0026-895X/01/6001-225–232$3.00
MOLECULAR PHARMACOLOGY
Copyright © 2001 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 60:225–232, 2001

Vol. 60, No. 1
859/915933
Printed in U.S.A.

Mechanism of Mitotic Block and Inhibition of Cell Proliferation by the Semisynthetic *Vinca* Alkaloids Vinorelbine and Its Newer Derivative Vinflunine

VIVIAN K. NGAN, KRISTA BELLMAN, BRIDGET T. HILL, LESLIE WILSON, and MARY ANN JORDAN

Department of Molecular, Cellular, and Developmental Biology and Neuroscience Research Institute, University of California, Santa Barbara, California (V.K.N., K.B., L.W., M.A.J.) and Division de Cancerologie Experimentale, Centre de Recherche Pierre Fabre, Castres, France (B.T.H.)

Received February 5, 2001; accepted April 16, 2001

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

The two second-generation *Vinca* alkaloids, vinorelbine and vinflunine, affect microtubule dynamics very differently from vinblastine, a first generation *Vinca* alkaloid. For example, vinblastine strongly suppresses the rate and extent of microtubule shortening in vitro, whereas vinorelbine and vinflunine suppress the rate and extent of microtubule growing events. We asked whether these differences result in differences in mitotic spindle organization that might be responsible for the superior antitumor activities of the two second-generation *Vinca* alkaloids. IC_{50} values for inhibition of HeLa cell proliferation for vinflunine, vinorelbine, and vinblastine were 18, 1.25, and 0.45 nM, respectively, similar to the concentrations that induced mitotic block at the metaphase/anaphase transition (38, 3.8, and 1.1 nM, respectively), indicating that mitotic block is a major contributor to antiproliferative action for all three drugs. Mitotically blocked cells exhibited aberrant spindles, consistent with induction of block by suppression of microtubule dynamics. Despite differences in their actions on individual dynamic instability parameters, morphologically detectable differences in spindle effects among the three drugs were minimal, indicating that overall suppression of dynamics may be more important in blocking mitosis than specific effects on growth or shortening. We also found that the peak intracellular drug concentration at the mitotic IC_{50} value was highest for vinflunine (4.2 $\pm$ 0.2 μM), intermediate for vinorelbine (1.3 $\pm$ 0.1 μM), and more than 10-fold lower for vinblastine (130 $\pm$ 7 nM), suggesting that intracellular binding reservoir(s) may be partially responsible for vinflunine's high efficacy and minimal side effects.

Vinca alkaloids, including the natural products vincristine and vinblastine and the semisynthetic derivatives vindesine and vinorelbine, are antimitotic drugs that are widely used in cancer treatment (Donehower and Rowinsky, 1993). Vinorelbine, the most recent clinically approved *Vinca* alkaloid, shows improved efficacy and reduced toxicity. It is effective in non–small-cell lung cancer, metastatic breast cancer, and ovarian cancer and shows promise in lymphoma, esophageal cancer, and prostatic carcinoma (Johnson et al., 1996; Crown, 1997; Bunn and Kelly, 1998). Vinflunine is a new semisynthetic bifluorinated compound that, having completed phase I clinical trials, is now in phase II (Armand et al., 2001; Fumoleau et al., 2001). Vinflunine is more active than vinorelbine, vinblastine, or vincristine against a number of murine tumors and human tumor xenografts. For example, vinflunine exhibited high or moderate antitumor efficacy in 64% (7 of 11) tumor models, whereas vinorelbine exhibited, at best, moderate activity in 27% (3 of 11) (Kruczynski et al., 1998a,b; Hill et al., 1999).

This work was supported by Grants from the Institut de Recherche Pierre Fabre and National Institutes of Health (CA57291).

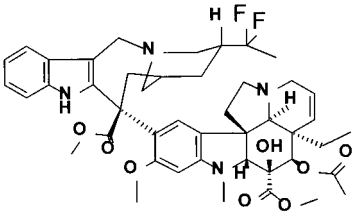
Vinflunine and vinorelbine differ structurally from vinblastine in the velbanamine "upper" portion of the molecule (Fig. 1). Both drugs were synthesized by a novel method resulting in a ring with eight members rather than nine within the velbanamine portion (Langlois et al., 1976; Mangeney et al., 1979). Vinflunine was derived by further modification of vinorelbine, using superacidic chemistry to introduce two fluorine atoms (Fahy et al., 1997; Jacquesy and Fahy, 2000).

The mechanism of action of the *Vinca* alkaloids was initially thought to involve depolymerization of spindle microtubules and induction of paracrystalline tubulin-*Vinca* alkaloid arrays. At relatively high concentrations (micromolar), the *Vinca* alkaloids inhibit microtubule polymerization (Binet et al., 1990, Jordan et al., 1991; Kruczynski et al., 1998a). However, they also have a more subtle and powerful action on microtubules; they inhibit their dynamics at concentrations below those required to inhibit polymerization (Jordan et al., 1985; Toso et al., 1993; Dhamodharan et al., 1995). For example, low concentrations of vinblastine (8–32 nM) block mitosis in BSC-1 cells in association with suppression of microtubule dynamics, in the absence of appreciable changes

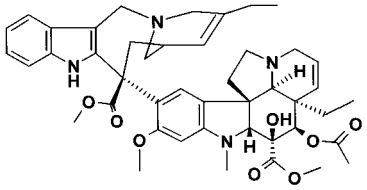
ABBREVIATIONS: PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

in microtubule mass or spindle microtubule organization (Dhamodharan et al., 1995). Vinblastine inhibits chromosome congression (the prometaphase movement of chromosomes to the spindle equator) and the transition from metaphase to anaphase, by binding with high affinity to microtubule ends and suppressing microtubule dynamics (Jordan et al., 1991; Jordan and Wilson, 1998).

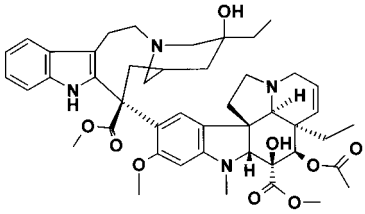
Microtubules display two dynamic behaviors, dynamic instability, and treadmilling, which are important for cell cycle progress (Mitchison and Kirschner, 1984, Margolis and Wilson, 1998; Rodionov et al., 1999). Dynamics play critical roles in the equi-partitioning of chromosomes to the two daughter cells by the mitotic spindle. For example, microtubules emanating from the spindle poles at prometaphase make vast growing and shortening excursions (dynamic instability), probing the cytoplasm until they "find" and attach to the kinetochores of chromosomes. Failure of the microtubules to capture all the chromosomes leads to mitotic block (Rudner and Murray, 1996) and apoptosis (Jordan et al., 1996). In addition, chromosomes aligned at the metaphase plate oscillate under tension produced by motor molecules and dynamic kinetochore-attached microtubules. Superimposed on the oscillations is microtubule treadmilling (Mitchison, 1989), in which tubulin undergoes net addition to microtubule ends at the kinetochores and balanced net loss at the poles. These forces are important in signaling at the metaphase/anaphase checkpoint (Nicklas et al., 1995).



