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Preclinical report

Vinflunine, a new vinca alkaloid: cytotoxicity, cellular accumulation and action on the interphasic and mitotic microtubule cytoskeleton of PtK2 cells

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Vinflunine, a newly synthesized derivative, possesses marked *in vivo* antitumor properties and, like other alkaloids, inhibits *in vitro* tubulin assembly at μM concentrations. However, in contrast to other vinca alkaloids, vinflunine exhibits relatively low *in vitro* cytotoxic potency. The aim of this report was to investigate whether the action(s) of vinflunine on the microtubule cytoskeleton could account for its cytotoxicity or if its cellular action requires another molecular target. Four vinca alkaloids used in cancer therapy and vinflunine were studied using PtK2 cells. Their activities on the most dynamic microtubules were investigated in mitosis and in interphase by evaluating the disturbance of the metaphase plate and the splitting of the diplosome, respectively. No correlation was observed between the cellular accumulation of these compounds and either their cytotoxicity or their action(s) on the microtubule cytoskeleton. In contrast, cytotoxicity, mitotic disturbance and diplosome splitting were observed in the nM range for vinblastine, vincristine, vindesine and vinorelbine, although these events occurred at 10 times higher concentrations in the case of vinflunine. Hence, dynamic modifications of both the mitotic and interphasic microtubule cytoskeleton are compatible with *in vitro* cytotoxicity of vinflunine, raising questions about the conventional biochemical screening of these vinca alkaloids. [© 1999 Lippincott Williams & Wilkins.]

Key words: Cytoskeleton, cytotoxicity, microtubule, vinca alkaloids, vinflunine, vinorelbine.

Introduction

Vinca alkaloids are important clinically useful anticancer agents.[1] Vinblastine and vincristine interact with α/β tubulin heterodimers, and modify microtubule dynamics and assembly, leading to the arrest of cell division at metaphase.[2] In certain cases acquired resistance to vincas occurred concomitantly with a modification of the tubulin isotypes.[3] However, it is noticeable that a discrepancy frequently appears to exist *in vitro* between the concentrations leading to cytotoxicity (in the nM range) and those preventing the assembly of purified microtubule proteins (in the μM range).[4,5] In the past this has been attributed to cellular accumulation processes.[6,7] More recently, it has been observed that mitotic inhibition occurred with little microtubule disassembly.[8] This could result from limited modification of overall microtubule dynamics,[9] although the possibility that vinca alkaloids could interact with other proteins appears not to have been ruled out.

Recent studies with vinflunine, a new fluorinated derivative of vinorelbine obtained by hemisynthesis employing superacid conditions,[10] have once again highlighted this enigma. Vinflunine possesses marked antitumor activity in preclinical studies[11,12] and is currently undergoing phase I clinical testing in France. Vinflunine proved 3- to 17-fold less cytotoxic than vinorelbine, vinblastine or vincristine against L1210 and P388 murine leukemia cells *in vitro*, and 2- to 100-fold less potent against a panel of eight human tumor cell lines;[13] yet all four vincas prevented the *in vitro* assembly of tubulin with IC_{50} values in the μM range, differing only by factors of between 5 and less than 2.[13] Moreover, the extent of specific binding of each

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radiolabeled vinca to tubulin differed markedly, with that of vinorelbine being greatly reduced relative to vinblastine and that of vinflunine proving undetectable.[13] Such discrepancies clearly raise questions about the true pharmacological target of these newer molecules.

