Cancer Chemother Pharmacol (1991) 28: 434–440
034457049100130K

Cancer Chemotherapy and Pharmacology

Differential in vitro action of S-12363, a new vinblastine derivative, and of its epimer on microtubule proteins

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Received 23 February 1991/Accepted 10 June 1991

Summary. The action of two epimers of a new vinblastine derivative that differ in their in vivo antitumor activity and their cytotoxicity was studied in vitro in brain microtubule proteins. These two compounds, called S-12363 and S-12362, could not be distinguished from one another or from other active vinca alkaloids by their ability to prevent microtubule assembly. However, they differed strongly both from one another and from vincristine and vinblastine in their ability to induce the formation of tubulin paracrystals and in the stability of the paracrystals following temperature shifts from 0° to 37°C and vice versa. The most potent drug, S-12363, induced considerable tubulin aggregation, which was even more pronounced than that observed in the presence of vincristine. Previous results have shown that S-12363, in contrast to vincristine, induces no neurotoxic effects. This observation is in disagreement with a direct relationship between tubulin aggregation and neurotoxicity.

Introduction

Since the discovery of the antitumor effects of alkaloids that were extracted from the Madagascar periwinkle (*Catharanthus roseus*), this class of agents, known as *Vinca* alkaloids, has been the origin of intense research that initially led to the development of vinblastine and vincristine (Fig. 1), which remain the clinically most widely used of these drugs [9]. Numerous vinblastine derivatives exhibiting promising pharmacological properties have recently been synthesized. Some are characterized by the presence of a carrier amino acid at the vindoline moeity [6] that is intended to confer optimal polarity and, thus, to facilitate cellular uptake. The new vinca alkaloids S-12363 and its epimer S-12362 (Fig. 1) have been obtained according to this strategy by grafting an optically active α-aminophosphonate bioisoster of valine at the C23 position of O4-desacetyl vinblastine [17]. S-12363 is on average 36–72 times more cytotoxic than vinblastine and vincristine, respectively, for human tumor cell lines [31, 34] and exhibits a wide spectrum of activity in vivo in both murine tumors and human tumor xenografts, with its optimal doses being 10–40 times lower than those of vinblastine and vincristine [4, 16]. In contrast, S-12362, which differs only in the absolute configuration of the asymmetric carbon of the side chain, is 270 times less cytotoxic and 1000 times less potent in vivo [34].

The mechanisms responsible for the cytotoxic and antitumor activity of vinca-alkaloid derivatives represent a controversial topic. The major antitumor effects appear to be related to their high affinity to tubulin, a major protein constituting the microtubule cytoskeleton, resulting in the disruption of the mitotic spindle apparatus and the arrest of cells as metaphase-like figures [13, 14]. In vitro, at low concentrations, vinca alkaloids inhibit tubulin assembly into microtubules [30, 44], but at higher concentrations [2, 3, 5, 7, 27] they induce the aggregation of tubulin into spirals and paracrystalline structures (Fig. 2). Therefore, we compared the in vitro activity of the two epimers S-12362 and S-12363 in mammalian brain microtubule proteins so as to determine whether they differ in their action at the molecular level. We evaluated in vitro the ability of these new vinblastine derivatives to inhibit microtubule assembly and to induce the formation of paracrystalline aggregates.

Materials and methods

Chemicals. S-12363 is the 1 S epimer and S-12362 the 1 R epimer of 1-[3-(O4 deacetyl-3-demethoxycarbonyl vinca-leukoblastinyl)-carbonylamino]-2 methyl-propylphosphonic acid diethylester sulfate. They were synthesized and purified as described by Lavielle et al. [17]. They were separated by HPLC and were found to be 98%–99% pure. Vinblastine sulfate (VLB) and vincristine sulfate (VCR) were purchased from Deshors Laboratories (France). Their structures are shown in Fig. 1. All

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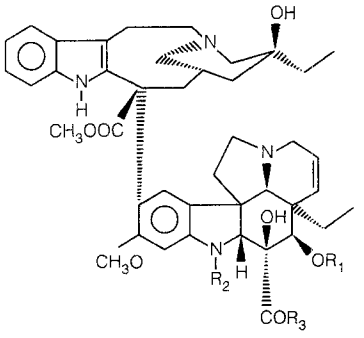


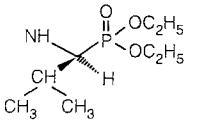
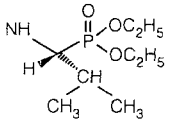
	R 1	R 2	R 3
Vinblastine	$COCH_3$	CH_3	OCH_3
Vincristine	$COCH_3$	CHO	OCH_3
S 12363	H	CH_3	
S 12362	H	CH_3	

Fig. 1. Structures of vinblastine, vincristine, S-12362 and S-12363

compounds were dissolved in a 1 : 1 (v/v) water: ethyl alcohol mixture. The maximal concentration of ethyl alcohol in the assays was $\leq 0.5\%$. These concentrations of alcohol enabled the complete solubilization of the drugs and had no effect on the extent or the kinetics of microtubule assembly and disassembly.

