Mechanism of Inhibition of Cell Proliferation by *Vinca* Alkaloids

Mary Ann Jordan, Douglas Thrower, and Leslie Wilson

Department of Biological Sciences, University of California, Santa Barbara, California 93106

**ABSTRACT**

We have used a structure-activity approach to investigate whether the *Vinca* alkaloids inhibit cell proliferation primarily by means of their effects on mitotic spindle microtubules or by another mechanism or by a combination of mechanisms. Five *Vinca* alkaloids were used to investigate the relationship in HeLa cells between inhibition of cell proliferation and blockage of mitosis, alteration of spindle organization, and depolymerization of microtubules. Indirect immunofluorescence staining of microtubules and 4,6-diamidino-2-phenylindole staining of chromatin were used to characterize the effects of the drugs on the distributions of cells in stages of the cell cycle and on the organization of microtubules and chromosomes in metaphase spindles. The microtubule polymer was isolated from cells and quantified using a competitive enzyme-linked immunoadsorbent assay for tubulin. We observed a nearly perfect coincidence between the concentration of each *Vinca* derivative that inhibited cell proliferation and the concentration that caused 50% accumulation of cells at metaphase, despite the fact that the antiproliferative potencies of the drugs varied over a broad concentration range. Inhibition of cell proliferation and blockage of cells at metaphase at the lowest effective concentrations of all *Vinca* derivatives occurred with little or no microtubule depolymerization or spindle disorganization. With increasing drug concentrations, the organization of microtubules and chromosomes in arrested mitotic spindles deteriorated in a manner that was common to all five congeners. These results indicate that the antiproliferative activity of the *Vinca* alkaloids at their lowest effective concentrations in HeLa cells is due to inhibition of mitotic spindle function. The results suggest further that the *Vinca* alkaloids inhibit cell proliferation by altering the dynamics of tubulin addition and loss at the ends of mitotic spindle microtubules rather than by depolymerizing the microtubules. The specific alterations of spindle microtubule dynamics appear to differ among the five *Vinca* congeners, and such differences may be responsible for differences in the antitumor specificities of the drugs.

**INTRODUCTION**

The *Vinca* alkaloids, potent inhibitors of cell proliferation, are widely used in cancer chemotherapy [reviewed in (1)]. Despite numerous studies, the mechanism responsible for their ability to inhibit cell proliferation has not been established firmly. The antiproliferative activity of *Vinca* alkaloids has been attributed in large part to inhibition of mitosis (2, 3), as a result of depolymerization of mitotic spindle microtubules (4).

The interactions of the *Vinca* alkaloids with tubulin and microtubules *in vitro* have been studied extensively. They bind to tubulin and at high concentrations can inhibit polymerization of tubulin into microtubules *in vitro* and *in vivo* (4–8). The *Vinca* alkaloids induce tubulin to self-associate (9) and at appropriate conditions they can induce formation of tubulin paracrystals in cells and *in vitro* (10, 11). Also under certain conditions they can depolymerize microtubules *in vitro* by fraying and peeling protofilaments at both microtubule ends (6, 12). At the lowest effective concentrations *in vitro*, the *Vinca* alkaloids kinetically stabilize microtubules by reducing the rate of tubulin addition and loss at microtubule ends (13).

However, the ability of *Vinca* congeners to inhibit cell proliferation does not correlate well with their ability to interact with tubulin and microtubules *in vitro* [reviewed in (1, 8), also see "Discussion"]. This may be due to the fact that the *Vinca* alkaloids are concentrated intracellularly (14, 15) and accumulate differentially in some cells (14). Alternatively, one or all of the *Vinca* alkaloids may act on targets other than tubulin or microtubules. The *Vinca* alkaloids are known to affect several cellular processes that appear unrelated to microtubules, including RNA, DNA, and lipid biosynthesis (16–18), cyclic nucleotide and glutathione metabolism (19–21), and calmodulin-dependent ATPase activity (22).

In the present work, we used a structure-activity approach with a human tumor cell line, HeLa, to determine the mechanism responsible for inhibition of cell proliferation by the *Vinca* alkaloids. The 5 dimeric *Vinca* congeners chosen for this study were vincristine, vinblastine, vindesine, vinepidine (LY119863), and vinrosidine (Fig. 1), which encompassed a broad range of antiproliferative potencies. We determined the antiproliferative activity of the 5 *Vinca* congeners and their effects on microtubules and mitosis *in vivo* and examined how well inhibition of cell proliferation by the congeners correlated with their abilities to depolymerize microtubules and to alter mitotic spindle organization and function. Specifically, we investigated the concentration dependence for inhibition of proliferation, the distribution of cells in stages of the cell cycle, the organization of spindle microtubules and chromosomes in metaphase by immunofluorescence microscopy, the production of multinucleated cells, and the mass of assembled microtubule polymer for the 5 *Vinca* alkaloids.