This study aimed to investigate the perturbation of the microtubule cytoskeleton using the same ranges of concentrations as those leading to cytotoxicity both in mitosis and in interphase. During mitosis, the formation of a typical chromosomal plaque requires an undisturbed mitotic spindle.[14,15] In the presence of low concentrations of microtubule poisons some chromosome bivalents remain away from the chromosomal plaque.[2] Their number increases with the concentration of microtubule poison preventing the formation of a normal spindle, and leading to the disappearance of the metaphase and post-metaphase figures. During interphase, the microtubule cytoskeleton is disassembled only in the presence of high concentrations of vinca alkaloids, in agreement with its reduced dynamic state.[13] However, in several interphasic mammalian cells, the centrosome, typically constituted by two adjacent centrioles, and the associated pericentriolar material (diplosome) splits in the presence of low concentrations of microtubule poisons which destabilize the most dynamic microtubules. The two centrioles, although remaining within the proximity of the nuclear envelope, migrate and reach opposite locations distant by several μm.[16] Both the impairment of the formation of the chromosomal plaque and the separation of the centrosome units constitute two assays which rely upon a mild destabilization of the mitotic and interphasic microtubule cytoskeleton, respectively. Using these two assays, the action of the four vinca alkaloids used in the clinic and of vinflunine currently undergoing clinical trials was compared with their *in vitro* cytotoxicities and cellular drug accumulation capacities.

Materials and methods

Reagents

Vinblastine and vincristine were extracted, while vinorelbine and vinflunine were synthesized and obtained from P. Fabre Médicaments (Castres, France). Rat kangaroo (Potoroo) cells (PtK2) were grown in Dulbecco's medium modified by Iscove (Gibco/BRL, Gergy Pontoise, France).

Immunofluorescence labeling

For labeling γ-tubulin and microtubules, cells were fixed with formaldehyde and processed for immunocytochemistry.[17] The rat monoclonal antibody YL1/2[18] was used to label α-tubulin. Rabbit polyclonal antibodies R75 were affinity purified on overexpressed *Xenopus* γ-tubulin and used to label γ-tubulin, as detailed earlier.[17] Nuclei and chromosomes were stained with 4′,6-diamidino-2-phenylindole (0.2 μg/ml; DAPI). Preparations were observed by epifluorescence with a Zeiss Axiophot microscope equipped with a $\times 40$ (NA: 1.30) plan-neofluar objective, an Optovar varying from $\times 1.25$ to $\times 2.5$, a $\times 4$ TV camera. Images recorded by a Nocticon camera (LH 4015; Lheritiar, Saint Oven L'Aumone, France) were digitized (100 frames averaging) with an image processing system (Sapphire; Quantel, Montigny Les Bratonneux, France). The linear distance between centrosomes was determined with the 'measurement' program in 50 recorded interphase cells, while the percentage of post-mitotic cells (metaphase+anaphase+telophase) was determined for 50 mitotic cells.

Cytotoxicity evaluations and drug accumulation measurements

Exponentially growing cells were studied. Cells were exposed to a range of concentrations of each vinca alkaloid for a 48 h period and their proliferation determined using a standard MTT-based colorometric assay detailed earlier.[13] IC_{75} values, i.e. concentration of compound required to reduce absorbance to 75% of that of control cells, were determined by interpolation between data points.

For accumulation studies cells were incubated with 10 nmol and 1.25 μCi of tritiated vinca at 37°C. After predetermined time intervals, drug uptake was stopped and the radioactivity associated with the cell pellets quantified, as detailed earlier.[19]

Results

Action of vinblastine on PtK2 cells

Cells were treated with different concentrations of vinblastine for 6 h, an incubation time allowing for both the accumulation of abnormal mitotic figures and the interphasic separation of the centrosome. Vinblastine concentrations as low as 5 nM induced the abnormal location of chromosome bivalents and prevented the formation of the chromosomal plate

leading to the disappearance of typical metaphase and post-metaphase stages (Figure 1). Abnormal mitotic figures were apparent after 30 min of incubation (Figure 2). The separation of the diplosome in two centrosomal units observed after immunofluorescence staining with γ-tubulin antibodies was quantified by determining the linear distance between the two γ-tubulin dots. This separation became detectable in the presence of 5 nM vinblastine (Figure 1) and the distance between the two was maximal (12 μm) in the presence of 50 nM vinblastine. Although it has been reported that high concentrations of colcemid completely inhibited the separation of the centrosome,[16] this inhibition was not observed in the presence of high vinblastine concentrations. Average distances of 5 μm between the two γ-tubulin dots were observed after 6 h in the presence of 250 nM vinblastine.

