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VINBLASTINE SULFATE

ITS REVERSIBLE THERMAL AGGREGATION AND INTERACTION WITH HYDROPHOBIC GROUPS*

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Abstract—Vinblastine sulfate (10^{-3} M) in an aqueous solution at a pH of 7·4 aggregates readily when heated. This aggregation, which involves a sharp transition over a short temperature range, is pH dependent, can be reversed by cooling, is inhibited by various proteins and by sodium dodecyl sulfate, but is not affected by ionic strength or hydrophilic substances. The nature of its interaction with microtubular proteins is dependent on the relative concentration of the reactants. Excess protein inhibits heat aggregation, whereas an excess of vinblastine results in co-precipitation. Since the assembly of microtubules involves an endothermic polymerization of protein subunits, similar to that exhibited by vinblastine over similar pH and temperature ranges, it is concluded that the inhibition of mitosis caused by vinblastine is related to its strong lipophilic character and to its hydrophobic interaction with the microtubular system

VINBLASTINE is a dimeric alkaloid obtained from the leaves of the *Vinca rosea* Linn, a common flowering herb known as the periwinkle.[1] It is a complex molecule containing both indole and dehydroindole moieties, and observation of its structure reveals that it has a strong hydrophobic character. Similarly to colchicine and griseofulvin, it arrests mitosis at the metaphase and this effect seems to be mediated by a reversible interaction with the subunits of the mitotic spindle.[2] A colchicine-binding protein was isolated from the eggs and mitotic apparatus of the sea urchin[3] with physical and chemical properties quite similar to microtubular proteins isolated from other species.[4–7] More recently it was shown that vinblastine is capable of precipitating microtubular protein from a homogenate of HeLa cells and pig brain,[8] as well as causing an intracytoplasmic sequestration of microtubular protein in cultured fibroblasts and leukocytes, leading to the appearance of microcrystalline inclusions.[9] Microtubules can be dissociated into native subunits by treating with a detergent, sodium lauryl sarcosine.[10]

In living cells, mitotic spindle fibers are in a dynamic state of equilibrium with the soluble protein subunits.[11–13] Under physiological conditions this association is very weak ($\Delta G^\circ_{25^\circ} < 1$ kcal mole^{-1}) and the equilibrium can be shifted toward dissociation by the presence in the medium of low concentrations of colchicine, vinblastine, griseofulvin and other closely related structures. The mechanism by which the mitotic inhibitors affect these processes are not clearly understood and it is felt that some of our findings will help to further elucidate their mode of action.

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While investigating the influence of these compounds on the polymerization kinetics of tropocollagen, a relatively simple self-assembling system, it became apparent that vinblastine had unusual solubility characteristics which could be directly associated to its action on the microtubular system. We therefore decided to investigate further this self-associating phenomenon exhibited by vinblastine, as well as the nature of its interaction with proteins and other substances of partial hydrophobic and hydrophilic character.

METHODS

Reagents. Vinblastine sulfate was a gift from Eli Lilly & Company. Radioactive colchicine was obtained from New England Nuclear Corp; crystalline bovine serum albumin, cryst. and pepsin from CalBiochem; sodium dodecyl sulfate from Mallinckrodt, and dextran (Rheomacrodex), average molecular weight approximately 40,000, from Pharmacia.

Isolation of protein from microtubules and mitotic apparatus. Flagella were obtained from the sperm of the sea urchin, *Lytechinus pictus* (Paciffc BioMarine Supply Company, Venice, Calif.), following the procedure of Stevens *et al.*[14] Sperm cells were homogenized in sea water containing 10^{-4} M EDTA. The homogenate was sedimented at low speed, and the pellet obtained was resuspended and the centrifugation repeated. The combined supernatants were centrifuged at 10,000 *g* for 5 min. The final supernatant containing the flagellar protein (and no contaminating sperm heads as judged by phase-contrast microscopy) was dialyzed against a buffer containing tris-HCl (10^{-3} M), EDTA (10^{-4} M) and KCl (3×10^{-3} M), pH 8·0. The soluble protein was converted to an acetone powder and dried under vacuum.