Microtubule proteins. Sheep-brain microtubule proteins (15–20 mg/ml) were obtained by two cycles of assembly-disassembly at 37° and 0°C, respectively [38]. The extraction buffer (buffer I) contained 100 mM piperazine-*N*,*N*′-bis[2-ethanesulfonic acid] (PIPES), 1 mM ethylene glycol-bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 0.5 mM $MgCl_2$ and 1 mM guanosine triphosphate (pH 6.9). Glycerol (4 M) was added for microtubule assembly (buffer II). Microtubule proteins, consisting of tubulin (84%) and associated microtubule proteins (16%), were stored in liquid nitrogen. They were thawed, centrifuged at 0°C and diluted to 2.8 mg/ml buffer I prior to their use.

Turbidimetric assays. The assembly and disassembly of microtubule proteins into microtubules and the assembly of microtubules into spiral aggregates were followed using turbidimetric measurement. The variation of absorbance of the solution of microtubule proteins (2.8 mg/ml) was determined in buffer I using a Beckman spectrophotometer equipped with thermostatically controlled cuvettes. The absorbance due to light scattering [12] was measured at 400 nm at both 37° and 0°C, and the reassembly buffer in the absence of microtubule proteins was used as a blank. The IC_{50} for assembly inhibition, i. e. the drug concentration that reduces the assembly of microtubules by 50% as compared with untreated controls, was determined graphically.

Sedimentable proteins. Sedimentable protein corresponding to paracrystals of tubulin spirals was measured by the Lowry procedure [19] following centrifugation in a Beckman airfuge (Rotor type 30°, 27 pounds/inch^2) for 5 min at 0° or 37°C.

Microscopy. Tubulin assemblies were observed by electron microscopy (Phillips EM 301, 80 kV) following negative staining with 2% uranyl acetate. Spiral paracrystals were observed by interference-contrast microscopy (Zeiss Axiophot; oil condenser na, 1.4; X 63 oil objective na, 1.4; X2 Optovar and X4 video lens) and images were recorded by a Lhesa camera (Pasecon) and were treated using an image-processing system (Sapphire from Quantel) by integrating 200 frames and applying shading, histogram and stretch functions.

Results

Inhibition of tubulin assembly

Microscopic observation revealed that purified sheep-brain tubulin assembled into microtubules when it was incubated at 37°C. The rate and extent of microtubule assembly could be quantitatively followed according to the increase in absorbance at 400 nm [12] and to the increase in sedimentable proteins [37]. Low concentrations of vinca alkaloids are known to inhibit microtubule assembly in vitro [30, 44]. Vinblastine concentrations of 0.25–2.5 μM decreased both the rate and the total amount of microtubule assembly in a dose-dependent manner. An identical observation was made for the two vinblastine derivatives S-12362 and S-12363. In all cases, the microtubules that had assembled in the presence of these drugs disassembled following a decrease in temperature to 0°C. The IC_{50} values for vinblastine, S-12362 and S-12363 were similar as determined either by the rate of microtubule assembly measured according to the variation in absorbance (0.8, 1 and 0.7 μM, respectively) or by the total amount of assembled tubulin measured according to either the final plateau in absorbance (1.5, 2 and 1.3 μM, respectively) or the final amount of sedimentable proteins (1.2, 2.1 and 1.4 μM, respectively).

Induction of tubulin spirals and spiral aggregates at 0°C

The addition of vinblastine to a preparation of purified sheep-brain microtubule proteins at 0°C had no apparent effect on the absorbance when the concentration was <5 μM. In contrast, in the presence of increasing concentrations of vinblastine and vincristine that varied from 10 to 300 μM, a large increase in absorbance was observed at 400 nm (Fig. 3). Concomitantly, tubulin spirals and larger spiral aggregates (Fig. 2) were observed under electron microscopy. Although the IC_{50} value for the inhibition of microtubule assembly in the presence of vinblastine and vincristine did not differ significantly, higher concentrations of vincristine induced more extensive spiralisation and tubulin aggregation than did vinblastine (Fig. 3). Therefore, we studied the effects of high concentrations of S-12362 and S-12363 so as to determine whether these two compounds would differ from one another and from other vinca alkaloids.

Tubulin aggregation was quantitated by the increase in turbidity, as turbidity is a linear fuction of protein concentration [40]. S-12362 induced an increase in absorbance that was slightly lower (23%) than that produced by vinblastine (Fig. 3), and the concentrations of drug required to