Vinflunine



Vinorelbine



Vinblastine

Fig. 1. Chemical structures of vinflunine, vinorelbine, and vinblastine.

We recently compared the effects of vinflunine, vinorelbine, and vinblastine on dynamic instability and treadmilling of purified bovine brain microtubules (Ngan et al., 2000). Interestingly, the inhibited parameters differed significantly, and in some ways oppositely, for vinflunine and vinorelbine compared with vinblastine. An important question is whether these differences result in significantly different modes of mitotic inhibition. Hence, in the present study, we asked whether the differences between the actions of these compounds on microtubule dynamics result in important differences in spindle organization that may be responsible for the superior antitumor activities of the two newer drugs.

We found that each drug blocked mitosis in HeLa cells at a concentration similar to that which inhibited proliferation, indicating that the mitotic block induced by all three drugs is a major contributor to their antiproliferative action. Although the intracellular drug concentrations of vinflunine, vinorelbine, and vinblastine varied 32-fold at the media concentrations that blocked mitosis, each drug produced remarkably similar effects on spindle organization. These results indicate that the differential inhibition of specific parameters of microtubule dynamic instability by the three drugs is not as important as their ability to suppress overall microtubule dynamics.

Materials and Methods

Cell Culture. HeLa S3 cells [epithelial-like cells from epithelioid carcinoma of human cervix (American Type Culture Collection, Manassas, VA)] were cultured at 37°C in the presence of 5% CO_2 using Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and nonessential amino acids (Sigma Chemicals, St. Louis, MO) in 250-ml tissue culture flasks or 35-mm six-well plates (doubling time, 18–20 h). For studies evaluating mitotic block, cells were grown on poly-L-lysine-treated (50 μg/ml, 2 h, 37°C, washed once with sterile water) sterile glass coverslips in six-well plates. Plating densities ranged from 1 to 5 $\times$ 10^4 cells/ml to maintain cultures in log growth during drug incubation. Cells were incubated with drugs by replacing the original medium with an equal volume of medium containing the required concentration of drug, or no drug (control), and incubation was continued at 37°C for 20 h.

Cell Proliferation. At the time of initiation and termination of drug incubation, duplicate cultures were detached from the culture vessel by incubation with trypsin (0.5 mg/ml in phosphate-buffered saline: 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, and 0.5 mM EDTA, pH 7.2) and live cells were counted using a hemocytometer. Trypan blue dye was used to distinguish living from dead cells (Loo and Rillema, 1998). Cell proliferation was calculated from the difference in cell number at the beginning and the end of drug incubation, relative to the increase in cell number for control cultures during the same period.

Mitotic Progression. To evaluate mitotic indices, HeLa cells grown for 20 h in the absence or presence of drug in six-well plates were detached by incubation with trypsin, washed with phosphate-buffered saline, and fixed with 100% methanol. Cells were incubated with 10% normal goat serum to block nonspecific antibody staining, and then with a rat anti-tubulin monoclonal antibody (YL1/2; Harlan Sera-Labs, Inc., Crawley Down, Sussex, UK) (1 h, 37°C) followed, after washing, by Rhodamine Red-X-conjugated goat anti-rat IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 h at room temperature. Chromosomes and chromatin were stained with 0.1 to 1 μg/ml 4,6-diamidino-2-phenylindole for 2 to 5 min. Stained cells were mounted on slides using Vectashield (Vector Laboratories) antifade reagent.

To evaluate microtubule and chromosome organization and the

stage of mitosis at which the cells were blocked, cells grown on poly-L-lysine-coated coverslips (to enhance cell attachment) were incubated with drug for 20 h, fixed with 10% formalin (25°C), followed by 100% methanol containing 2 mM EGTA (4°C) (Jordan et al., 1991), and stained as described above with the addition of mouse anti-tubulin monoclonal antibody (GTU-88; Sigma Chemical, St. Louis, MO) and fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibody (Sigma Chemicals). Analysis of spindle organization is more accurate using attached cells because the cells attach with their spindle axes lying parallel to the glass surface, and it becomes relatively easy to discern changes in numbers of chromosomes congressed to the metaphase plate and changes in the number of spindle poles. Because only mitotic cells were enumerated, any differential attachment between interphase and mitotic stages was unimportant. Stained cells were mounted on slides using Prolong (Molecular Probes, Eugene, OR) antifade reagent. Cells were examined using an Eclipse E800 microscope (Nikon, Melville, NY) fitted with 60× and 100× (numerical aperture 1.4 for both) objectives and an Orca II CCD (Hamamatsu, Bridgewater, NJ) camera supported by MetaMorph (version 4.0) imaging software (Universal Imaging Corp., Downington, PA).

Quantification of Intracellular Drug Accumulation. The time course of drug accumulation in HeLa S3 cells was determined as described previously (Jordan and Wilson, 1999). Briefly, cells were grown in poly-L-lysine-coated sterile scintillation vials, incubated for varying time intervals with trace amounts of [^{3}H]vinflunine (specific activity, 3.0 Ci/mol), [^{3}H]vinorelbine (3.0 Ci/mol), or [^{3}H]vinblastine (15.5 Ci/mol) (Amersham Pharmacia Biotech UK, Ltd., Little Chalfont, Buckinghamshire, UK), washed quickly with 100 mM PIPES, 1 mM EGTA, 1 mM $MgSO_4$, pH 6.9 (2.5 ml) after media removal, and lysed with water (1.0 ml), and scintillation fluid (10 ml; Ready Protein$^+$, Beckman Coulter, Fullerton, CA). The media at time of drug addition was sampled to determine specific activity. Intracellular drug concentration was calculated by dividing the total drug contained in the cells by total cell volume. Total cell volume was determined by counting cells in a duplicate set of scintillation vial cultures incubated with an equivalent concentration of nonradioactive drug and using 2.4 pl as the average volume of a HeLa cell (Jordan et al., 1991).

Results

Effects of Vinflunine and Vinorelbine on HeLa Cell Proliferation and Mitotic Progression. The effects of vinflunine and vinorelbine on HeLa cell proliferation were compared with those of vinblastine by culturing cells in the absence or presence of vinflunine (1–30 nM), vinorelbine (0.5–5 nM), or vinblastine (0.1–1.5 nM) for one cell cycle and counting the increase in cell number 20 h later relative to the number of cells present at the time of drug addition (Fig. 2). Vinflunine and vinorelbine inhibited cell proliferation by 50% (IC_{50}) at concentrations of 18 and 1.25 nM, respectively. In comparison, the IC_{50} value for vinblastine was 0.45 nM, as reported previously (Jordan et al., 1991).