We found that inhibition of cell proliferation coincided with arrest of cells in a metaphase-like stage of the cell cycle with all 5 *Vinca* alkaloids, strongly suggesting that their antiproliferative activity is due predominantly or entirely to inhibition of mitotic spindle function. Contrary to our expectations, whereas inhibition of proliferation by vinblastine and vincristine appeared to be due to spindle microtubule perturbation, it was not due to depolymerization of the microtubules. The results suggest that by subtly modulating the dynamics of tubulin addition and loss at microtubule ends, the *Vinca* alkaloids act specifically on spindle microtubules to alter their dynamic behavior and, thereby, their ability to function properly in chromosome movement.

**MATERIALS AND METHODS**

Cell Culture. HeLa S3, epithelial-like cells from epithelioid carcinoma of human cervix, were provided by Dr. Jeannette Bulinski (Columbia University, New York, NY) or American Type Culture Collection (Rockville, MD). Cells were grown in monolayer in either Falcon (Becton Dickinson, Lincoln Park, NJ) T175 or Corning (Corning, NY) 225-cm² tissue culture flasks, or 35-mm 6-well plates in Dulbecco's modified Eagle's medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (Sigma) at 37°C in 5% carbon dioxide in the absence of antibiotics. Cell plating densities were arranged so that cells were in log phase of growth for the duration of
approximately 40 h later, fresh medium with polylysine (500 μM/ml, 2 h, 37°C, followed by a rinse with water and vinrosidine.

The same whether cells were grown on polylysine-coated coverslips or in control and drug-treated cells were counted to determine the increase in cell number (inhibition of proliferation by drug was found to be the same whether cells were grown on polylsine-coated coverslips or in monolayer in flasks as described above). Simultaneously, parallel coverslips of cells were fixed for immunofluorescence microscopy.

Immunofluorescence Microscopy. Cells grown on coverslips were fixed for immunofluorescence microscopy. Cells that had been fixed and stained as described above, using a human serum containing pellets by homogenization and incubation at 0°C for 1 h in depolymerization buffer (0.1 M 2-(N-morpholino)ethanesulfonic acid, 1 mM MgSO4, 10 mM CaCl2, 5 mM GTP, 1 mM dithiothreitol, 1 mM tosyl arginine methyl ester, 0.05 mg/ml aprotinin, 0.02% sodium azide, 0.125% Nonidet P-40, pH 6.9), followed by centrifugation (200,000 x g, 90 min, 37°C) to collect the cytokeskets. Subsequently, tubulin was solubilized from microtubule-containing pellets by homogenization and incubation at 0°C for 1 h in depolymerization buffer (0.1 M 2-(N-morpholino)ethanesulfonic acid, 1 mM MgSO4, 10 mM CaCl2, 5 mM GTP, 1 mM dithiothreitol, 1 mM tosyl arginine methyl ester, 0.05 mg/ml aprotinin, 0.02% sodium azide, pH 6.9) (24). Whole cell lysates were prepared by sonication (100 W, low setting, 2 x 15 s, Bionico IV; WVR Scientific, San Francisco, CA) at 0°C in depolymerization buffer (24). Tubulin in microtubules or total cell tubulin was determined by enzyme-linked immunosorbent assay (24) using a monoclonal antibody to β-tubulin (IgM, 1:1:1; a gift from Dr. David Asai, Purdue University, West Lafayette, IN) (25). Bovine brain tubulin served as the tubulin standard (26).

Determination of Drug Uptake and Cell Volume. Cells were seeded as described above. Twenty-four h later, fresh medium was added containing [3H]vinblastine sulfate (0.6-100 nM, specific activity 62-10,000 Ci/mole; Moravek, Brea, CA) or [3H]vinristine sulfate (0.5-100 nM, specific activity 60-3400 Ci/mole; New England Nuclear, Wilmington, DE). Media were sampled for determination of specific activity at the time of drug addition. Twenty h later, [3H]hydroxymethyl inulin from cells was verified by washing some pellets in buffer [0.1 M 2-(N-morpholino)ethanesulfonic acid, 1 mM MgSO4, 1 mM EGTA, pH 6.5] and recentrifugation; following this procedure, 2-4% of the initial hydroxymethyl inulin in pellets was retained, and this was subtracted from the radioactivity used to determine extracellular volume.

Miscellaneous Procedures. Protein concentrations were determined by the method of Lowry et al. (27) using bovine serum albumin as a standard. Vinblastine, vincristine, vinodesine, and vinrosidine were generous gifts from Eli Lilly and Co., Lilly Research Laboratories, Indianapolis, IN. All other chemicals were reagent grade.

RESULTS

Inhibition of HeLa Cell Proliferation by Vinca Alkaloids. We measured the concentration dependence for inhibition of HeLa cell proliferation by vincristine, vinblastine, vinodesine, and vinrosidine to determine the K_i (Table 1). All of the congeners inhibited HeLa cell proliferation in a concentration-dependent manner (Fig. 2, open circles). The potencies of the 5 congeners differed by more than 3 orders of magnitude. Vinblastine was the most potent derivative (K_i = 0.45 nM; Table 1) and its epimer, vinrosidine, was the weakest (K_i = 2200 nM; Table 1). No more than 5-10% reduction in cell viability occurred at any concentration examined with all of the drugs except for vinrosidine, which caused 15% cell death at a concentration of 33 μM and 97% cell death at a concentration of 100 μM.
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Table 1 Relationship among reduction in microtubule mass, accumulation at metaphase, and inhibition of cell proliferation for 5 Vinca alkaloids