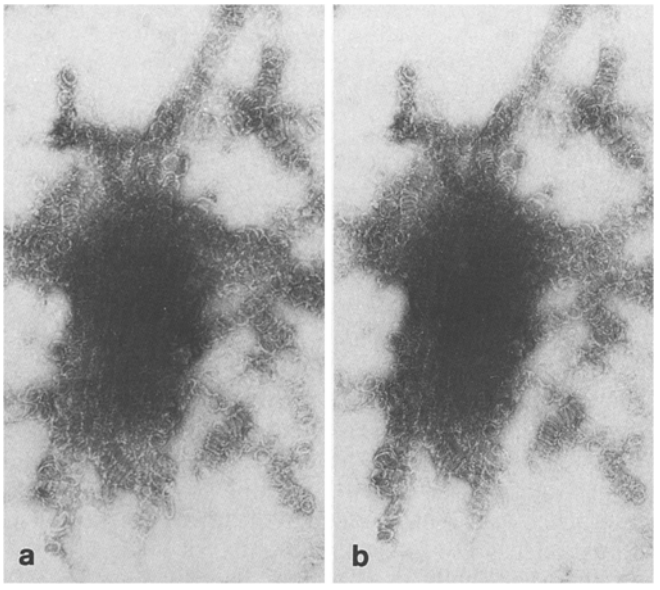


Fig. 2. Stereoscopic image pair of tubulin spirals and tubulin paracrystal induced by the action of vinca alkaloids. Microtubule proteins were incubated for 1 h at 0°C in the presence of 150 μM vincristine, and tubulin spirals and paracrystals were observed by electron microscopy. A small paracrystal is shown to illustrate that aggregations of tubulin spirals are formed by the association of tubulin spirals that form the paracrystals. Tubulin spirals and paracrystals observed in the presence of vincristine, vinblastine, S-12363 and S-12362 exhibit the same overall ultrastructure. The steroscopic image pair was obtained from two photographs of the same field taken at an angle of $\pm 6° \times 98{,}000$

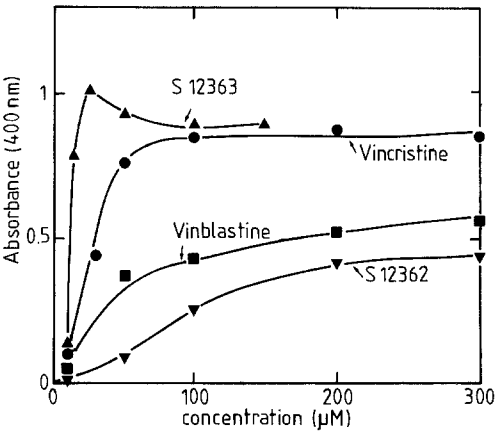


Fig. 3. Effects of increasing concentrations of vinca alkaloids on the formation of tubulin paracrystals at 0°C. For each experimental point, the absorbance of microtubule proteins was determined at the plateau after a 1-h incubation at 0°C in the presence of increasing concentrations of *Vinca* alkaloids

achieve maximal absorbance were higher for S-12362 than for vinblastine. In contrast, S-12363 and vincristine induced a large increase in absorbance (Fig. 3), but maximal absorbance was reached at a concentration of S-12363 that was 5 times lower than that of vincristine. In all cases, spirals were observed by electron microscopy (Fig. 2), but spiral aggregates observed by interference-contrast microscopy were larger in the presence of S-12363 and vincristine than in the presence of S-12362 and vinblastine (Fig. 4). Moreover, spiral paracrystals were numerous in the presence of S-12363 and vincristine, whereas they occurred rarely and were evenly distributed in the presence of S-12362 and vinblastine. The rate of absorbance increase was a linear function of drug concentration but differed markedly for the four vinca alkaloids (Fig. 5). The maximal increase in the initial rate of absorbance according to drug concentration was observed for S-12363 and was 11-fold that found for vincristine. In contrast, the increase in the rate of absorbance was extremely low for both S-12362 and vinblastine, although the increase observed in the presence of vinblastine was twice that found in the presence of S-12362 (Fig. 5).

Effect of temperature shifts on tubulin spirals and spiral aggregates at 0° and 37°C

It has previously been reported that the increase in absorbance observed at 0°C after the addition of vinblastine (40 μM) to microtubule proteins was reversible and disappeared when tubulin assemblies were further warmed to 37°C [22]. Since vinblastine, vincristine, S-12362 and S-12363 differed in the rate and extent of the increase in absorbance of the microtubule proteins at 0°C, we first studied, the relative effect of these compounds at 0° and 37°C and then examined the effects of a shift in the incubation temperature upwards (from 0° to 37°C) and downwards (from 37° to 0°C) on the absorbance.

In the first part of the experiment, each of the vinca alkaloids was added to a preparation of microtubule proteins that was either kept at 0°C or immediately incubated at 37°C. As previously shown (Fig. 3), the addition of 150 μM vinca alkaloid at 0°C led to an increase in absorbance (Fig. 6A). An increasing effect was observed for the drugs in the following order: S-12362, vinblastine, vincristine and S-12363. As expected, S-12363 and vincristine induced a plateau that corresponded to similar absorbance, whereas the plateau in absorbance observed in the presence of vinblastine and S-12362 was 1.6 and 8 times lower, respectively (Fig. 6A). In contrast, when 150 μM vinca alkaloid was added to microtubule proteins just before the temperature was raised to 37°C, the overall effects of these drugs were different (Fig. 6B). In agreement with previously reported observations for vinblastine [22], neither vinblastine, S-12362 nor vincristine could induce a large increase in absorbance at 37°C such as that observed at 0°C for vincristine and vinblastine. However, S-12363 was equally potent at 37°C and 0°C in inducing an increase in absorbance following its addition to the solution of microtubule proteins. These observations were further confirmed by the morphology and the density of spiral paracrystals as observed using interference-contrast microscopy (Fig. 4). The findings obtained using the variation of absorption at 400 nm and the observation of paracrystals by interference-contrast microscopy were further supported by determinations of the amount of sedimentable proteins. In all cases except in the presence of vincristine at 37°C, low absorption values correlated with levels of sedimentable proteins that were <1.3 mg/ml, whereas high absorption