The relationship between cell cycle progression and inhibition of cell proliferation was examined by determining the mitotic index at a range of *Vinca* alkaloid concentrations. HeLa cells were incubated for 20 h in the absence or presence of a range of vinflunine, vinorelbine, or vinblastine concentrations, then both detached and attached cells were collected, fixed, and their microtubules and chromosomes stained. The numbers of cells in mitosis and in interphase were then determined by microscopy (*Materials and Methods*). As shown in Fig. 3A, all three drugs blocked cell cycle progression in mitosis at the same range of drug concentrations that inhibited proliferation. The IC_{50} values at which 50% of the cells were found to be in mitosis were 38, 3.8, and 1.1 nM for vinflunine, vinorelbine, and vinblastine, respectively, just slightly higher than the IC_{50} values for inhibition of proliferation for the three drugs.

The stage of mitosis at which the block occurred was determined by counting relative numbers of cells at each stage of mitosis after staining. As shown in Fig. 3B, the ratio of the number of cells in anaphase to those in metaphase decreased to zero over the concentration range that induced mitotic block, indicating a block specifically in metaphase. At concentrations of 75 nM vinflunine, 8 nM vinorelbine, and 2.2 nM vinblastine, no cells were in anaphase. Thus, the block occurred specifically at the transition from metaphase to anaphase. The potency of the three compounds with respect to both inhibition of cell proliferation and mitotic block was vinblastine > vinorelbine > vinflunine.

Effects of Vinflunine and Vinorelbine on Spindle Organization. Vinflunine and vinorelbine affect microtubule dynamics very differently from vinblastine. We wanted to determine whether these differences would result in significantly different alterations in spindle organization and might be responsible for the superior antitumor activities of the two newer drugs. Thus, we compared the alterations in the arrangement of microtubules, centrosomes, and chromosomes induced by each drug at the IC_{30}, IC_{50}, and 2× IC_{50} concentrations for mitotic block, using immunofluorescence microscopy.

As shown in Fig. 4, A-C, control cells in mitosis contained well-organized bipolar spindles with two distinct, well-separated spindle poles and a few astral microtubules. At metaphase, all of the chromosomes were organized in a compact equatorial metaphase plate (Fig. 4C). A small percentage of cells (18%) contained multipolar spindles (generally tripolar or quadripolar spindles). The percentages of normal, abnormal, and multipolar spindles in controls are shown graphically in Fig. 6, A-C (unshaded bars) (discussed below).

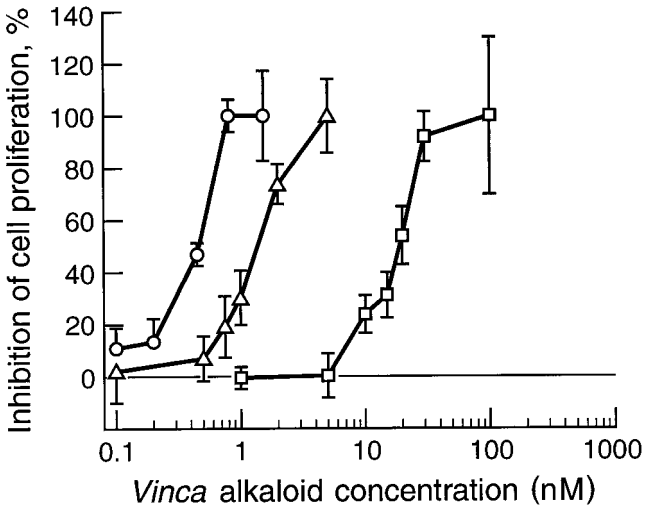


Fig. 2. Concentration dependence for inhibition of HeLa cell proliferation by vinflunine (□), vinorelbine (△), and vinblastine (○). The percentage inhibition of cell proliferation is the increase in cell number that occurred during 20 h of continuous incubation with drug compared with the increase in cell number in a parallel culture without drug (see *Materials and Methods*). The cell number approximately doubled in control cultures during the 20-h incubation. Results represent mean ± S.E. of three to four independent experiments.

Cells after incubation with *Vinca* alkaloids are shown in Figs. 4 and 5, quantitated in Fig. 6 A-C and shown diagrammatically at the bottom of Fig. 6. At concentrations of vinflunine, vinorelbine, and vinblastine ranging from the IC_{30} to twice the IC_{50} for mitosis, spindles were often abnormal. The abnormalities mostly fell into a series of increasingly aberrant types that we categorized in an earlier study of the *Vinca* alkaloids vinblastine, vincristine, vindesine, and vinepidine (Jordan et al., 1991). Abnormal spindle Types I and II consisted of bipolar spindles with few or many uncongressed chromosomes, respectively (see Fig. 4, arrows). Type III consisted of monopolar spindles enclosed in a ball of chromosomes (Figs. 4 and 5, asterisks).

Even after drug incubation, some metaphase spindles appeared normal and were bipolar with completely congressed chromosomes; they were indistinguishable from spindles of control cells (see Fig. 4, A-C). For example, at the IC_{30} for mitotic block (28 nM vinflunine, 2.6 nM vinorelbine, and 0.7 nM vinblastine), 10 to 25% of the spindles appeared normal (Fig. 6, lightly shaded bars). At the IC_{30}, all categories of spindles existed in approximately equal numbers, except that the Type III spindles predominated after incubation with vinorelbine (57% were of Type III with vinorelbine). At the IC_{50} (38 nM vinflunine, 3.8 nM vinorelbine, and 1.1 nM vinblastine, Fig. 6, medium-shaded bars) normal spindles were rare, whereas Type III spindles predominated (52 to 63%). At the 2× IC_{50} concentration (heavily-shaded bars), there were no normal spindles (Fig. 6, A-C, left column), and 75 to 87% of the spindles were Type III after incubation with each of the three drugs. The percentages of multipolar spindles did not vary significantly with increasing drug concen-

