulin by iodo[^{14}C]acetamide [31] showed that with vinflunine, inhibition of tubulin alkylation was only transitory, being reversed after two hours of incubation, unlike the persistent inhibition noted with vinblastine and vincristine. Interestingly, vinorelbine appeared to act in the same manner as vinflunine. An alternative explanation for these data might be that only a very low proportion of vinflunine is 'available' to bind to free tubulin and that the drug's cytotoxicity is mediated by another mechanism. In this respect, the report by De Arruda *et al.* [40] on a new synthetic derivative of dolastatin 10, LU103793, is of interest. LU103793, while inducing similar overall biological effects on tubulin as vinblastine, could not be detected as binding to tubulin, nor as inhibiting the binding of vinblastine or colchicine to tubulin. However, by video microscopy, it was shown that LU103793 bound to microtubule ends with high affinity at low concentrations, suggesting that its cytotoxic activity was associated primarily with a disruption of microtubule dynamics [41].

In considering the capacity of a compound to bind to tubulin or to interfere with the binding of [^{3}H]*Vinca* alkaloids to tubulin under our experimental conditions, our data indicate that the various *Vinca* alkaloids tested can be classified as follows: vincristine > vinblastine > vinorelbine > vinflunine. These results are in agreement with other published data, since vincristine is known to bind to tubulin with a higher overall affinity than vinblastine [15, 42, 43]. More recently, Lobert *et al.* [16] found the same order in comparing the interaction of vinorelbine with tubulin with that of vincristine and vinblastine. Therefore, it would appear that vinflunine may further extend this *Vinca* alkaloid classification based on overall binding affinities.

However, the strength of binding to tubulin is not necessarily related to antitumour efficacy. For example, Singer and Himes [15] reported that the relative binding affinities to bovine brain tubulin of vincristine, vindesine,

vinblastine and vinepidine were inversely correlated with their effects on B16 tumour cell proliferation, with vinepidine interacting with tubulin with the highest affinity, yet being the least efficient inhibitor of proliferation. In this respect, it may be noteworthy that while vinorelbine and vinflunine differ in terms of their catharanthine moities, vinblastine, vincristine and vindesine are modified in their vindoline portions. Our results highlight the fact that single structural changes either in the vindoline or in the catharanthine parts of the molecule can independently alter the overall tubulin binding affinities/interactions. Interestingly, these effects seem to be additive for vinepidine, which is modified in both moieties. Furthermore, Jordan *et al.* [44] showed that retardation of tumour growth resulting from B16 melanoma cells treated with drug before being injected into mice was best achieved with vindesine, one of the weaker inhibitors of net addition of tubulin dimers at assembly ends of steady-state microtubules, relative to vincristine, vinblastine and vinepidine. This apparent lack of correlation may be due to the fact that the *Vinca* alkaloids are concentrated intracellularly and accumulate differentially in certain cells or that they actually target other intracellular sites, as discussed by Jordan *et al.* [45]. Ongoing investigations aimed at defining other parameters implicated in the antitumour activity of this new compound include monitoring the cellular uptake and accumulation, apoptosis induction, drug metabolism and pharmacokinetic properties of vinflunine, as well as identifying any non-tubulin-related targets.

If, however, tubulin is considered to be the primary target of *Vinca* alkaloids, it can be expected that differential interactions with tubulin should correlate to some extent with differences in their clinical utilities. The classification we have proposed above, based on their relative capacities to interfere with the binding of [^{3}H]*Vinca* alkaloids to tubulin, inversely correlates with the amount of drug required to induce mitotic accumulation of L1210 cells, to disassemble the microtubular network and to induce paracrystals in A-10 cells. Lobert *et al.* [16] suggested that since their defined order of overall tubulin binding affinities, namely: vincristine > vinblastine > vinorelbine, correlated well with the weekly intravenous drug dose of each *Vinca* alkaloid used in the clinic, namely 0.4–1.4, 4–20 and 25–35 mg/m^2, respectively, differential effects on microtubule dynamics might correlate clinically with the amount of drug needed to suppress tumour growth, which is determined not only by the potency of the drug but also by its dose-limiting toxicities.

In conclusion, we have synthesised a uniquely fluorinated vinorelbine derivative that appears to function as a specific inhibitor of tubulin, while exhibiting tubulin binding properties at least quantitatively different from those of the other *Vinca* alkaloids. These results with vinflunine therefore permit an extention of the spectrum of tubulin binding properties beyond that of vinorelbine relative to the more classic *Vinca* alkaloids. While these data may not fully account for the markedly superior spectrum of *in vivo* preclinical antitumour activity identified with vinflunine, results of clinical testing of this novel compound are eagerly awaited to establish whether its use can improve on the major clinical activity already identified with vinorelbine [46].

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