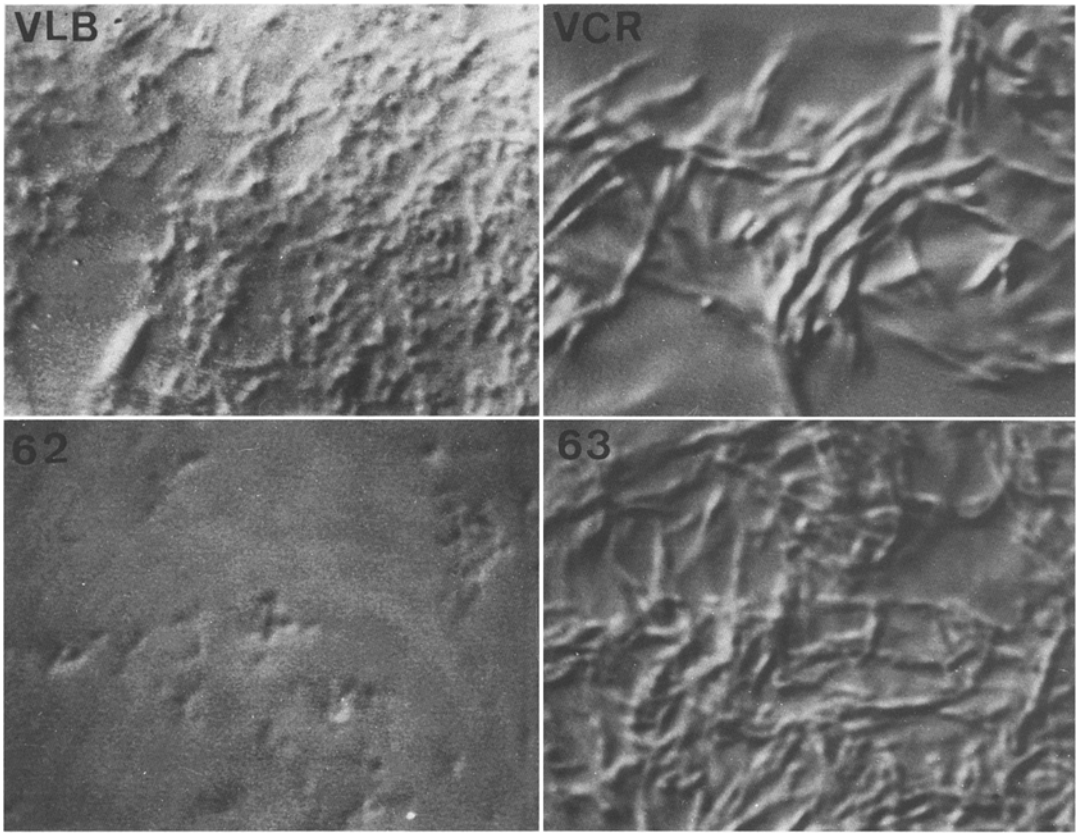


Fig. 4. Interference-contrast observation of spirals and paracrystals formed by microtubule proteins in the presence of vinca alkaloids. Microtubule proteins were incubated for 1 h at 0°C in the presence of 100 μM vinca alkaloid and were observed by interference-contrast microscopy. *VLB*, Vinblastine, *VCR*, vincristine; *62*, S-12362; *63*, S-12363. ×3,100

values corresponded to amounts of sedimentable proteins varying from 2.1 to 2.7 mg/ml (Fig. 6).

In the second part of the experiment, the mixtures of vinca alkaloids and microtubule proteins that had previously been incubated at 0° and 37°C were subjected to a temperature shift up to 37°C and to another shift down to 0°C. The plateau of absorbance of microtubule proteins that had been preincubated at 0°C in the presence of S-12363 remained stable when the temperature was increased to 37°C (Fig. 6A), whereas the plateau showed a marked decrease when the preincubation had been performed in the presence of vinblastine and S-12362 (Fig. 6A). However, in the presence of vincristine, a transient decrease in absorbance was observed after a temperature shift up to 37°C and was followed by a slow increase in absorbance until the optical density had reached the original plateau level observed at 0°C (Fig. 6A). Thus, the behavior of microtubule proteins that had been preincubated at 0°C in the presence of vincristine and then further warmed to 37°C differed markedly from that observed when microtubule proteins were warmed directly after the addition of this drug (cf. Fig. 6A and B). The plateau of absorbance of microtubule proteins that had been preincubated at 37°C with S-12363 remained stable when the incubation temperature of the mixture was shifted down to 0°C (Fig. 6B), whereas the same experimental protocol applied to microtubule proteins in the presence of vincristine, vinblastine and S-12362 led to a clear increase in absorbance, which in the case of vinblastine and S-12362 was even higher than that expected from direct incubation at 0°C (cf. Fig. 6A and B). In all cases, low absorption values correlated with amounts of sedimentable proteins varying from 1.1 to 1.6 mg/ml, whereas high values correlated with quantities varying from 2.2 to 2.6 mg/ml.