<table>
<thead>
<tr>
<th>Vinca alkaloïd</th>
<th>Inhibition of proliferation ($K_{prol}$, nM)</th>
<th>Accumulation at metaphase ($K_{meta}$, RM)</th>
<th>Depolymerization of microtubules ($K_{dep}$, RM)</th>
<th>$K_{dep}/K_{prol}$</th>
<th>$K_{dep}/K_{meta}$</th>
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<td>9</td>
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<tr>
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<td>0.8</td>
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<td>24</td>
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<tr>
<td>Vindesine</td>
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<td>5.5</td>
<td>8</td>
<td>1.6</td>
<td>1.5</td>
</tr>
<tr>
<td>Vinepidine</td>
<td>90</td>
<td>200</td>
<td>110</td>
<td>1.2</td>
<td>0.6</td>
</tr>
<tr>
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<td>2200</td>
<td>2700</td>
<td>1.2</td>
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* Drug concentration that inhibits cell proliferation by 50%. Derived from the data of Fig. 2.
* Drug concentration required for 50% decrease in cellular microtubule mass. Derived from the data of Figs. 8 and 9.
* Drug concentration required for 50% accumulation of cells at a metaphase-like stage. Derived from the data of Fig. 2.

Accumulation of Inhibited Cells at a Metaphase-like Stage of the Cell Cycle. We examined the organization and distribution of microtubules in untreated (control) and drug-treated cells by antitubulin immunofluorescence microscopy, and examined the organization and distribution of the chromosomes by DAPI staining ("Materials and Methods"). All of the Vinca alkaloids caused the cells to accumulate at a stage of the cell cycle either identical to or closely resembling mitotic metaphase (described further below). We found that the ability of each Vinca congener to inhibit cell proliferation closely coincided with the ability of each congener to cause accumulation of cells at a metaphase-like stage of mitosis (Fig. 2, open squares). The concentration of each congener that caused 50% accumulation of cells at a metaphase-like stage was determined from the data of Fig. 2 and is shown in comparison to the $K_{prol}$ in Table 1.

At the lowest effective concentrations of all of the Vinca alkaloid congeners examined, cells that had accumulated at the metaphase stage of mitosis exhibited either normal or nearly normal-looking spindles. At higher drug concentrations, the organization of the spindles deteriorated progressively in a manner that was similar for all 5 of the congeners. Four abnormal spindle types could be discerned that were characterized by increasing degrees of disorganization (designated types I, II, III, and IV). At any specific drug concentration, a mixture of 2 or 3 abnormal spindle types was usually observed, with the more distorted configurations gradually predomninating as the drug concentrations were increased.

The metaphase spindle morphology characteristic of normal metaphase spindles and of types I–III abnormal spindles in cells incubated with Vinca alkaloids are shown in Figs. 3–5. The metaphase spindles of control cells were bipolar, with very few or no detectable astral microtubules, and the chromosomes were well-organized into compact metaphase plates (Fig. 3, curved arrows). After exposure for 18–20 h to any of the 5 Vinca alkaloids at concentrations slightly less than the concentration of each congener that caused 50% accumulation of cells at a metaphase-like stage, the metaphase spindles that accumulated were predominantly bipolar (Fig. 4). Many of the metaphase spindles that accumulated in the drug were indistinguishable from metaphase spindles of untreated cells. However, the organization of some of the metaphase spindles was slightly altered. These were designated type I abnormal metaphase spindles.

Two differences between normal and type I spindles could be discerned. First, the astral microtubules of the type I spindles were more prominent, longer, and more numerous than those of normal spindles (Fig. 4, a and e, small arrows). Second, most of the metaphase chromosomes of type I spindles were organized into compact metaphase plates, but often one or more chromosomes were found at one or both spindle poles (polar chromosomes) rather than at the metaphase plate (Fig. 4, b, d, f, h, and j, small arrows). These abnormally located chromosomes were almost always associated with the exaggerated tufts of astral microtubules (compare Fig. 4, a to b, c to d, and e to f).

Polar chromosomes often were distributed unequally between the 2 spindle poles (Fig. 4, b, d, f, h, and j, small arrows). We observed many cells with one or several chromosomes at one pole and no chromosomes at the opposite pole. Cells in such configurations appeared to be incomplete metaphases rather...
caused microtubule depolymerization (see below), the microtubules consisted of a ball of condensed chromatin that enclosed one or more star-shaped aggregates of microtubules or other polymeric form of tubulin (Figs. 4, e and f, and 5, a and b, asterisks). As the drug concentration was increased through the range that caused microtubule depolymerization (see below), the microtubules of the star-shaped aggregates changed from long and wispy to short and stubby. These remaining microtubules sometimes appeared to have larger than normal diameter (data not shown). At concentrations of drug that caused accumulation of 70–90% of all cells in a metaphase-like stage, most of the accumulated spindles were type II or III. The final state of the progression of Vinca-induced abnormalities in spindle organization was designated type IV. Microtubules were virtually non-existent and the chromosomes formed nondescript aggregates (data not shown). Both types III and IV abnormal spindles resembled the aberrant spindles classically described in colchicine-induced mitosis (called "c-mitosis" (3,4,28–30)).