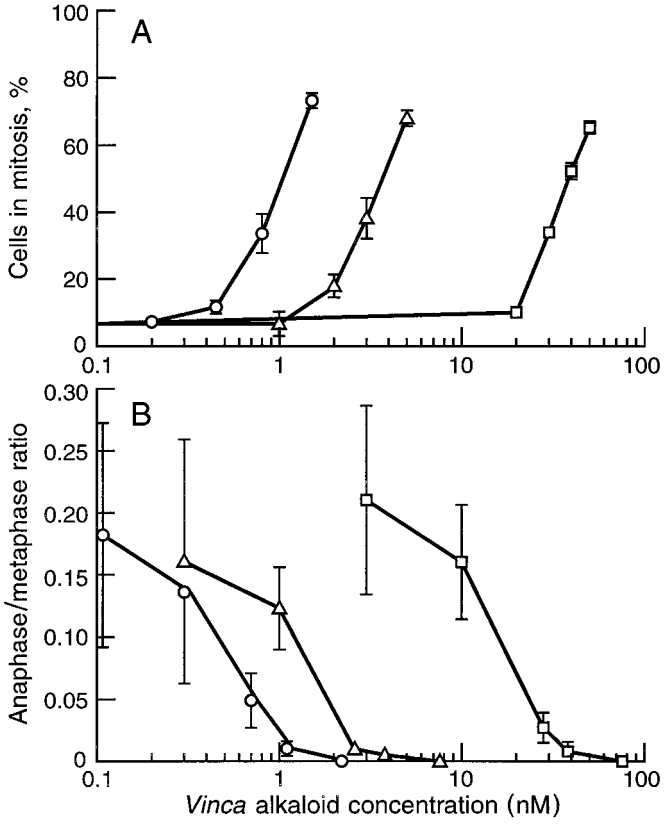


Fig. 3. Concentration dependence for accumulation of HeLa cells in mitosis (A) and for a mitotic block specifically at the transition from metaphase to anaphase (B). Vinflunine (□), vinorelbine (△), and vinblastine (○). The mitotic index was determined by counting the percentage of cells in each stage of mitosis (prometaphase through telophase) after a 20-h continuous incubation with drug. Both floating and attached cells were collected, their microtubules and chromosomes stained (see *Materials and Methods*), and cells counted by fluorescence microscopy. Data in B are the ratio of the number of cells in anaphase to the number of cells in metaphase; the ratio in the absence of drug was 0.21 ± 0.02. Between 200 and 400 cells were counted for each condition in each experiment. Results represent means ± S.E. of two to three independent experiments.

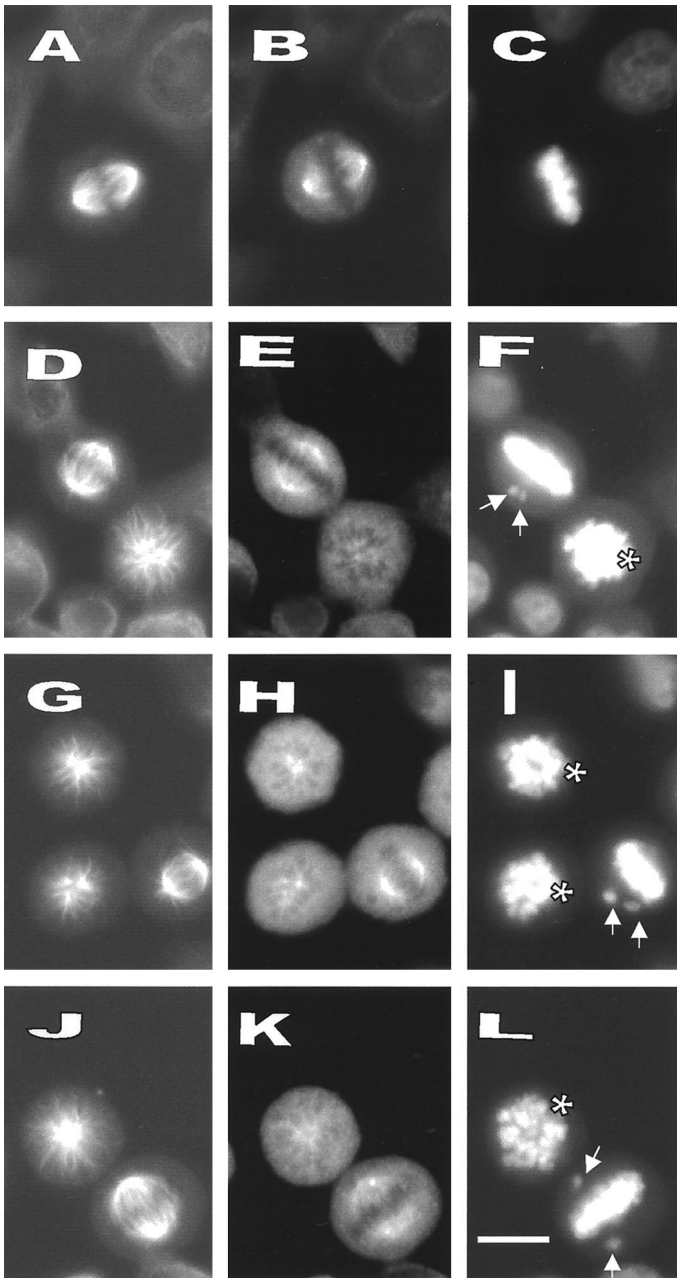


Fig. 4. Mitotic HeLa cells in the absence (A, B, and C) and presence of the IC_{50} concentration of 38 nM vinflunine (D, E, and F), 3.8 nM vinorelbine (G, H, and I), and 1.1 nM vinblastine (J, K, and L). Cells were fixed and incubated with an anti-β-tubulin antibody to stain microtubules (A, D, G, and J) (first column), with an anti- γ -tubulin to stain centrosomes (B, E, H, and K) (second column), and 4,6-diamidino-2-phenylindole (DAPI) to stain chromosomes and and nuclei (C, F, I, and L) (third column). Type I and II abnormal spindles consist of bipolar spindles with one or more uncongressed chromosomes (arrows). Type III abnormal spindles consist of monopolar spindles with chromosomes arranged in a ball (asterisks). Bar, 10 μm.

trations, remaining at or below 20% of all spindles. Thus, as shown in Figs. 5 and 6 and summarized in the histogram in Fig. 6, all three drugs showed the same sequence of concentration-dependent changes in spindle morphology, albeit over different concentration ranges.

In addition, staining for γ-tubulin indicated that centrosomes in Type III spindles were frequently more fragmented after incubation with vinorelbine and vinflunine than with vinblastine; that is, the punctate masses of centrosomal staining were further apart from one another within the sphere of chromosomes (data not shown; illustrated diagrammatically in Fig. 6).

Measurement of the distance between poles of bipolar spindles after drug incubation indicated that the pole-to-pole distance was shorter at the IC_{50} concentrations of the three drugs (Table 1). Vinorelbine had the greatest effect on the spindle length, reducing it by 29%, from 9.7 ± 0.3 μm in controls to 6.9 ± 0.1 μm. The smaller size of spindles after vinorelbine exposure is clearly visible in Fig. 4, G and H. The pole-to-pole distance was reduced 20% by vinflunine (to 7.8 ± 0.3 μm) and by vinblastine (to 7.6 ± 0.1 μm). Shortening of the spindle may be caused by net microtubule depolymerization or by changes in the balance of dynamics between microtubule subsets in the spindle. In support of the latter idea, the drug paclitaxel, which enhances microtubule polymerization, also induces spindle shortening (Jordan et al., 1993).