The decrease in the percentage of proliferating cells after different incubation periods in the presence of increasing concentrations of vinblastine was used to define its cytotoxicity (Figure 3). Since this percentage reached a plateau in the presence of high alkaloid concentrations, the concentration leading to 75% of

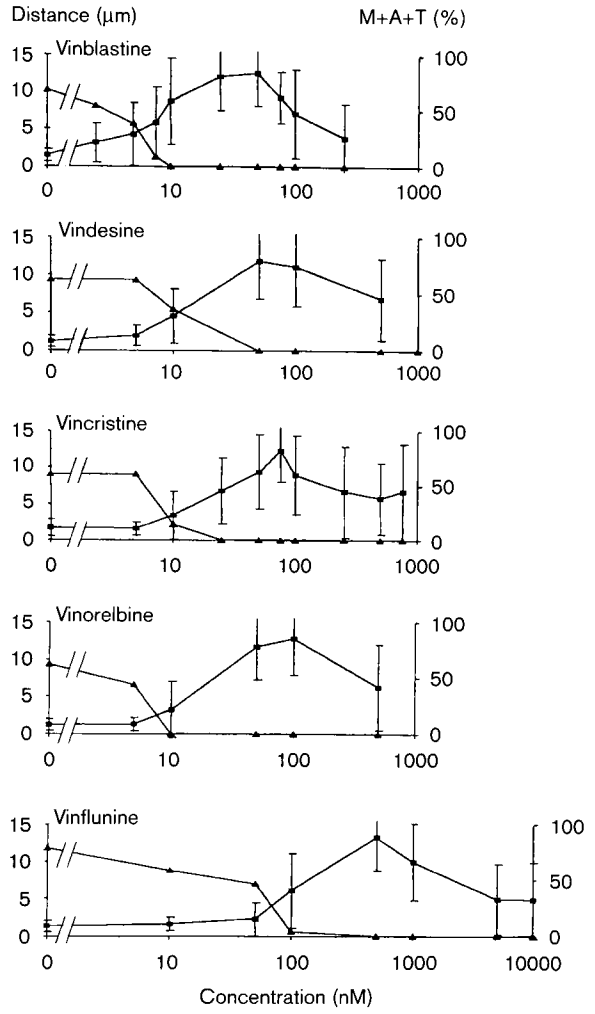

Figure 1. Effects of increasing concentrations of the different vinca alkaloids on the mitotic and interphasic microtubule cytoskeletons in PtK2 cells. Cells were incubated for 6 h in the presence of various concentrations of the different vinca alkaloids. Then their microtubule cytoskeleton was observed by immuno-fluorescence. ▲, Percentage of metaphase (M), anaphase (A) and telophase (T) figures (M+A+T); ■, distance between the two γ-tubulin dots constituting the diplosome; the standard deviation indicates the variability of the distance observed between the two separated γ-tubulin dots.