Less than 3% of untreated cells were in metaphase, and of those cells in metaphase, less than 2% of the spindles exhibited detectable abnormalities. The numbers of cells in metaphase, the number exhibiting abnormal metaphase spindles, and the extent of the spindle abnormalities increased as the concentrations of each of the 5 Vinca congeners increased (Fig. 6). For example, at 1 nM vincristine, 9.4% of the cells were in metaphase. Of those metaphase spindles, 69% exhibited no detectable abnormalities, 29% were type I and type II abnormal spindles (Fig. 6, closed circles), and 1.5% were type III and type IV abnormal spindles (Fig. 6, open circles). The frequency of type III and IV metaphases increased approximately coincidentally with the accumulation of cells in metaphase (Fig. 6, squares). Thus, at 3.3 nM vincristine, when 61% of the cells were arrested in metaphase, approximately 69% of the metaphase spindles were types III and IV. Ten % of the metaphase spindles appeared normal, and 21% were types I and II abnormal spindles. At 33 nM vincristine, 79% of the cells were arrested in metaphase, and 100% of the metaphases were types III and IV, and no normal, type I, or type II spindles were observed. At sufficiently high drug concentrations, all mitotic cells exhibited type IV spindle configurations.

Concentrations of the Vinca alkaloids that produced metaphase arrest also induced formation of multinucleate interphase cells (Fig. 7). In control cultures, between 1 and 4% of interphase cells exhibited multiple nuclei. The number of multinucleated interphase cells increased approximately in parallel with the frequency of metaphase arrest. The maximum occurrence of multinucleate interphase cells (between 24 and 43% with the 5 congeners) occurred at approximately the same drug concentrations that caused metaphase arrest (Fig. 7).

Formation of Tubulin Paracrystals. Paracrystal formation occurred at substantially higher concentrations than those required to completely depolymerize the microtubules. For example, no microtubules remained at 100 nM vinblastine and higher. However, when vinblastine-treated cells were examined by immunofluorescence microscopy, paracrystals were observed only at vinblastine concentrations of 10 μM and higher (data not shown). These results indicate that paracrystals do not form by direct conversion of microtubules into paracrystals.

Relationship between Inhibition of Cell Proliferation by the Vinca Alkaloids and Spindle Microtubule Depolymerization. The total mass of tubulin in the form of microtubules and the total cell tubulin levels were determined by extraction of stabilized cytoskeletons and measurement of total and cytoskeletal tubulin levels after cells were exposed for 20 h to different concentrations of each of the Vinca congeners ("Materials and Methods"; Fig. 8). The mean total cell tubulin of untreated HeLa cells was 3.6% of the total cell protein, in close agreement with previous results of Bulinski and Borisy (31) and the mean microtubule

![Fig. 3. Microtubule and chromosome organizations in untreated (control) cells.](image-url)
MECHANISM OF ACTION OF THE VINCA ALKALOIDS

Fig. 4. Microtubule and chromosome organizations in cells after incubation for 18–20 h with Vinca alkaloid congeners at approximately the lowest concentrations of each congener that inhibited cell proliferation. a, c, e, g, and i, antitubulin immunofluorescence; b, d, f, h, and j, DAPI stain of chromosomes and chromatin in the same cells. a and b, 2 nM vincristine; c and d, 0.8 nM vinblastine; e and f, 6 nM vinbesine; g and h, 60 nM vinepidine; i and j, 1000 nM vinrosidine. The spindles of many inhibited cells were bipolar (types I and II, small and large arrows, respectively) although a few had collapsed to a monopolar star-like arrangement of microtubules surrounding a ball of chromatin (type III, asterisks in e–j) (some bipolar spindles were tipped on end and resembled monopolar spindles; this configuration was distinguished by focusing up and down on the original preparation). Bipolar spindles in drug-treated cells often had prominent tufts of astral microtubules (arrows in a, c, and e); these were often associated with chromosomes that were not included in the compact metaphase plates but, rather, were found near the spindle poles (arrows in b, d, f, h, and j). × 800.
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Fig. 5. Highly aberrant spindles characteristic of drug-treated HeLa cells after incubation for 20 h with Vinca alkaloids at concentrations that produced nearly complete inhibition of cell proliferation. a, 10 nM vincristine, immunofluorescence microscopy with an antitubulin monoclonal antibody; b, DAPI staining of chromatin and chromosomes in the same cells. Type II spindles (large arrows) had very long astral microtubules and shortened pole-pole distances; their chromosomes were arranged in very incomplete metaphase plates with many chromosomes found at the spindle poles. *, type III (ball-shaped) spindles, which retained no discernible bipolarity. x 800.

polymer mass was 1.1% of the total cell protein, or 31% of the total tubulin content (24).

Each of the 5 Vinca alkaloids produced a concentration-dependent decrease in the mass of microtubules and an approximately parallel decrease in the total quantity of cell tubulin (Fig. 8). The decrease in microtubule mass as determined by measurement of tubulin levels in extracted, stabilized microtubule cytoskeletons agreed completely with the decrease observed by immunofluorescence microscopy (data not shown). The concentration of each Vinca congener required for 50% reduction in the mass of microtubule polymer was determined from the data of Fig. 8, and is presented in Table 1.