Although the *Vinca* alkaloids exerted substantial effects on spindle organization at the IC_{50} values, these same concentrations did not significantly affect the appearance of microtubules in interphase cells. As shown in Fig. 7A, control cells in interphase were flat, well spread, and uniform in size, with a fine filamentous array of microtubules radiating from the centrosomes and generally a single nucleus that was spherical or ovoid in shape. After incubation with the mitotic IC_{50}

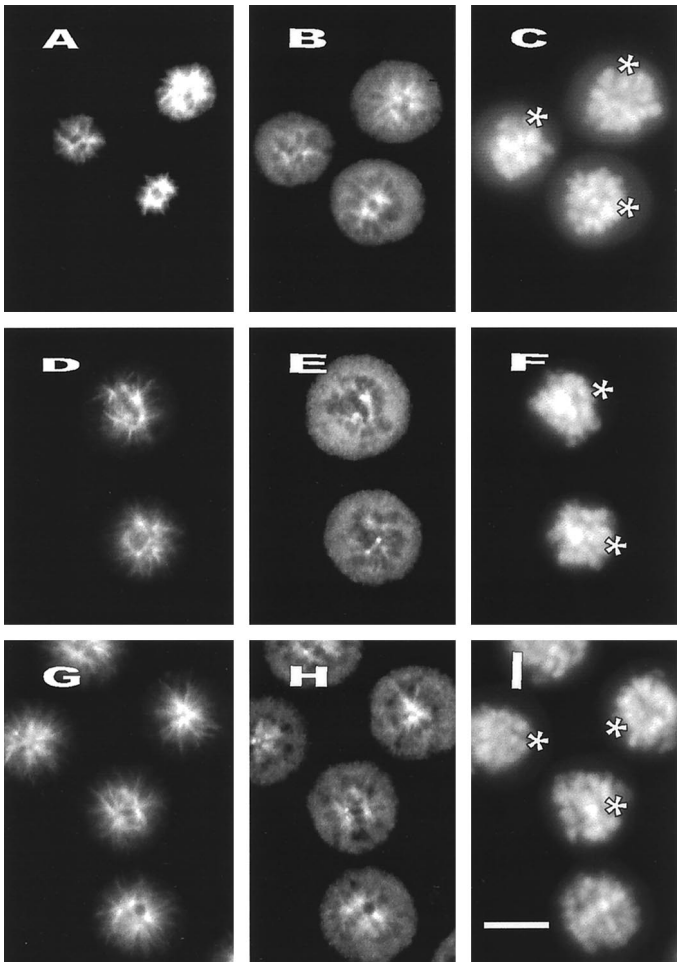


Fig. 5. Mitotic HeLa cells in the presence of 2× IC_{50} concentration of 76 nM vinflunine (A, B, and C), 7.6 nM vinorelbine (D, E, and F), and 2.2 nM vinblastine (G, H, and I). Cells were fixed and stained as in Fig. 4 for microtubules (A, D, and G) (first column), centrosomes (B, E, and H) (second column), and chromosomes and nuclei (C, F, and I) (third column). *, type III spindles. Bar, 10 μm.

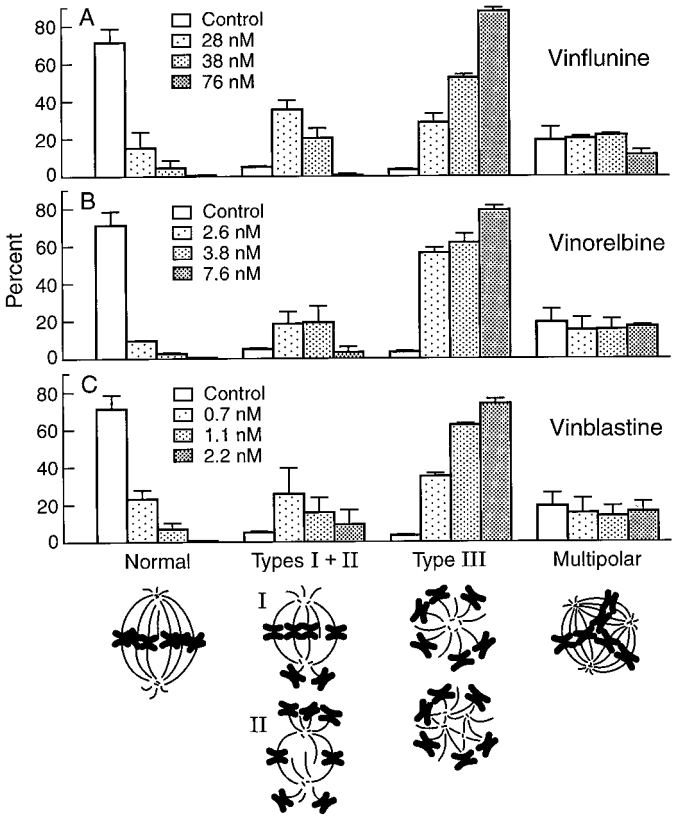


Fig. 6. Frequency of spindle abnormalities induced in HeLa cells by vinflunine, vinorelbine, or vinblastine at concentrations of 0.7× IC_{50}, 1× IC_{50}, and 2× IC_{50} for mitotic block. Bottom, diagram of four general spindle types. "Normal" consisted of bipolar metaphase spindles with completely congressed chromosomes; types I and II were bipolar spindles with one or more uncongressed chromosomes; type III were monopolar spindles with chromosomes arranged in a ball around the spindle and containing centrosomes that appeared fragmented or with centrioles that had separated some distance apart. All three drugs showed the same sequence of concentration-dependent changes in spindle morphology. Data represent means ± S.E. of two independent experiments and evaluation of at least 450 mitotic cells per condition.

TABLE 1

Effect of *Vinca* alkaloids at the IC concentration for mitotic block on spindle length in HeLa cells

Spindle length was measured as the interpolar distance, namely the distance between centrosomes at opposite spindle poles of bipolar spindles after fixing and staining cells with tubulin antibodies (mean ± S.E.M. of at least 50 spindles from two independent experiments for each test condition).

Drug (IC_{50} value)	Pole-to-Pole Distance
	μm
No drug (control)	9.7 ± 0.3
Vinflunine (38 nM)	7.8 ± 0.3
Vinorelbine (3.8 nM)	6.9 ± 0.1
Vinblastine (1.1 nM)	7.6 ± 0.1

concentration of each of the *Vinca* alkaloids for 20 h, cells frequently contained more than one nucleus (Fig. 7, B-D, arrows); that is, 15.9, 10.5, and 8.6% of interphase cells were multinucleate after incubation with 38 nM vinflunine, 3.8 nM vinorelbine, and 1.1 nM vinblastine, respectively, compared with 6.9% of the control populations. The drug-exposed cells appeared slightly more rounded than control cells, but their microtubules resembled the microtubules of control cells and were not depolymerized to any detectable extent, as indicated by anti-γ-tubulin staining (Fig. 7).

Intracellular Drug Concentration. We wanted to know whether the quantitative differences in the effects of vinflunine, vinorelbine, and vinblastine, on cell proliferation and mitotic block might result from differences in the levels of cellular drug accumulation. That is, we wanted to determine whether less vinblastine is required than vinflunine or vinorelbine to inhibit proliferation and to block mitosis because it accumulates to a higher concentration in cells. Thus HeLa cells were incubated in media containing [^{3}H]vinflunine (30 nM), [^{3}H] vinorelbine (3 nM), or [^{3}H] vinblastine (1 nM) for periods of 2 to 24 h, and then the intracellular drug concentrations were measured (*Materials and Methods*). The concentrations added to the media were chosen to approximate the IC_{50} values for mitotic block (Fig. 3) aiming to induce equivalent biological effects in cells with each drug.