Protein from the mitotic apparatus was isolated by the method of Kane[15] as modified by Mangan *et al.*[16] Washed eggs were fertilized, pelleted and resuspended in 1·0 M urea to remove the fertilization membrane. This last step was repeated, the pellet was washed twice with sea water and finally resuspended in Ca^{2+}-free sea water. At metaphase, zygotes were suspended in a solution containing 1·0 M hexanediol, 10^{-3} EDTA and 0·01 M potassium phosphate, pH 6·3, to free the mitotic apparatus. After vigorous shaking, the material which sedimented by low speed centrifugation was washed with the hexanediol solution, suspended in 0·15 M NaCl and converted into an acetone powder. Solubilization of microtubular proteins was accomplished by extracting the acetone powder with 6·0 M urea containing 10^{-2} M mercaptoethylamine (pH 7·4). After alkylation with an excess of iodoacetic acid, the solution was dialyzed against 0·15 M NaCl buffered to pH 7·4 with Na_2HPO_4 (2×10^{-2} M).

Soluble collagen. Native monomeric collagen was extracted from rat skin by 0·45 M NaCl, pH 7·0, and purified by repeated reprecipitations as previously described.[17] The final product was lyophilized and reconstituted prior to use by dissolving in the starting buffer (0·02 M sodium phosphate, 0·15 M NaCl, pH 7·4) and centrifuging at 100,000 *g* for 2 hr.

Aggregation and co-precipitation studies. The heat-induced aggregation studies described were performed using a Kintrac-DU-spectrophotometer, equipped with an automatic sample changer and a thermocirculator with a refrigerated accessory, which allowed accurate temperature control. The temperature inside the cuvettes was monitored by a Telethermistor. The rate of heating (or cooling) was set at 0·2° per min. The aggregation of vinblastine was followed by measuring turbidity at

400 mμ. When measuring the partition coefficients between water and octanol, the ultraviolet absorption of the aqueous phase was read at 270 mμ where both vinblastine and colchicine show significant absorption. Protein concentration was determined by the Lowry method,[18] and in the case of collagen, its concentration was established on the basis of its hydroxyproline content.[19] Co-precipitation of labeled colchicine, glycine and dextrose with vinblastine was measured by mixing these compounds (2×10^{-4} M) in 0·15 M NaCl with increasing molar ratios of vinblastine. The mixtures were warmed to 37° and the precipitates and supernatants analyzed for radioactivity.

RESULTS

Figure 1 summarizes the effect of pH on the behavior of an aqueous 0·001 M solution of vinblastine sulfate buffered with 0·02 M sodium phosphate and heated at a constant rate. The aggregation elicited by a rise in temperature and pH between pH 6·8 and 7·6 can be clearly seen. Variations in ionic strength (between $\mu = 0{\cdot}08$ and 0·6 with NaCl) did not significantly affect this pattern.

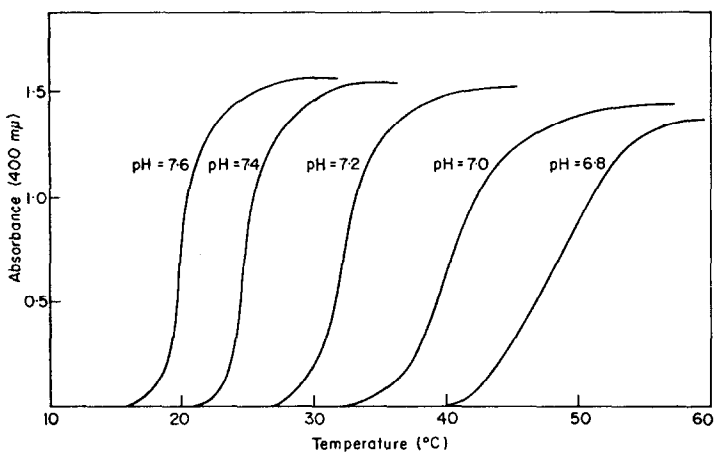

FIG. 1. Heat precipitation of vinblastine sulfate ($1{\cdot}1 \times 10^{-3}$ M) at various pH levels in water buffered with 0·02 M sodium phosphate. The tracings reflect the changes in optical density which occur during the heating period.

The specificity with which vinblastine interacts with hydrophobic sites is illustrated in Fig. 2. Whereas an amphoteric substance like glycine and a hydrophyllic compound like dextran (and monosaccharides and dissaccharides) did not interact with vinblastine to prevent endothermic aggregation, various proteins such as pepsin and bovine serum albumin (BSA), as well as sodium dodecyl sulfate (SDS), bind vinblastine quite readily, preventing it from undergoing subsequent heat aggregation. An acid-hydrolyzed preparation of bovine serum albumin at pH 7·4 did not behave like the intact protein, reflecting a stereo-specific requirement for stabilization. At a lower concentration of BSA (10^{-5} M), a co-precipitation of the protein with vinblastine occurred. It should be emphasized that all the compounds tested in this connection (BAS, SDS, dextrans, etc., and subsequently, alkylated microtubular proteins) were quite soluble at the pH levels and temperature ranges employed in this study. In those instances where