The extent of microtubule depolymerization by the 2 most potent congeners, vincristine and vinblastine, did not correlate with inhibition of proliferation (Fig. 9). Vincristine and vinblastine inhibited cell proliferation by 50% at concentrations that were, respectively, 4.1-fold and 24-fold lower than the concentrations that reduced the microtubule polymer mass by 50% (Table 1). Thus, inhibition of cell proliferation, and disruption of mitotic spindle progression from metaphase to anaphase, occurred in the absence of significant microtubule depolymerization with these 2 Vinca alkaloid derivatives. In contrast, the microtubule polymer mass was reduced 50% by vindesine, vinepidine, and vinrosidine at approximately the same concentrations of the derivatives that inhibited cell proliferation by 50% (Table 1). Thus, inhibition of cell proliferation with the 3 least potent Vinca alkaloid derivatives correlated closely with the quantitative depolymerization of microtubules.

Determination of Intracellular Vincristine and Vinblastine Concentrations. The effects we observed on spindle morphology and microtubule polymer mass occurred at Vinca alkaloid concentrations significantly below the concentrations shown previously to affect microtubule polymerization in vitro (5–8). One possible explanation for the discrepancy is that the Vinca alkaloids are concentrated intracellularly (14, 15). We determined the intracellular concentrations of vincristine and vinblastine at the extracellular drug concentrations that caused abnormal spindle morphology and microtubule depolymerization. The intracellular concentrations of vincristine were between 16- and 19-fold higher than the concentrations in the culture media, and the intracellular concentrations of vinblastine were between 31- and 63-fold higher than the concentrations in the culture media (Table 2).

Interestingly, the intracellular concentrations of both drugs (33 nM vincristine, 38 nM vinblastine) at the extracellular concentrations that inhibited cell proliferation by 50% were similar to the concentrations that inhibit the dynamics of tubulin addition and loss at microtubule ends in vitro (5–8), see...
MECHANISM OF ACTION OF THE VINCA ALKALOIDS

Vincristine

Fig. 7. Induction of multinucleate interphase cells by the Vinca alkaloids. The percentage of interphase cells having more than one nucleus (•) and the frequency of metaphase arrest (D; data are from Fig. 2) after exposure to Vinca alkaloids for 18–20 h are shown in relation to concentration for vincristine, vinblastine, vindesine, vinepide, and vinrosidine. The percentage of multinuclear interphase cells was determined by scoring a minimum of 50 cells from 2 or 3 replicate experiments (“Materials and Methods”). In control cell populations, multinucleate interphase cells were always less than 4% of the total interphase cells (n=459 interphase cells were scored for each control value).

"Discussion"). In addition, calculation of the intracellular tubulin concentration using values for the mass of tubulin per cell (derived from data of Fig. 8) and for average cell volume (2.3–2.5 picoliters per cell, determined by microscopic measurements of suspended cells and by included and excluded cell volume in pellets) indicated that the intracellular tubulin concentration was 2 mg/ml. This concentration is similar to that used in studies on the effects of Vinca alkaloids on microtubule assembly dynamics in vitro (e.g., 8, 13).

DISCUSSION

We used a pharmacological structure-activity approach to investigate the mechanism(s) responsible for inhibition of HeLa cell proliferation by the Vinca alkaloids. We chose a series of Vinca derivatives with widely different potencies, and determined for each derivative the concentration dependence for inhibition of cell proliferation, for arresting cells at the metaphase stage of mitosis, and for decreasing the microtubule polymer mass (inducing microtubule depolymerization) (Table 1; Figs. 2, 8, and 9). Analysis of the data strongly indicates that inhibition of cell proliferation by these Vinca derivatives was caused by inhibition of the cell cycle at metaphase of mitosis. Inhibition of mitotic spindle function at metaphase at the lowest effective concentrations of all Vinca derivatives occurred with little or no disruption of spindle organization or depolymerization of the spindle microtubules. The present data, together with results of our recent studies on the effects of vinblastine on the dynamics of tubulin addition and loss at microtubule ends in vitro (13), suggest that the Vinca alkaloids act selectively at the ends of spindle microtubules during metaphase to kinetically stabilize their assembly and disassembly dynamics. Further, the results suggest that such vinblastine-sensitive dynamics are critical to the ability of cells to progress from metaphase to anaphase.

Inhibition of Cell Proliferation by Vinca Alkaloids at Metaphase. Using criteria of chromosome condensation and rounding of cells, vinblastine, vincristine, and other active Vinca alkaloids were shown previously to arrest cells in mitosis (2, 3, 19, 32–34). For example in one of the earliest studies, Palmer et al. (2) found that exposure of J-96 human monocytic leukemia cells to approximately 5 μM vinblastine for 53 h caused 45% accumulation of cells in mitosis. The drug prevented chromatid separation, so the mitotic block was deduced to occur at metaphase. Cuts (3) obtained similar results in Ehrlich ascites tumors in mice and in normal rat bone marrow cells. Also, Bruchovsky et al. (33) found that inhibition of L-cell proliferation by vinblastine correlated closely with accumula-