As shown in the time course of uptake in Fig. 8, all three drugs entered the cells gradually, reaching maximal levels within 4 h. The maximum intracellular concentrations were significantly higher than the extracellular levels in the media: 4.2 μM, 1.3 μM, and 130 nM for vinflunine, vinorelbine, and vinblastine, respectively; these represented concentrations increased by factors of 140-, 430-, and 130-fold for vinflunine, vinorelbine, and vinblastine, respectively, over those added to the media. Thus, vinblastine accumulated intracellularly to a degree similar to that of vinflunine. Therefore, the increased potency of vinblastine compared with vinflunine cannot be accounted for by preferential cellular accumulation of vinblastine. However, preferential accumulation of vinorelbine relative to vinflunine may account for at least a portion of the increased potency of vinorelbine in HeLa cells.

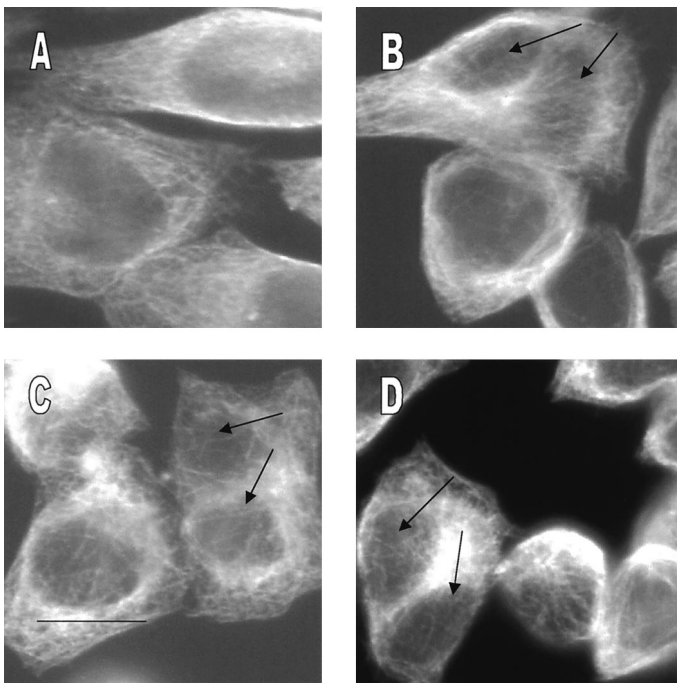


Fig. 7. Effects of *Vinca* alkaloids on the interphase array of microtubules at the IC_{50} concentration for mitotic block. In controls (A), cells are well spread, have a single nucleus, and their microtubules are in a fine filamentous array. In the presence of 38 nM vinflunine (B), 3.8 nM vinorelbine (C), or 1.1 nM vinblastine (D), cells are more rounded, often have more than one nucleus (arrows), and their microtubules are clumped or slightly bundled. The mass of microtubule polymer is not detectably diminished. Cells were fixed and incubated with an anti-tubulin antibody to stain microtubules. Bar, 10 μm.

Discussion

We have found that vinflunine, vinorelbine, and vinblastine inhibit proliferation of HeLa cells in parallel with mitotic block at the metaphase/anaphase transition. At the concentrations that inhibited mitotic progression, each of the three drugs induced similarly aberrant spindle organization. Taken together, these results indicate that inhibition of cell proliferation by all three *Vinca* alkaloids results primarily from mitotic block induced by suppression of microtubule dynamics.

Does Inhibition of Particular Parameters of Dynamic Instability Determine the Effectiveness of Mitotic Block? We previously determined the effects of equal concentrations (400 nM) of vinflunine, vinorelbine, and vinblastine on the dynamic instability behavior of bovine brain microtubules in vitro. Interestingly, we found that vinflunine and vinorelbine affected dynamic instability differently, often oppositely, from vinblastine (Ngan et al., 2000). For example, neither vinflunine nor vinorelbine inhibited the rate of shortening significantly, whereas vinblastine inhibited it by 44%. The duration of growing excursions was increased 53 to 59% by vinflunine and vinorelbine but was not altered significantly by vinblastine. The duration of pause (attenuated dynamics) was unaffected by the two newer drugs, whereas it was increased 60% by vinblastine. Thus vinblastine mainly decreased the shortening rate and increased pause duration and total time in pause phase. In contrast, vinflunine and vinorelbine mainly decreased the growing rate, its duration, and the total time growing while greatly

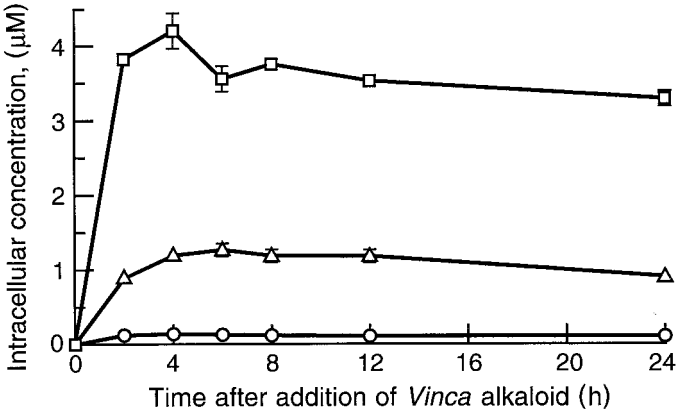


Fig. 8. Time course of uptake of 30 nM vinflunine (□), 3 nM vinorelbine (△), and 1 nM vinblastine (○) into HeLa cells. ^{3}H-labeled compound at the IC_{50} value for mitotic block was added to HeLa cells growing exponentially in scintillation vials. At intervals from 10 min to 24 h, samples were prepared for determination of cell-associated radioactivity (see *Materials and Methods*). Values are mean ± S.E. of two independent experiments.

decreasing the time in pause. Overall effects on dynamicity [a measure of tubulin addition and loss from microtubule ends (Toso et al., 1993)] were similar; all three drugs decreased dynamicity between 21 and 32%. Another difference is that vinflunine and vinorelbine inhibited microtubule treadmilling 1.5- to 6.4-fold less potently than vinblastine (Ngan et al., 2000).

In view of these differences among the three *Vincas* in terms of their inhibition of dynamic instability and treadmilling, it became important to ask whether the abnormalities they induced in spindle organization differed and, if so, might help to explain differences in antitumor potency. However, only minimal differences in spindle organization were detected. At the concentrations that inhibited mitotic progression, each of the three drugs induced similarly aberrant spindle organization. At the IC_{30} values for mitotic block for all three drugs, 10 to 23% of the spindles had a normal configuration of microtubules and chromosomes and the proportions of abnormal types I+II, type III, and multipolar spindles were similar. As the concentration of each drug was increased, the proportion of type III spindles increased, whereas proportions of normal spindles and spindles of types I+II decreased. Aberrant spindle morphology is most likely to result from suppression of microtubule dynamics. However, an important result of this study is that inhibition of different subsets of dynamic instability parameters by these three drugs does not seem to result in differences in spindle abnormalities. Rather, the overall suppression of microtubule dynamicity resulted in spindle abnormalities and mitotic block.