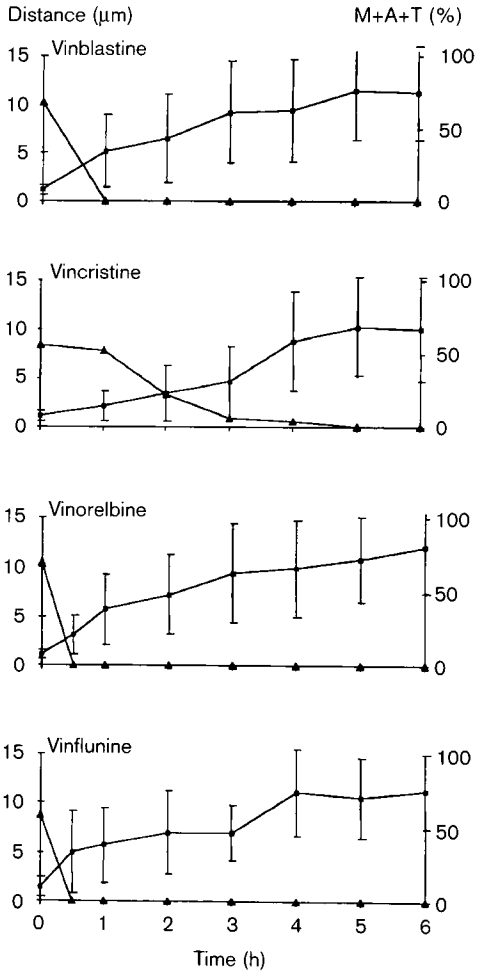

Figure 2. Kinetics of action of the different vinca alkaloids on the mitotic and interphasic microtubule cytoskeletons in PtK2 cells. Cells were incubated in the presence of the concentration of vinca alkaloid leading to the maximal separation of the two centrosomal γ-tubulin dots after 6 h (vinblastine, 25 nM; vincristine, 50 nM; vinorelbine, 100 nM; and vinflunine, 500 nM). The legend is the same as in Figure 1.

control survival (IC_{75}) was determined rather than the more conventionally used IC_{50} value. The IC_{75} value determined after 48 h of incubation in the presence of vinblastine was in the same range as the concentrations which led to the disappearance of the metaphase and post-metaphase mitotic figures after 6 h (Table 1). This IC_{75} value was 17-fold lower than the vinblastine concentration leading to the maximal separation of the two centrosomal dots, but similar to that inducing the first signs of separation after a 6 h incubation (Table 1). Hence the effects of vinblastine on the mitotic and the interphasic microtubule cytoskeleton are generally compatible with its cytotoxic action on PtK2 cells.

Comparative action of vinca alkaloids on the formation of the chromosomal plaque and on the splitting of the centrosome in PtK2 cells

In order to extend these observations, the actions of vincristine, vindesine, vinorelbine and vinflunine on the microtubule cytoskeleton were next compared with their cytotoxicities (Table 1 and Figure 3). In all cases, the concentrations leading to the disappearance of the metaphase and post-metaphase figures were similar to those leading to the first signs of separation of the two γ-tubulin dots in interphase, but were 5–10 times lower than the concentrations resulting in their maximal separation (Table 1). Hence, in each case, the cellular toxicity was compatible with an action on tubulin.

Despite the similarities between the effects of these various vinca alkaloids, clear differences were also observed. (i) The concentrations leading to the disappearance of the metaphase and post-metaphase stages varied from 5 nM for vinblastine to 50 nM for vinflunine, with vincristine, vindesine and vinorelbine exhibiting intermediate values (Figure 1 and Table 1). (ii) The concentrations leading to the maximal centrosomal distances varied from 50 nM for vinblastine to 500 nM for vinflunine, while intermediate concentrations were observed for the other vincas (Figure 1 and Table 1). (iii) The first signs of centrosome separation which occurred in the presence of only 5 nM vinblastine were observed in the presence of more than 75 nM vinflunine, again with the other vincas exhibiting intermediate values (Figure 1 and Table 1). (iv) At the optimal concentration, the almost complete disappearance of the metaphase and post-metaphase stages occurred after 3 h of incubation in the presence of vincristine, 30 min of incubation in the presence of vinblastine and vinorelbine, and after only 15 min in the presence of vinflunine (Figure 2).

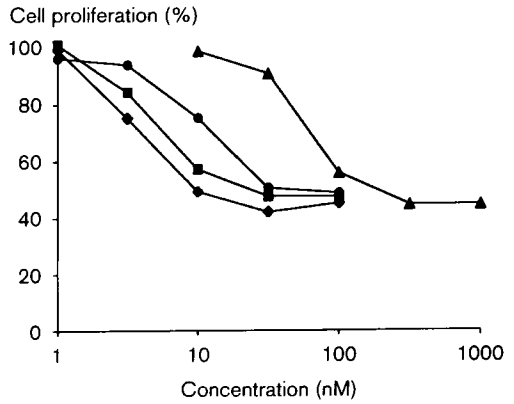

Figure 3. Cytotoxicity of the different vinca alkaloids on PtK2 cells. Exponentially growing cells were exposed to a range of concentrations of each test compound for 48 h and then their proliferation was determined using a standard MTT-based colorimetric assay, as detailed in Materials and methods. ◆, Vinblastine; ■, vincristine; ●, vinorelbine; ▲, vinflunine.