Fig. 8. Effect of Vinca alkaloids on the mass of tubulin in microtubules (D) and on the total cellular tubulin (O). Drugs were added to exponentially growing cells in culture and harvested 18–20 h later for determination of total protein, total tubulin, and tubulin in microtubule polymer (“Materials and Methods”). Results are presented as μg of tubulin per 100 μg of cell protein. From 2 to 8 independent isolations were performed for each drug concentration, and non-drug-treated controls were included in each experiment. Arrows on the ordinates, mean values for control cultures. The microtubule polymer mass consisted of between 31 and 36% of the total cellular tubulin in control cells (range of mean values for all controls performed). The mean values for the ratio of total tubulin/total protein in control cells ranged from 2.4 ± 0.4% (n = 22) for the control cells of the vinblastine experiments to 3.9 ± 0.4% (n = 7) for the control cells of the vinrosidine experiment. The mean values for polymerized tubulin/total protein in control cultures ranged from 0.9 ± 0.1% (n = 18) for control cells of the vincristine experiments to 1.3 ± 0.1% (n = 8) for control cells of the vinrosidine experiments.
distinguishable from normal bipolar metaphase spindles when the lowest effective concentrations of each derivative were in vindesine, vinepidine, and vinrosidine arrested cell proliferation percent reduction in cell proliferation as compared with proliferation of untreated cells after exposure for 20 h to Vinca alkaloids is presented as a percentage of cell proliferation by the Vinca alkaloids. The mass of microtubules remaining in the cells in mitosis or in a multinucleate condition in formation for each concentration.* Increase, intracellular concentration/concentration in medium.

Surprisingly, the spindles of many arrested cells incubated at high concentrations of the Vinca alkaloids accumulated at the metaphase stage (Fig. 2). We do not know whether all of the functions of the interphase microtubule network were un-

teraphase-like fashion to the opposite pole.

Another indication of altered spindle microtubule function at low Vinca alkaloid concentrations was that one or more chromosomes often were found at one or both spindle poles (Fig. 4). We do not know whether the chromosomes at the poles were unable to congress to the metaphase plate during prophase, or were transported to the poles by anaphase-like movement. Failure to congress to the metaphase plate could occur, for example, by the inability of microtubules to capture kinetochores or elongate properly during congression. Movement of one or a few chromosomes to the poles could occur by breakage of the attachment of kinetochore microtubules at one pole, and movement of the still-linked chromatid pairs in anaphase-like fashion to the opposite pole.

The Vinca alkaloids significantly reduced the quantity of microtubules as the concentration of each Vinca derivative was increased. While the relationship between the reduction of microtubule mass and arrest of cells at metaphase was quantitatively different for the 5 Vinca derivatives (Table 1), the manner in which disruption of spindle microtubule organization occurred was qualitatively similar for all 5 Vinca congeners (discussed below) (Figs. 3–6). As the drug concentration was increased, spindle organization was disrupted severely. Spindles collapsed into monopolar configurations and the chromosomes aggregated into a ball, usually in association with a star-shaped monoaster of long microtubules or, as microtubules depolymerized, with short residual microtubule fragments or possibly abnormal polymeric forms of tubulin (Fig. 5). At sufficiently high concentrations of all 5 derivatives, total microtubule depolymerization occurred. These were typical of the c-mitotic configurations described in the early studies on the Vinca alkaloids [e.g., (2, 3)].

Interphase cells with apparently normal organizations were often observed in the same cultures along with cells blocked at metaphase (data not shown). Further, many cells exposed even to high concentrations of the Vinca alkaloids accumulated at the metaphase stage (Fig. 2). We do not know whether all of the functions of the interphase microtubule network were un-

Table 2 Intracellular and extracellular drug concentrations

<table>
<thead>
<tr>
<th></th>
<th>Intracellular*</th>
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<tr>
<td>Vincristine</td>
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<tr>
<td>0.5 nM</td>
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<td>19</td>
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<td>10 nM</td>
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* Intracellular drug concentration was measured after incubating exponentially growing cells in monolayer culture in media containing radiolabeled drug for 20 h ("Materials and Methods"). Between 2 and 5 independent assays were performed for each concentration.

^ Increase, intracellular concentration/concentration in medium.

^ Mean ± SE.

Fig. 9. Relationship between reduction in microtubule mass and inhibition of cell proliferation by the Vinca alkaloids. The mass of microtubules remaining in growing cells in monolayer culture in media containing radiolabeled drug for 20 h ("Materials and Methods"). Between 2 and 5 independent assays were performed for each concentration.* Increase, intracellular concentration/concentration in medium.
perturbed at these concentrations, but any functions associated with progress of cells from interphase to mitosis clearly were not inhibited by the drugs. Also, prophase functions of the spindles including congressing of the chromosomes to the metaphase plate at the lowest effective Vinca alkaloid concentrations occurred normally or nearly normally. Thus, the most Vinca-sensitive process may be that required for progression of mitosis from metaphase to anaphase. Our results agree with those of Palmer et al. (2), who concluded that vinblastine blocked cells at metaphase because the drug inhibited chromatid separation in the mitotically arrested cells. Our results also suggest that mitotic spindle microtubules are more sensitive to the Vinca alkaloids than interphase microtubules.