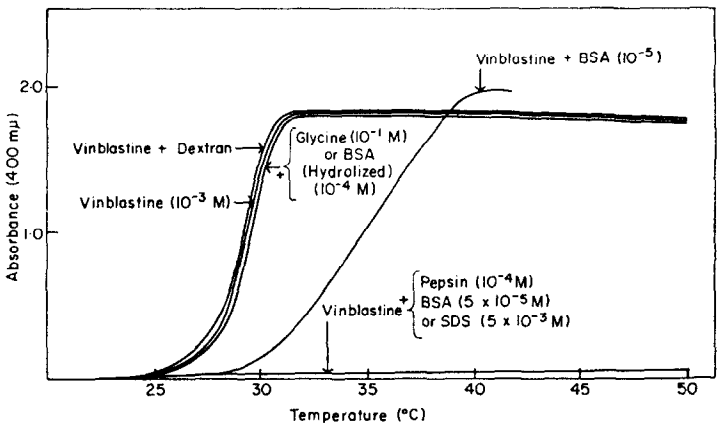

FIG. 2. Interaction of vinblastine sulfate with various hydrophilic and hydrophobic substances at pH 7·4 and at increasing temperatures. Dextran (10^{-4} M; average molecular weight, 40,000), glycine (10^{-1} M) or hydrolyzed bovine serum albumin (BSA), 10^{-4} M, did not prevent the endothermic aggregation of vinblastine. Pepsin (10^{-4} M), BSA (5×10^{-5} M) and sodium dodecyl sulfate (SDS) 5×10^{-3} M, completely inhibited the aggregation, whereas a lower concentration of BSA (10^{-5} M), caused it to co-precipitate with vinblastine over an extended temperature range.

co-precipitations occurred, they could be attributed exclusively to an association phenomenon with the vinblastine molecule. Similarly, urea (an ambivalent type of compound as far as its ability to disrupt H-bonds and hydrophobic interactions), at low concentrations, extends the temperature range of equilibrium between monomer and aggregate, and at higher concentrations, greatly enhances the stability of the monomeric form at elevated temperatures.

The interaction of SDS with vinblastine is summarized in Fig. 3. A concentration of SDS of 10^{-3} M (a 1 : 1 M ratio with vinblastine) caused a co-precipitate to form which could not be solubilized at any temperature. Increasing the number of molecules of SDS successively increases the stability of the micellar structure and progressively

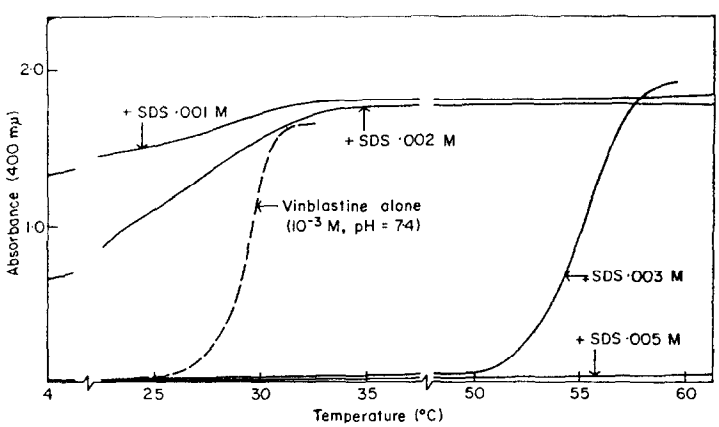

FIG. 3. Interaction of sodium dodecyl sulfate (SDS) at various concentrations with vinblastine sulfate (10^{-3} M, pH 7·4). The behavior of vinblastine without any additive is reflected by the dotted line. The solid lines represent the turbidity of the solutions containing increasing amounts of SDS.

retards its heat aggregation. A 5:1 M excess of SDS completely prevents heat precipitation. The marked affinity of vinblastine for nonpolar environments is also reflected by its rate of partition between water and octanol. If an aqueous solution of vinblastine (0·001 M, pH 7·4) is shaken at 18° with an equal volume of octanol using a Vortex mixer, 99·98 per cent of the vinblastine is transferred to the nonpolar phase within 2 min. A comparable experiment for colchicine showed a 98·1 per cent transfer within a similar period of time. At equilibrium, 98·7 per cent of the colchicine was present in the octanol. Colchicine, studied over a wide range of pH and concentration, does not aggregate like vinblastine, probably due to its greater solubility in polar solvents. Nevertheless, using [^{14}C]colchicine, we found that it will partially co-precipitate with vinblastine (Table 1). This may explain the enhanced binding of colchicine to microtubular protein in the presence of vinblastine.[20] Other compounds tested in this connection, such as glycine and glucose, did not co-precipitate under any conditions.