How Does Inhibition of Microtubule Dynamics by *Vinca* Alkaloids Result in Aberrant Spindle Organization and Block Mitosis? Uncongressed chromosomes in the drug-treated cells are most probably those that are inaccessible to attachment by relatively nondynamic microtubules emanating from the distant spindle pole. Type III spindles, which seem collapsed with both centrosomes contained in a ball of chromosomes, may result from suppression of treadmilling/dynamic instability, leading to spindle poles not being held apart. Thus, if the treadmilling addition of tubulin at the kinetochore ends of microtubules is inhibited in the absence of balanced inhibition of loss at the opposite ends, the kinetochore microtubules would shorten, resulting in shortening of the entire spindle. Similarly, unbalanced suppression of growing and shortening dynamics of kinetochore microtubules might lead to net shortening of the spindle.

During mitosis, chromosomes that are attached in a bipolar fashion to the metaphase spindle oscillate about the spindle equator. The kinetochores of sister chromatids are under tension, are periodically stretched apart by shortening of dynamic microtubules, and then relax back together as tension is relieved. The signal to progress from metaphase to anaphase seems to involve the development of sufficient tension or the attachment of a sufficient number of microtubules to kinetochores (Hays and Salmon, 1990; Nicklas et al., 1995). Very low concentrations of vinblastine that block the cells in metaphase inhibit the stretching, thereby reducing the tension (J. Kelling, L.W., K. Sullivan, and M. A.J., unpublished observations). Tension on kinetochores may also be provided by the treadmilling of kinetochore microtubules, a process that transports tubulin subunits along the lengths of the microtubules from their attachment at the kinetochores toward the spindle poles (Mitchison, 1989; Waters et al., 1996). Our results indicate that suppression of different combinations of dynamics parameters can lead to mitotic block.

Potential Therapeutic Significance of the High Intracellular Levels of Vinflunine and Vinorelbine. Measurements of radiolabeled *Vinca* alkaloid uptake into cells indicated that peak intracellular drug concentrations were considerably higher than the concentrations added to the medium. The peak concentration was highest for vinflunine (4.2 ± 0.2 μM), almost as high for vinorelbine (1.3 ± 0.1 μM), but more than 10-fold lower for vinblastine (130 ± 7 nM). The implications of these findings are especially interesting because similar (micromolar) concentrations of vinflunine and vinorelbine significantly inhibit polymerization in vitro (Kruczynski et al., 1998a; Ngan et al., 2000). For example, 4 μM vinflunine reduced polymerization of brain tubulin by 88%, and 1 μM vinorelbine reduced it by 60%. In contrast, 130 nM vinblastine reduced polymerization by less than 5% (Ngan et al., 2000). If all intracellular vinflunine or vinorelbine at their IC_{50} values were free to bind tubulin, one would predict that most microtubules would be depolymerized. In contrast, the IC_{50} concentration of vinblastine would have little effect on microtubule polymer mass. However, the data in Figs. 4 and 7 indicate that with all three drugs, most microtubules remain intact (although spindles were somewhat reduced in size in the presence of vinorelbine). These results suggest that not all intracellular vinflunine and vinorelbine is available to bind to tubulin or microtubules, and thus much of the drug must be sequestered in intracellular reservoirs, such as membrane compartments. Such reservoirs might be important in the antitumor activity of these drugs, providing a continuous intracellular source of drug and prolonging their therapeutic effects. Vinflunine was previously found to diffuse freely out of cells; however, it is not clear that the experimental conditions accurately mimicked those in vivo (Jean-Decoster et al.1999). We suggest that intracellular binding reservoir(s) may be responsible for the exceptional breadth of vinflunine's therapeutic index (Kruczynski et al., 1998b; Jacquesy and Fahy, 2000) providing a reservoir for excess drug and enabling its gradual release, thereby prolonging its antitumor effects with associated low toxicity.

Explanations for the exceptionally high intracellular concentrations required for the effectiveness of vinflunine and vinorelbine include their unique effects on microtubule dynamic instability and treadmilling and their low affinity for binding to tubulin. Using sedimentation velocity, Lobert et al. (1998) found that vinflunine has a 3- to 16-fold lower overall affinity for tubulin than vinorelbine, whereas vinorelbine has lower overall affinity than vinblastine under the conditions of the experiments (Lobert et al., 1996, 1998). These results suggest that the lower affinity of vinflunine and vinorelbine for tubulin, may explain, at least in part, the high concentrations of drug required to block mitosis and cell proliferation (Lobert et al., 2000). However, they do not explain the relatively high therapeutic efficacy of these newer *Vincas*.

On the basis of its binding affinity for tubulin and its potent effects on microtubule dynamics, vinblastine would be predicted to be the most potent mitotic inhibitor of the three *Vincas* alkaloids. This prediction is borne out by the data.

One might also predict that vinblastine would be the most effective antitumor agent. However, based on the preclinical and clinical efficacy of the three drugs, this seems not to be the case (Kruczynski et al., 1998a,b; Hill et al., 1999). Thus, the most potent drug is not the most efficacious, because vinorelbine and vinflunine seem to have clear therapeutic advantages. The reasons for this are unknown; it may be caused by other features of their modes of action or different pharmacokinetic characteristics.

The lower potency and low tubulin binding affinity of vinflunine correlates well with the finding that it is the least cytotoxic of the *Vinca* alkaloids studied (Kruczynski et al., 1998a). The therapeutic index of a relatively weakly potent drug may be broader than that of an extremely potent drug. Thus, if a drug acts selectively on tumor tissue, a drug of low potency may be more effective than one of high potency because of reduced toxic side effects. The balance between efficacy and toxicity for these first- and second-generation *Vinca* alkaloids remains to be clarified mechanistically.

References

Armand JP, Fumoleau P, Marty M, Variol P, Pinel M-C, Picard M and Puozzo C (2001) Pharmacokinetics of vinflunine, a novel Vinca alkaloid, during the Phase I dose escalation study (D1 Q 3 WEEKS). *Proc Am Assoc Cancer Res* **42:**381.

Binet S, Chaineau E, Fellous A, Lataste H, Krikorian A, Couzinier JP and Meininger V (1990) Immunofluorescence study of the action of navelbine, vincristine and vinblastine on mitotic and axonal microtubules. *Int J Cancer* **46:**262–266.

Bunn A Jr and Kelly K (1998) New chemotherapeutic agents prolong survival and improve quality of life in non-small cell lung cancer: a review of the literature and future directions. *Clin Cancer Res* **4:**1087–1100.

Crown J (1997) Optimising treatment outcomes: a review of current management strategies in first-line chemotherapy of metastatic breast cancer. *Eur J Cancer* **33:**S15–S19.

Dhamodharan RI, Jordan MA, Thrower D, Wilson L and Wadsworth P (1995) Vinblastine suppresses dynamics of individual microtubules in living cells. *Mol Biol Cell* **6:**1215–1229.