Relationship among Antitumor Activity of the Vinca Alkaloids, Their Antiproliferative Activity, and Their Ability to Cause Metaphase Arrest. Significant differences have been observed in the antitumor activities and toxicities of the clinically useful Vinca alkaloid congeners. In addition, the antitumor activities of the clinically valuable Vinca alkaloids do not correlate positively with their abilities to inhibit microtubule polymerization (1, 8, 15). A number of actions of the Vinca alkaloids have been described that are not related in any obvious way to an action on tubulin or microtubules [e.g., (16-18)]. Thus, one possible explanation for these differences is that the antiproliferative actions of the Vinca alkaloids are brought about by a mechanism that does not involve tubulin or microtubules or by a combination of mechanisms. Another possible explanation is that the differences are due in part to differential rates and extents of cellular uptake and/or release of the Vinca alkaloids.

The present study was designed to circumvent complications due to differences in uptake and retention of the 5 congeners by examining the degree of correlation between cell proliferation and several important cellular actions of the congeners. We observed a nearly perfect coincidence over a broad range of concentrations between the concentration of 5 Vinca derivatives that produced 50% inhibition of proliferation and the concentration of the 5 derivatives that caused 50% accumulation of cells at metaphase. This was true despite the fact that the potencies of the drugs varied over a very broad range of concentrations (Fig. 2; Table 1). The strong correlation between inhibition of cell proliferation and accumulation of cells at metaphase suggests strongly that in HeLa cells, all 5 of the Vinca congeners inhibit cell proliferation by the same mechanism, by disruption of mitotic spindle function.

It is interesting and perhaps significant that a high percentage of HeLa cells was arrested after a single cell cycle of exposure to the Vinca alkaloids (Fig. 2). Such a high degree of mitotic arrest has not been observed in all cell types exposed to the Vinca alkaloids. For example, a maximum of only 50% accumulation of cells at mitosis occurred after exposure of L1210 leukemia cells to a number of different Vinca derivatives (19) and after exposure of J-96 (human monocytic leukemia) cells to vinblastine (2). In our own unpublished experiments, we observed a maximum of only 3% accumulation of cells at metaphase in BSC cells, and 5% accumulation in PtK1 cells after incubating the cells with concentrations of vinblastine (100 nm) that produced nearly total inhibition of proliferation. A similar high degree of mitotic arrest in HeLa cells in response to Vinca alkaloids or other antimitotic drugs has been reported by other investigators (34-36).

The reason for the differences in response of different cell types is not known. Perhaps metaphase arrest by the Vinca alkaloids is more complete or persists longer in HeLa cells than other cell lines. A proportion of the HeLa cells became multinuclear (Fig. 8), suggesting that blocked HeLa cells either revert or progress to an interphase condition without completing mitosis and cytokinesis. Differences in the abilities of specific cell types to progress through cytokinesis to interphase or to revert to interphase without undergoing cytokinesis might play an important role in the antitumor effectiveness of the Vinca alkaloids.

Relationship between Mitotic Arrest and Microtubule Disruption. The immunofluorescence data suggesting that disruption of mitotic spindle function at the lowest effective Vinca concentrations occurred without significant depolymerization of mitotic spindle microtubules were strongly supported by quantitative measurements of microtubule polymer mass (Figs. 8 and 9). For example, the ratio of the concentration of Vinca alkaloid required to reduce the total microtubule polymer mass by 50% to the concentration required to inhibit mitosis by 50% was remarkably high with vinblastine and vincristine (Table 1). Interestingly, the ratio was much lower with the other Vinca derivatives examined. Thus, even though the effects of all of the derivatives on mitosis and microtubule organization were qualitatively similar at the lowest effective concentrations, the actions diverged quantitatively at higher concentrations of the congeners. Thus, differences in the effects of the 5 derivatives on the dynamics of tubulin addition and loss of spindle microtubules must exist.

Such divergence may be due to differences in the ability of different Vinca derivatives to alter the balance of on-and-off rate constants at opposite microtubule ends (13). Such divergence may also be due to differences in the relative ability of the 5 Vinca derivatives to interact with the 2 different affinity classes of binding sites that exist on microtubules (7, 12, 13, 37) as suggested by Potier et al. (38). For example, at relatively low concentrations, vinblastine binds to a small number of high-affinity binding sites at one or both microtubule ends (mean Kd, 1.9 M, 16-17 sites/microtubule) (7). Interaction of vinblastine with these sites results in the kinetic stabilization of microtubule ends and little depolymerization (13). At higher concentrations, vinblastine binds to a large number of vinblastine binding sites on the surface of the microtubule (1.4 to 1.7 vinblastine binding sites/mole tubulin in microtubules; Kd, 0.25-0.33 M) (37). Interaction of vinblastine with these sites appears to cause microtubule depolymerization by endwise spaying and peeling of protofilaments. Thus, a differential ability of the Vinca derivatives to interact with the 2 different classes of binding sites would result in different degrees of separation between microtubule depolymerization and kinetic stabilization of microtubule ends for the various derivatives examined.