The initial separation of the two centrosomal γ-tubulin dots followed a similar relative timing (Figure 2). (v) Although an average distance between the centrosome of 5 μm was reached after 3 h in the presence of vincristine, this distance was observed after 1 h in the presence of vinblastine and vinorelbine, and after only 30 min with vinflunine (Figure 2). (vi) The concentrations leading to cytotoxicity varied from 3 nM for vinblastine to 54 nM for vinflunine (Table 1). Despite these differences, the cytotoxicity of each vinca alkaloid was compatible with their action on the interphasic and mitotic cytoskeleton (Table 1).

Kinetics of action of the various vinca alkaloids

In contrast to a previous report based on the action of colcemid,[16] a partial inhibition of the separation of the centrosomes was observed in the presence of high concentrations of vinca alkaloids. In order to determine whether this effect could result from the kinetics of the destabilization of the microtubule cytokeleton, the separation of the centrosome was followed comparatively at sub- and supra-optimal concentrations. Vincristine was selected for this investigation since it exhibited the slowest kinetics (Figure 2). In the presence of 75 nM vincristine, the separation of the two γ-tubulin dots constituting the centrosome was sigmoidal with a maximal apparent rate of 2.9 μm/h, reaching an average distance of 11 μm after 5 h (Figure 4). In the presence of a sub-optimal concentra-

tion of vincristine (20 nM), the average distance between the two γ-tubulin dots increased linearly over 5 h at a slow rate (1.1 μm/h). In contrast, in the presence of a supra-optimal concentration of vincristine (500 nM), separation of the two γ-tubulin dots occurred with an apparent maximal rate of 6.3 μm/h, but did not increased further after 45 min. Hence, the kinetics of the separation of the two γ-tubulin dots constituting the centrosome which results from the destabilization of the microtubule cytoskeleton likely reflects the variation of the intracellular concentration of vinca.

These differing kinetics of action of these five vinca alkaloids might be explained, at least in part, by a differential uptake. This hypothesis was checked by measuring their accumulation by PtK2 cells using radiolabeled compounds. Both the intracellular accumulation and the apparent kinetics of uptake were strongly dependent on the vinca alkaloid studied (Figure 5). After a 90 min incubation the cellular accumulation of vincristine was 4-fold lower than that of vinblastine and vinorelbine, and 9-fold lower than vinflunine.

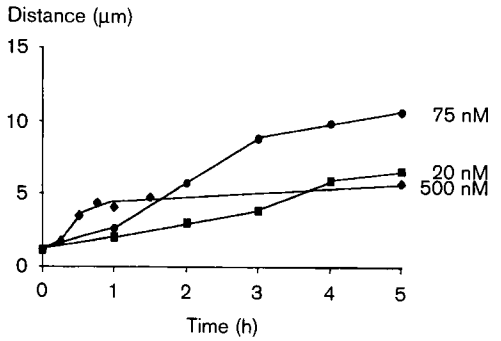

Figure 4. Kinetics of action of different concentrations of vincristine on diplosome splitting in PtK2 cells. The separation kinetic of the two centrosomal units was monitored in the presence of different concentrations of vincristine (■, 20 nM; ●, 75 nM; ◆, 500 nM).

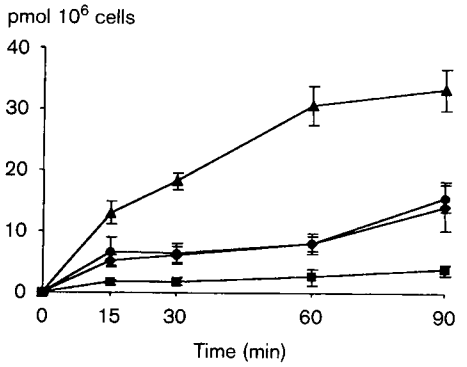

Figure 5. Accumulation kinetics of vinca alkaloids in PtK2 cells. Cells were incubated in the presence of the same concentration of radiolabeled vinca alkaloid (10 nmol and 1.25 μCi) and their accumulation was determined as detailed in Materials and methods. ◆, Vinblastine; ■, vincristine; ●, vinorelbine; ▲, vinflunine.