The reversibility of the heat aggregation process undergone by vinblastine is shown in Fig. 4. Dissolution of the aggregate by cooling followed the same pattern, except that a slight lag caused the return curve to be shifted slightly toward the left.

TABLE 1. INTERACTIONS OF INCREASING AMOUNTS OF VINBLASTINE SULFATE WITH [^{14}C]COLCHICINE, [U-^{14}C]GLYCINE OR [U-^{14}C]GLUCOSE (5×10^{-4} M) MEASURED BY THE DEGREE OF CO-PRECIPITATION OF THESE COMPOUNDS AT pH 7·4 AND 37°

Vinblastine to additive ratio	Per cent of additive co-precipitated with vinblastine		
	Colchicine	Glycine	Glucose
1:1	4·8	0	0
5:1	10·0	0	0
10:1	16·2	0	0
20:1	28·0	0	0

The interactions of vinblastine with microtubular proteins obtained from sperm flagella and mitotic apparatus are shown in Fig. 5. The material obtained as described in the experimental procedure tends to aggregate and become almost insoluble in physiological salt solutions. After precipitation by acetone, its solubility was slightly increased, but still a very significant amount remained undissolved. Because of this, the acetone powders were solubilized with 6·0 M urea containing 10^{-2} M mercaptoethylamine as described in the experimental section.

At low concentrations of microtubular protein, co-precipitation of vinblastine with the protein was induced at relatively low temperatures. Increasing the concentration of flagellar protein (6 mg/ml) retarded the heat-induced aggregation of vinblastine, while higher concentrations (10 mg/ml) completely inhibited the heat precipitation of vinblastine over the temperature range investigated.

Because of our desire to investigate this phenomenon in the presence of a native protein known to undergo a sol → gel transformation *in vitro*, we looked at the interaction of vinblastine with soluble collagen. Native soluble collagen at pH 7·4 undergoes a sharp tropocollagen → fiber transition at 37°. In the presence of vinblastine (10^{-3}

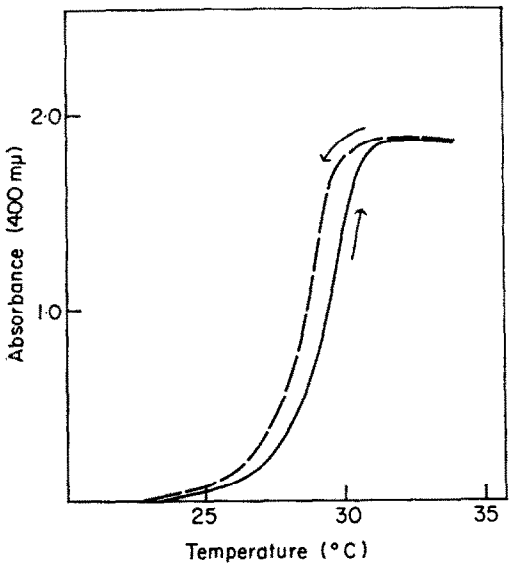

FIG. 4. Reversibility of the heat precipitation of vinblastine caused by subsequent cooling. Vinblastine (10^{-3} M) was heated until maximum precipitation occurred and then allowed to cool at the same rate.

M), the precipitation was very gradual and began to occur at room temperature. At higher concentrations of vinblastine (10^{-2} M), the precipitation of collagen could be accomplished almost quantitatively at lower temperature, and this has yielded a method which we have since used successfully to separate native collagen from mixtures with other proteins (Fig. 6).