Discussion

The molecular properties of S-12363, a new derivative of vinca alkaloids, were studied in mammalian-brain microtubule proteins in vitro. Mammalian-brain tubulin is more easily purified than are microtubule proteins from cultured tumor cell lines [8, 28, 29]. Therefore, tubulin from mammalian brain is largely used in vitro for characterization of the pharmacological action of microtubule poisons [5, 15, 24, 42, 43] and for screening of new active derivatives [44]. Although it must be borne in mind that mammalian-brain microtubule proteins cannot entirely account for the microtubule proteins in tumor cells, there is no evidence that the various mammalian isotubulins differ in their susceptibility to vinblastine derivatives. Moreover, the role of microtubule-associated proteins in the interaction between vinca alkaloids and tubulin remains controversial [20, 21, 27, 36]. In the present preliminary study, we used preparations containing the microtubule-associated proteins so as

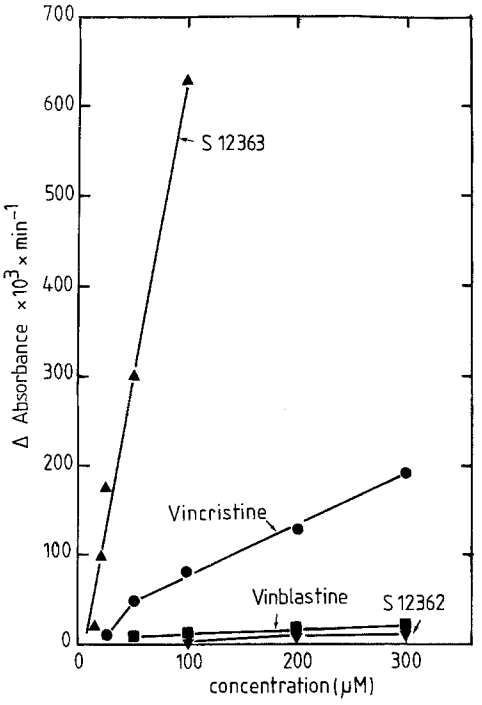


Fig. 5. Variation of the rate of increase in the absorbance of a solution of microtubule proteins at 0°C in the presence of increasing concentrations of vinca alkaloids, The increase in absorbance was recorded as a function of time for each drug concentration at 0°C in a thermostatted cuvette and was used to calculate the initial rate of the increase in absorbance (variation in absorbance per minute)

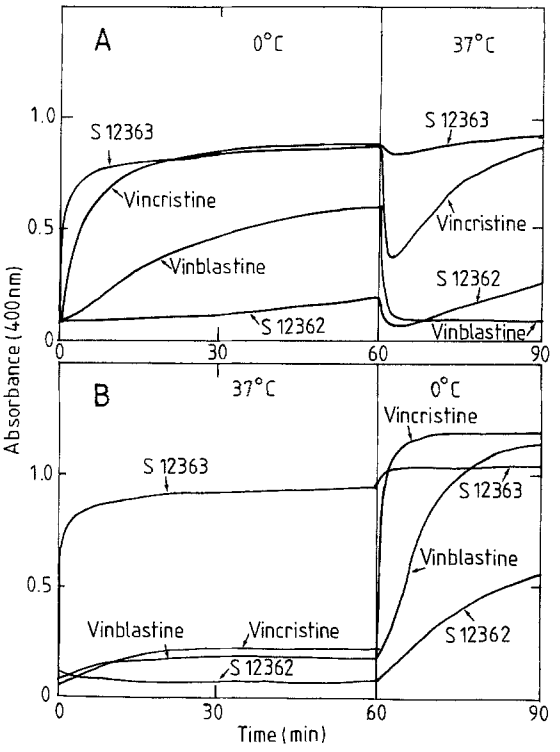


Fig. 6 A, B. Variation of the absorbance of microtubule proteins in the presence of vinca alkaloids. **A** Preincubation at 0°C for 1 h followed by a temperature shift to 37°C **B** Preincubation at 37°C for 1 h followed by a temperature shift to 0°C. In each case, the absorbance of microtubule proteins was recorded in the presence of 150 μM vinca alkaloids for 1 h at the initial temperature and was then followed for 30 min after the temperature shift. **A** The amounts of sedimentable proteins at the end of both the preincubation at 0°C and the incubation at 37°C were the following: S-12363, 2.3 and 2.2 mg/ml, respectively; vincristine, 2.7 and 2.6 mg/ml respectively; vinblastine, 2.2 and 1.6 mg/ml, respectively; S-12362, 0.9 and 1.1 mg/ml, respectively, **B** The amounts of sedimentable proteins at the end of both the preincubation at 37°C and the incubation at 0°C were the following: S-12363, 2.1 and 2.4 mg/ml, respectively; vincristine, 1.9 and 2.4 mg/ml, respectively; vinblastine, 1.3 and 2.2 mg/ml, respectively; S-12362, 0.9 and 1.5 mg/ml, respectively

to enable the possible action of some of these proteins during the aggregation of tubulin in the presence of vinca alkaloids.