The quantitative data on total microtubule polymer in Vinca alkaloid-inhibited cells was compared with the quantity of microtubule polymer in a nonsynchronous control cell population, where the majority of cells were in interphase. Thus, we are assuming for our analysis that the mass of assembled microtubule polymer in metaphase cells does not differ significantly from that of cells in interphase. We attempted to make such a measurement experimentally. However, we were unable to produce and process for microtubule mass determinations a sufficiently high proportion of cells in metaphase using a double thymidine block plus physiological “shake-off” method, and artificial methods for inducing cell synchrony such as use of mitotic inhibitors were not feasible because of the possible effects they might exert on the quantity of microtubule polymer.
However, the quantitative enzyme-linked immunoadsorbent assay data agreed well with the observations made on individual cells by immunofluorescence microscopy. We detected no change in the levels of assembled polymer or the size of the soluble tubulin pool until relatively high concentrations of the Vinca alkaloids were used (Fig. 8). It seems reasonable to conclude that the quantity of assembled polymer is probably not substantially different at mitosis than during interphase, and that our interpretations of the quantitative data on tubulin and microtubule pools are reasonable.

Because the quantity of microtubule polymer was not reduced when cells were blocked at metaphase at the lowest effective concentrations of all congeners, one might question whether the Vinca alkaloids are acting on a metaphase-sensitive non-microtubule target. We think this is unlikely. First, changes in the astral microtubules and shortening of the interpolar distance were observed in blocked spindles even at the lowest effective concentrations of the 5 Vinca derivatives (Figs. 3-5), suggesting that the block was associated with a perturbation of microtubule dynamics. Second, as already indicated, results of recent studies on the dynamics of tubulin addition and loss at the ends of bovine brain microtubules in vitro indicate that the Vinca alkaloids can strongly affect the dynamics of tubulin exchange at microtubule ends without causing significant effects on the polymer mass. For example, 0.15 μM vinblastine reduced the apparent dissociation and association constants at the net assembly ends of the microtubules in vitro by 27 and 41%, respectively, and reduced the flux rate by 50%, while causing less than 7% reduction in microtubule polymer mass (13).

Effects of the Vinca Alkaloids on Cellular Tubulin Pools. A decrease in the total cellular tubulin pool was observed in parallel with the increase in microtubule depolymerization (Fig. 8). This is consistent with the results of Cleveland et al. (39), who found that the total tubulin pool decreased after depolymerization of microtubules by colchicine and nocodazole. In addition, our results clarify the initially puzzling observations of Cleveland et al. (39) made with vinblastine. They found, and we corroborated (data not shown), that exposure of cells to 10 μM vinblastine caused the total tubulin pool to increase rather than decrease. Cleveland et al. (39) suggested that the vinblastine concentration they used might have caused tubulin to aggregate into paracrystalline arrays, and suggested that, in general, regulation of tubulin pools occurs in response to changes in the concentration of soluble tubulin. In the present study, as they predicted, paracrystalline arrays were found at the concentrations used by Cleveland et al. (data not shown). By extending the measurement of tubulin pools to lower vinblastine concentrations, we found that, consistent with their general conclusion and consistent with the effects of other drugs that depolymerize microtubules, Vinca alkaloids appear to cause down-regulation of tubulin synthesis in the concentration range that causes microtubule depolymerization.

Relationship between the Extracellular and Intracellular Concentrations of the Vinca Alkaloids. The concentrations of Vinca alkaloids required to inhibit dynamics of tubulin exchange at the ends of microtubules in vitro are considerably higher than the concentrations in the external medium required to inhibit cell proliferation and mitosis in HeLa cells (4, 6–8). For example, 150 nm vinblastine is required to inhibit the rate of microtubule flux in vitro by 50% (7, 8), whereas 50% accumulation of HeLa cells at mitosis required only 0.8 nm vinblastine (Fig. 2), an almost 200-fold lower concentration. Lengsfeld et al. (14) found that vinblastine and vincristine were concentrated in HeLa cells to levels that were several-hundred-fold higher than the levels in the medium. We also found that vinblastine and vincristine accumulated to significantly higher concentrations intracellularly than were present in the external medium (Table 2). For example, at 0.6 nm extracellular vinblastine, the intracellular concentration was 38 nm. This is in the concentration range that affects the exchange of tubulin at the ends of steady-state microtubules in vitro (9). The same is true for vincristine. Thus, disruption of spindle function in HeLa cells by vincristine and vinblastine occurred in approximately the same concentration range that inhibits tubulin exchange at microtubule ends in vitro.

Finally, we note that the most sensitive antiproliferative and antimitotic effects that we have described in the present study for vincristine, vinblastine, and vindesine occurred at nanomolar concentrations of the drugs in vitro (Table 1). The steady-state serum concentrations of the three drugs (over a period of days) during continuous infusion or after intravenous bolus injection are in the range of 1–13 nm [reviewed in (40)]. Therefore, the Vinca alkaloids may exert their chemotherapeutic actions clinically by affecting the dynamics of mitotic spindle microtubules, rather than by depolymerizing the microtubules.

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MECHANISM OF ACTION OF THE VINCA ALKALOIDS


Mechanism of Inhibition of Cell Proliferation by *Vinca* Alkaloids

Mary Ann Jordan, Douglas Thrower and Leslie Wilson

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