Reversibility of the action of the vinca alkaloids

These differences in the accumulation of these vinca alkaloids by PtK2 cells might suggest that the reversiblity of their action on the microtubule cytoskeleton could differ. In contrast to the reappearance of the normal mitotic figures, the reversibility of the action of microtubule poisons can easily be quantified in interphase by following the decrease in the distance between the two separated centrosomal dots. Previous investigations have shown that the two

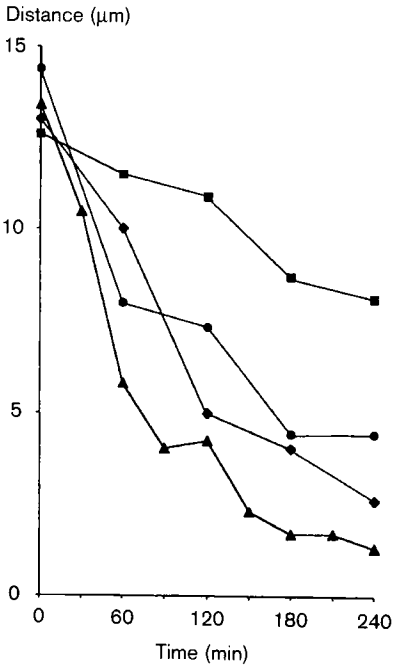

Figure 6. Reversibility of the action of vinca alkaloids: reassociation of the two γ-tubulin dots constituting the diplosome. PtK2 cells were incubated for 6 h in the presence of concentrations of vinca (vinblastine, 25 nM; vincristine, 50 nM; vinorelbine, 100 nM; vinflunine, 500 nM) in order to get the maximal diplosomal splitting (Time 0). Then cells were washed and incubated without vinca in order to follow the reassociation of the diplosomal units. ■, Vincristine; ●, vinorelbine; ◆, vinblastine; ▲, vinflunine.

separated centrosomal γ-tubulin dots present in colcemid-treated PtK2 cells migrate near one another and reconstitute a typical diplosome during the 2 h following washing.[16] A similar experiment was conducted with PtK2 cells (Figure 6). The kinetics of reversibility closely depend upon the vinca alkaloid studied. A typical diplosome was reformed 2.5 h after washing-out vinflunine, while the process was slower in cells treated with the three other vincas. Following washing the distance between the two centrosomal dots was reduced by half by 0.75 h for vinflunine-treated cells, by 1.5 h for both vinblastine- and vinorelbine-treated cells, and only after 4 h for those cells treated with vincristine.

Discussion

The concentrations of vinca alkaloids inducing the entire disassembly of the microtubule cytoskeleton are far in excess of their cytotoxic concentrations. Therefore, it was of pharmacological significance to determine whether these molecules modify the dynamics of the microtubule cytoskeleton at concentrations compatible with their cytotoxicity. Besides the vinca alkaloids of clinical interest, vinflunine, a new vinorelbine derivative possessing potent *in vitro* cytotoxicity[11] and marked *in vivo* antitumor properties,[12,13] albeit at higher doses than the other vinca reference compounds, has been studied here. The concentrations most able to perturb the microtubule dynamics during mitosis and in interphase were assessed, respectively, by monitoring the disappearance of normal metaphase and post-metaphase figures, and by following the separation of the two γ-tubulin dots constituting the centrosome. In all cases these concentrations were similar to those leading to cytotoxicity, although they varied over a 10-fold range depending of the molecule tested. Moreover, the vinca alkaloids studied acted at different concentrations on the microtubule cytoskeleton. Thus this *in vitro* inhibition of tubulin assembly which occurs in the μM range for all these derivatives is less sensitive than those assays involving the overall dynamics of the microtubule cytoskeleton and does not allow one to differentiate between the various vincas. The low sensitivity of this *in vitro* assay could result from various causes: (i) it is possible that the tubulin isoforms purified from mammalian brain are not representative of the tubulin isoforms present in cycling cells and/or the signal could overcome discrete modifications of the microtubule dynamics which are relevant to the stability of the microtubule cytoskeleton. *In vitro* the specific binding of vincristine and vinblastine to tubulin was readily detectable,[11] although the same assays demonstrated only minor tubulin-specific binding of vinorelbine and essentially none for vinflunine, perhaps due to the reversibility of their interaction(s) with tubulin in these latter cases. In contrast, the concentrations of vinorelbine and vinflunine leading to the disappearance of metaphase and post-metaphase figures and to the destabilization of the interphase microtubule cytoskeleton readily distinguished between these two compounds, as was also the case with their differing cytotoxicities.