At low concentrations, S-12363 inhibited tubulin assembly into microtubules at 37°C exhibiting an IC_{50} value of about 1 μM. In agreement with Pierré et al. [13, 33], this value was similar to those found for the epimer derivative S-12362 and for vinblastine and vincristine. Like vinblastine and vincristine, at high concentrations, S-12363 and S-12362 induced the assembly of tubulin spirals as shown by electron microscopy. Using interference-contrast microscopy and electron microscopy, numerous aggregates of tubulin spirals were observed in the presence of S-12363 and vincristine, whereas fewer were observed in the presence of S-12362 and vinblastine. The increase in absorbance was used to quantitate the effects of these drugs [40]. At 0°C, S-12363 and vincristine induced a large increase in absorbance as compared with that produced by vinblastine and S-12362. The maximal absorbance was reached using a concentration of S-12363 that was 5 times lower than that of vincristine. The increase in the rate of absorbance in the presence of increasing concentrations of S-12363 was 11-fold that observed in the presence of increasing concentrations of vincristine. The formation of paracrystals and their stability following temperature shifts (from 0° to 37°C and from 37° to 0°C) clearly differentiated the two epimers from one another and from vinblastine and vincristine. This observation contrasts with the similarity of the action of active vinca alkaloids on tubulin assembly. Thus, the effects of temperature shifts could be a useful signal for relating the in vitro action of these compounds to their structure and/or therapeutic activity.

Concentrations of vinca alkaloids used in vitro to inhibit microtubule assembly lie in the micromolar range [15, 43], whereas those used in vitro to induce tubulin aggregation are in the 10–100 μM range [3, 23, 27, 40]. In contrast, cytotoxic concentrations in cell cultures lie in the nanomolar range [18]. Three considerations could account for these differences. First, it is known that vinca alkaloids are strongly concentrated (150–500 times) by cells in culture [18]. Second, the instability of most cellular microtubules following drug treatment contrasts with their relative in vitro stability. The procedure of tubulin purification by assembly and disassembly could result in an enrichment in microtubule-associated proteins, enabling a stabilization of microtubules. Third, drug cytotoxicity could be mediated by drug action on a limited number of microtubules. In contrast, the action of microtubule poisons at the molecular level cannot be studied unless most tubulin molecules interact with the drug. In agreement with these observations, 100 μM R-anhydrovinblastine, an inactive vinca alkaloid

derivative, could neither inhibit microtubule assembly nor induce tubulin aggregation in vitro; moreover, even the highest concentrations that used failed to induce a non-specific aggregation of proteins at 0°C [5, 41].

Although compounds S-12363 and S-12362 differ only in the chirality of the aminophosphonate ester moiety, the configuration of this side chain has a dramatic influence on the activity of these drugs both in vivo and in vitro. S-12363 is 270 times more cytotoxic and 1000 times more potent than S-12362 in vivo. Similarly, S-12363 is more potent than S-12362 in vitro in inducing tubulin aggregation, whereas both compounds show similar activity in preventing microtubule assembly. Although the ability to prevent microtubule assembly is a requirement for the antitumor activity of a drug, other structural characteristics involved in other processes are clearly necessary for its cytotoxic effects and antitumor properties. Thus, further investigation of the biochemical mechanisms involved in tubulin aggregation in the presence of vinblastine derivatives is needed. The presence of multiple and different binding sites for some vinblastine derivatives [5, 15, 24, 42, 43], the induction of different transconformational changes in the tubulin molecule [26], the formation of different types of spiral paracrystals [1, 39] and the possible differential role of some microtubule-associated proteins [11, 20] are hypotheses than must be considered.

One of the main difficulties that hinder a clear understanding of the pharmacological properties of vinblastine and its derivatives at the molecular level involves partly the limited significance of the molecular models that are available in vitro and partly the limited number of derivatives that have been comparatively studied. Thus, reports based on a limited number of vinblastine derivatives could enable the exclusion of some hypotheses, as in the present study (see below). In contrast, it is necessary that caution be exercized in raising new hypotheses concerning the involvement of tubulin-aggregating properties in cell retention [18], cytotoxicity and in vivo pharmacological properties. Pierré et al. [32] have shown that S-12363 was strongly accumulated by murine and human tumor cells. Under the experimental conditions used by these authors, the surconcentration factors in L1210 cells were 12 for [^{3}H]-vincristine, 41 for [^{3}H]-vinblastine, 7 for [^{3}H]-S-12362 and 167 for [^{3}H]-S-12363. In addition, in contrast with vinblastine and S-12362, S-12363 and vincristine were strongly retained in these cells. This intracellular surconcentration of S-12363 could explained its high cytotoxicity, since this compound would be in contact with tubulin at a higher concentration and over a longer period than would the other compounds. However, this observation does not imply that either this surconcentration or its effects would be mediated by the highest tubulin-aggregating properties of this compound.