Donehower RC and Rowinsky EK (1993) Anticancer drugs derived from plants, in *Cancer: Principles and Practice of Oncology* (De Vita VT, Hellman S, and Rosenberg SA editors) pp 409–417, JB Lippincott, Philadelphia..

Fahy J, Duflos A, Ribet JP, Jacquesy JC, Berrier C, Jouannetaud MP and Zunino F (1997) *Vinca* alkaloids in superacidic media: a method for creating a new family of antitumor derivatives. *J Am Chem Soc* **119:**8576–8577.

Fumoleau P, Raymond E, Bennouna J, Armand J-P, Hocini A, Blanchot G, Pinel MC, De Bie J and Marty M (2001) Phase I trial of vinflunine (L0070) a novel fluorinated Vinca alkaloid in patients (pts) with advanced solid malignancies: final results. *Proc Am Assoc Cancer Res* **42:**834.

Hays TS and Salmon ED (1990) Poleward force at the kinetochore in metaphase depends on the number of kinetochore microtubules. *J Cell Biol* **110:**391–404.

Hill B, Fiebig H, Waud W, Poupon M, Colpaert F and Kruczynski A (1999) Superior in vivo experimental antitumour activity of vinflunine, relative to vinorelbine, in a panel of human tumour xenografts. *Eur J Cancer* **35:**512–520.

Jacquesy J-C and Fahy J (2000) Cancer superacid generation of new antitumor agents, in *Biomedical Chemistry: Applying Chemical Principles to the Understanding and Treatment of Disease* (Torrence PF, editor) pp 227–246, John Wiley and Sons, Inc., New York.

Jean-Decoster C, Brichese L, Barret J, Tollon Y, Kruczynski A, Hill B and Wright M (1999) Vinflunine, a new *Vinca* alkaloid: cytotoxicity, cellular accumulation and action on the interphasic and mitotic microtubule cytoskeleton of PtK2 cells. *Anti-Cancer Drugs* **10:**537–543.

Jordan MA, Himes RH and Wilson L (1985) Comparison of the effects of vinblastine, vincristine, vindesine, and vinepidine on microtubule dynamics and cell proliferation *in vitro*. *Cancer Res* **45:**2741–2747.

Jordan MA, Thrower D and Wilson L (1991) Mechanism of inhibition of cell proliferation by *Vinca* alkaloids. *Cancer Res* **51:**2212–2222.

Jordan MA, Toso RJ, Thrower D and Wilson L (1993) Mechanism of mitotic block and inhibition of cell proliferation by Taxol at low concentrations. *Proc Natl Acad Sci USA* **90:**9552–9556.

Jordan MA, Wendell KL, Gardiner S, Derry WB, Copp H and Wilson L (1996) Mitotic block induced in HeLa cells by low concentrations of paclitaxel (Taxol) results in abnormal mitotic exit and apoptotic cell death. *Cancer Res* **56:**816–825.

Jordan MA and Wilson L (1998) Microtubules and actin filaments: dynamic targets for cancer chemotherapy. *Curr Opin Cell Biol* **10:**123–130.

Jordan MA and Wilson L (1999) The use and action of drugs in analyzing mitosis. *Methods Cell Biol* **61:**267–295.

Johnson SA, Harper P, Hortobagyi GN and Pouillart P (1996) Vinorelbine: an overview. *Cancer Treatment Rev* **22:**127–142.

Kruczynski A, Barret J-M, Etievant C, Colpaert F, Fahy J and Hill BT (1998) Antimitotic and tubulin interacting properties of vinflunine, a novel fluorinated *Vinca* alkaloid. *Biochemical Pharmacol* **55:**635–648.

Kruczynski A, Colpaert F, Tarayre J, Mouillard P, Fahy J and Hill B (1998) Preclinical in vivo antitumor activity of vinflunine, a novel fluorinated *Vinca* alkaloid. *Cancer Chemother Pharmacol* **41:**437–447.

Langlois N, Gueritte F, Langlois Y and Potier P (1976) Application of the Polonovski reaction to the synthesis of vinblastine-type alkaloids. *J Am Chem Soc* **98:**7017–7024.

Lobert S, Ingram J, Hill B and Correia J (1998) A comparison of thermodynamic parameters for vinorelbine- and vinflunine-induced tubulin self-association by sedimentation velocity. *Mol Pharmacol* **53:**908–915.

Lobert S, Vulevic B and Correia J (1996) Interaction of *Vinca* alkaloids with tubulin: a comparison of vinblastine, vincristine, and vinorelbine. *Biochemistry* **35:**6806–6814.

Lobert S, Fahy J, Nill BT, Duflos A, Etievant C and Correia JJ (2000) *Vinca*-alkaloid-induced tubulin spiral formation correlates with cytotoxicity in the leukemic L1210 cell line. *Biochemistry* **39:**12053–12062.

Loo DT and Rillema JR (1998) Measurement of cell death. *Methods Cell Biol* **57:**251–264.

Mangeney P, Andriamialisoa RZ, Langlois N, Langlois Y and Potier P (1979) A new class of antitumor compounds: 5′-nor and 5′,6′-seco derivatives of vinblastine type alkaloids. *J Org Chem* **44:**3765–3768.

Margolis RL and Wilson L (1998) Microtubule treadmilling: what goes around comes around. *Bioessays* **20:**830–836.

Mitchison TJ (1989) Poleward microtubule flux in the mitotic spindle; evidence from photoactivation of fluorescence. *J Cell Biol* **109:**637–652.

Mitchison TJ and Kirschner M (1984) Dynamic instability of microtubule growth. *Nature (Lond)* **312:**237–242.

Ngan VK, Bellman K, Panda D, Hill BT, Jordan MA and Wilson L (2000) Novel actions of the antitumor drugs vinflunine and vinorelbine on microtubules. *Cancer Res* **60:**5045–5051.

Nicklas RB, Ward SC and Gorbsky GJ (1995) Kinetochore chemistry is sensitive to tension and may link mitotic forces to a cell cycle checkpoint. *J Cell Biology* **130:**929–939.

Rodionov VI, Nadezhdina E and Borisy GG (1999) Centrosomal control of microtubule dynamics. *Proc Natl Acad Sci USA* **96:**115–120.

Rudner AD and Murray AW (1996) The spindle assembly checkpoint. *Current Biology* **8:**773–780.

Toso RJ, Jordan MA, Farrell KW, Matsumoto B and Wilson L (1993) Kinetic stabilization of microtubule dynamic instability in vitro by vinblastine. *Biochemistry* **32:**1285–1293.

Waters JC, Mitchison TJ, Rieder CL and Salmon ED (1996) The kinetochore microtubule minus-end disassembly associated with poleward flux produces a force that can do work. *Mol Biol Cell* **7:**1547–1558.

Address correspondence to: Dr. Mary Ann Jordan, Department of Molecular, Cellular and Developmental Biology, University of California, Santa Barbara, CA 93106. E-mail: jordan@lifesci.ucsb.edu