The kinetics of intracellular uptake of vinca alkaloids were different: the accumulation of vinflunine by PtK2 cells was more rapid than that of the other vincas studied. Although this difference could account for the rapidity of the action of vinflunine on the microtubule cytoskeleton, it could not account for the high level of vinflunine necessary to destabilize the microtubule cytoskeleton nor for its relatively low cytotoxicity.

In vitro, a differential effect of vinca alkaloids on mammalian brain tubulin has been observed using sedimentation velocity experiments and a stopped flow light scattering technique.[20] Vincristine, vinblastine, vinorelbine and vinflunine were indistinguishable by their affinities for tubulin heterodimers. However, the respective liganded tubulin heterodimers exhibited a differential affinity for tubulin spirals and these substances exhibited a differential binding to unliganded tubulin assemblies. In these experiments, the order of magnitude of their affinities was: vincristine > vinblastine > vinorelbine > vinflunine.[20] Interestingly, the order deduced from their action on the microtubule cytoskeleton and from their relative cytotoxicities is similar, although vincristine seems to be less potent than vinblastine in the cellular assays. Hence it is likely that the interactions of vinca alkaloids with tubulin may be considered as largely responsible for their differential effects on the microtubule cytoskeleton in living cells. In agreement with this view, the reversibility of the action of vinca alkaloids is very definitely inversely proportional to these affinity parameters.

Besides sophisticated studies based on the determination of the affinity parameters on various tubulin assemblies[20] and the monitoring of microtubule dynamics by video microscopy,[21] the comparative action of vinca alkaloids on cultured cells appears to be a useful and easy alternative for selecting compounds with unusual properties such as vinflunine. The action of vinca alkaloids on PtK2 cells suggests also that mild destabilization of the microtubule cytoskeleton could occur at similar and low concentrations both in mitosis and in interphase. Although great emphasis has been placed on their action on cell division[2] and on the importance of

deregulation of the metaphase checkpoint(s) for cell viability,[15] the identified perturbation of the interphasic microtubule cytoskeleton raises the possibility that its mild destabilization in some tumor cells could also be significant in terms of cytotoxicity.

Conclusion

Vinflunine, a newly synthesized vinca alkaloid derivative, has marked *in vivo* antitumor properties and yet exhibits relatively low *in vitro* cytotoxic potency, although it inhibits *in vitro* tubulin assembly at μM concentrations. The comparative action of vinflunine and four vinca alkaloids widely used in cancer therapy on the most dynamic microtubules has been assessed both in mitosis and in interphase. Cytotoxicity, mitotic disturbance and interphase diplosome splitting were observed in the nM range for vinblastine, vincristine, vindesine and vinorelbine, but these occurred only at 10 times higher concentrations in the case of vinflunine. No correlation was observed between the cellular accumulation of these compounds and either their cytotoxicity or their action(s) on the microtubule cytoskeleton. Hence, dynamic modifications of both the mitotic and interphasic microtubule cytoskeleton are compatible with *in vitro* cytotoxicity of vinflunine. This finding (i) raises questions about the conventional biochemical screening of these vinca alkaloids, (ii) suggests that the action on the most dynamic microtubules could account for the cytotoxic action of vinflunine and of the four clinically used vinca alkaloids, and (iii) raises possibility that the cytotoxic action of vinca alkaloids could be mediated not only by mitotic disturbances, but also by an action on the interphase microtubule cytoskeleton.

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