It has been suggested that the neurotoxicity of vinca alkaloids is related to their ability to form tubulin aggregates in vitro [25, 35]. As S-12363 is the most potent vinca alkaloid known to induce tubulin aggregation, it appeared necessary that the neurotoxicity of this new drug first be evaluated. To this end, a comparative investigation of the neurotoxic effects of repeated intravenous administration of S-12363, vinblastine and vincristine was carried out in beagle dogs using electrophysiological recording and electron microscopic examination of the sciatic nerve [10]. S-12363 treatment caused no significant electrophysiological, clinical or histological modifications of the sciatic nerve. In contrast, significant alterations were observed after vincristine treatment. These recent findings are in disagreement with a direct relationship between tubulin aggregation and neurotoxicity. This observation could be explained in two ways: either tubulin aggregation is not sufficient and additional pharmacokinetic properties are also involved in the neurotoxic effects or neurotoxicity is simply not mediated by tubulin aggregation.

Acknowledgements. This work was supported by La Ligue Nationale Française contre le Cancer and by L'Association pour la Recherche sur le Cancer. The authors are endebted to Dr. T. Dunn for reading the manuscript and for helpful suggestions.

References

1. Amos L, Jubb, JS, Henderson R, Vigers G (1984) Arrangement of protofilaments in two forms of tubulin crystal induced by vinblastine. J Mol Biol 178: 711
2. Bensch KG, Malawista SE (1968) Microtubule crystals: a new biophysical phenomenon induced by *Vinca* alkaloids. Nature 218: 1176
3. Bensch KG, Marantz R, Wisniewski H, Shelanski M (1969) Induction in vitro of microtubular crystals by vinca alkaloids. Science 165: 495
4. Berlion M, Pierré A, Kraus-Berthier L, Cros S, Poupon MF, Atassi G, Bizzari JP (1990) Pre-clinical activity of the new vinca alkaloid, S-12363. Ann Oncol 1 [Suppl]: 40
5. Bhattacharyya B, Wolff J (1976) Tubulin aggregation and disaggregation: mediation by two distinct vinblastine-binding sites. Proc Natl Acad Sci USA 73: 2375
6. Bhushana Rao KSP, Collard MP, Trouet A (1985) Vinca-23-oyl amino acid derivatives as new anticancer agents. Anticancer Res 5: 379
7. Bryan J (1972) Vinblastine and microtubules: II. Characterization of two protein subunits from the isolated crystals. J Mol Biol 66: 157
8. Bulinski JC, Borisy GG (1979) Self-assembly of microtubules in extracts of cultured HeLa cells and the identification of HeLa microtubules-associated proteins. Proc Natl Acad Sci USA 76: 293
9. Creasey WA (1979) The *Vinca* alkaloids: In: Haker FC (ed) Antibiotics. Springer Verlag, New York Berlin Heidelberg, vol. 2, p 414
10. Delbarre B, Granger C, Grosse R, Wright M, Paraire M, Bizzari JP (1990) S 12363 a new vinca-alkaloid derivative inducing a particular spiralization of microtubules (abstract). Proc Am Assoc Cancer Res 31: 401
11. Donoso JA, Haskins KM, Himes RH (1979) Effect of microtubule-associated proteins on the interaction of vincristine with microtubule and tubulin. Cancer Res 39: 1604
12. Gaskin F, Cantor CR, Shelanski ML (1974) Turbidimetric studies of the in vitro assembly and disassembly of porcine neurotubules. J Mol Biol 89: 737
13. Himes RH, Kersey RN, Heller-Bettinger I, Samson FE (1976) Action of the vinca alkaloids vincristine, vinblastine and desacetyl vinblastine amide (vindesine) on microtubules in vitro. Cancer Res 36: 3798
14. Jordan MA, Himes RH, Wilson L (1985) Comparison of the effects of vinblastine, vindesine and vinedipine on microtubule dynamics and cell proliferation in vitro. Cancer Res 45: 2741
15. Jordan MA, Margolis RL, Himes RH, Wilson L (1986) Identification of a distinct class of vinblastine binding sites on microtubules. J Mol Biol 187: 61
16. Kraus-Berthier L, Visalli M, Seurre G, Bizzari JP, Pierré A (1990) Effects of a new vinca alkaloid, S 12363, on human tumor xenografts in nude mice. Proc Am Assoc Cancer Res 31: 415

17. Lavielle G, Hautefaye P, Schaeffer C, Boutin JA, Cudennec CA, Pierré A (1991) New α-aminophosphonic acid derivatives of vinblastine: chemistry and antitumor activity. J Med Chem (in press)
18. Lengsfeld AM, Dietrich J, Schultze-Maurer B (1982) Accumulation and release of vinblastine and vincristine by HeLa cells: light microscopic, cinematographic, and biochemical study. Cancer Res 42: 3798
19. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193: 265
20. Luduena RF, Fellous A, Francon J, Nunez J, McManus L (1981) Effect of tau on the vinblastine-induced aggregation of tubulin. J Cell Biol 89: 680
21. Luduena RF, Anderson WH, Prasad V, Jordan MA, Ferrigni KC, Roach MC, Hórówitz PM, Murphy DB, Fellous A (1986) Interactions of vinblastine and maytansine with tubulin. Ann NY Acad Sci 466: 718
22. MacKinlay SA, Luduena RF, Mackae TH (1986) Temperature effects on a solution of vinblastine-induced polymers assembled from brine shrimp (*Artemia*) tubulin. FEBS Lett 203: 301
23. Marantz R, Shelanski ML (1970) Structure of microtubule crystals induced by vinblastine in vitro. J Cell Biol 44: 234
24. Marantz R, Ventilla M, Shelanski M (1969) Vinblastine-induced precipation of microtubule protein. Science 165: 498
25. Marty M, Dieras V, Extra JM, Espie M, Leandri S, Besenval M, Krikorian A, Herrera A (1990) La vinorelbine (navelbine). Cahiers Cancer 2: 104
26. Morgan JL, Spooner BS (1983) Immunological detection of microtubule poison-induced conformational changes in tubulin. J Biol Chem 258: 13 127
27. Na GC, Timasheff SN (1982) In vitro vinblastine-induced tubulin paracrystals. J Biol Chem 257: 10 387
28. Nagle BW, Doenges KH, Bryan J (1977) Assembly of tubulin from cultured cells and comparison with the neurotubulin model. Cell 12: 573
29. Olmsted JB, Carlson K, Klebe R, Ruddle F, Rosenbaum J (1970) Isolation of microtubule protein from cultured mouse neuroblastoma cells. Proc Natl Acad Sci USA 65: 129
30. Owellen RJ, Hartke CA, Dickerson RM, Hains FO (1976) Inhibition of tubulin-microtubule polymerization by drugs of the vinca alkaloid class. Cancer Res 36: 1499
31. Pierré A, Léonce S, Anstett M, Hautefaye P, Lavielle G, Cudennec CA (1989) Cytotoxic properties of a new potent vinca-alkaloid derivative on human solid tumors in vitro. Proc Am Assoc Cancer Res 30: 581
32. Pierré A, Perez V, Molina M, Boutin JA, Anstett M, Lavielle G (1990) Relationships between binding to tubulin and cellular uptake and retention of S-12363. J Cancer Res Clin Oncol 116: 445
33. Pierré A, Lavielle G, Hautefaye P, Léonce S, Saint-Dizier D, Boutin JA, Cudennec CA (1990) Pharmacological properties of a new alpha-aminophosphonic acid derivative of vinblastine. Anticancer Res 10: 139
34. Pierré A, Kraus-Berthier L, Atassi G, Cros S, Poupon MF, Lavielle G, Berlion M, Bizzari JP (1991) Preclinical antitumor activity of a new *Vinca* alkaloid derivative, S-12363. Cancer Res 51: 2312
35. Potier P, Guénard D, Zavala F (1979) Résultats récents dans le domaine des alcaloides antitumoraux du groupe de la vinblastine. Etudes biochimiques. C R Soc Biol (Paris) 173: 414
36. Prakash V, Timasheff SN (1985) Vincristine-induced self-association of calf brain tubulin. Biochemistry 24: 5004
37. Schiff PB, Horwitz SB (1981) Taxol assembles tubulin in the absence of exogenous guanosine 5′-triphosphate or microtubule-associated proteins. Biochemistry 20: 3247
38. Shelanski ML, Gaskin F, Cantor CR (1973) Microtubule assembly in the absence of added nucleotides. Proc Natl Acad Sci USA 70: 765
39. Starling D (1976) Two ultrastructurally distinct tubulin paracrystals induced in sea-urchin eggs by vinblastine sulfate. J Cell Biol 20: 79
40. Ventilla M, Cantor CR, Shelanski ML (1975) Some features of the vinblastine-induced assembly of porcine tubulin. Arch Biochem Biophys 171: 154
41. Wilson L, Bryan J, Ruby A, Mezia D (1970) Precipation of proteins by vinblastine and calcium ions. Proc Natl Acad Sci USA 66: 807
42. Wilson L, Morse A, Bryan J (1978) Characterization of acetyl-[^{3}H]-labeled vinblastine binding to vinblastine-tubulin crystals. J Mol Biol 121: 255
43. Wilson L, Jordan MA, Morse A, Margolis RL (1982) Interaction of vinblastine with steady-state microtubules in vitro. J Mol Biol 159: 125
44. Zavala F, Guénard D, Potier P (1978) Interaction of vinblastine analogues with tubulin. Experientia 34: 1497