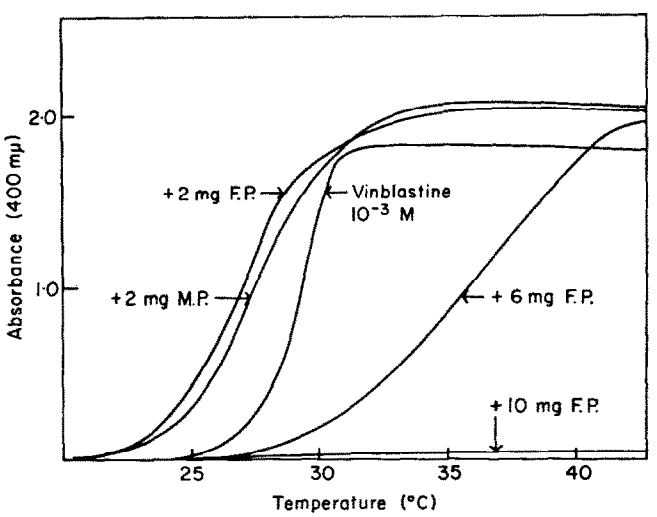

FIG. 5. Interaction of vinblastine with sperm flagella protein (F.P.) and mitotic protein (M.P.). All solutions contained vinblastine (10^{-3} M), pH 7·4, in the presence of various concentrations of F.P. (2, 6 or 10 mg/ml) or M.P. (2 mg/ml).

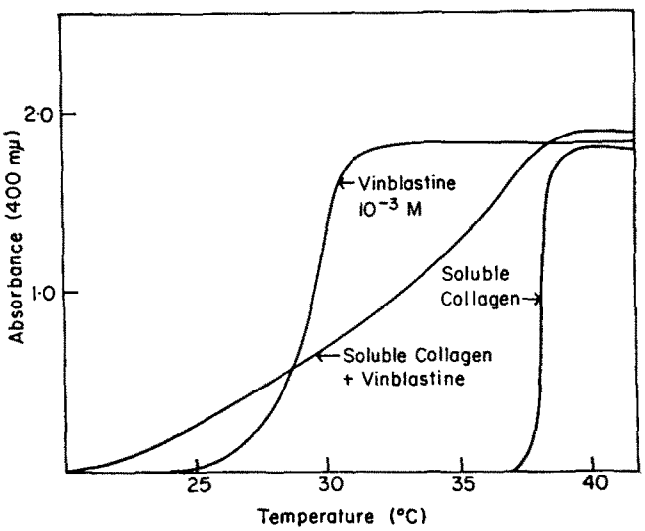

FIG. 6. Heat-induced polymerization of native neutral salt-soluble collagen at pH 7·4 (0·15 M NaCl, 0·027 M sodium phosphate) in the absence and presence of 10^{-3} M vinblastine.

DISCUSSION

The present experiments strongly suggest that the physiological effects of vinblastine are associated with its hydrophobic character and associated interactions.

This capability of vinblastine to aggregate endothermically under physiological conditions is a relatively rare property. Most of the compounds that behave in such a fashion are macromolecules which seem to require a proper balance of hydrophyllic and hydrophobic groups on their surface. It occurs with synthetic copolymers containing L-proline, α-aminoisobutyric acid and poly-ε-aminocaproyl-DL-alanine.[21] Earlier, Heyman[22] and Freundlich[23] reported that colloidal aqueous solutions of methyl cellulose showed an increase in volume of between 0·08 and 0·13 ml/100 g during gelation, an event which was favored by raising the temperature. Liquification of such a gel could be accomplished by cooling or by applying hydrostatic pressure to the system.

It is important to comment on the significance of these observations in relation to the phenomenon of self-assembly of biological microstructures, since the formation of the mitotic spindle and microtubular structures, the polymerization of G-actin to form fibrous structures, the aggregation of tropocollagen to form collagen fibers, and the assembly of the TMV protein also entail endothermic processes favored by heat and reversed by cooling.[24] The degree of association of the subunits of the mitotic spindle of various marine organisms has been monitored by measuring changes in birefringence. Using this technique, Inoué and Sato[11] were able to calculate the thermodynamic parameters involved in the monomer → polymer transition. For the *Pectinaria gouldi* they found a very low negative value for $\Delta G°$, a large enthalpy change ($\Delta H° = 83$ kcal mole^{-1}) as well as a marked increase in entropy ($\Delta S° = 286$ e.u.).[11]

Calculation of the thermodynamic parameters for the monomer to aggregate transition for vinblastine sulfate at pH 7·4 using the mathematical treatment of condensation

polymerization[25] or the van't Hoff equation following the criteria of Inoué yields values for $\Delta H°$ and $\Delta S°$ approximately twice as large as those encountered for the assembly of the mitotic spindle. Since these types of treatment have severe limitations because they can only be applied to the initial stages of the association process, accurate reliable values could not be calculated and are therefore not stated. The greater magnitude of the entropic gain seen in the vinblastine system is a direct consequence of the shorter temperature range over which the aggregation of vinblastine was completed. As is the case with hydrophobic binding, the reaction is driven by an increase in entropy which is sufficiently large to offset the endothermic nature of the association.

Amphoteric amino acids and hydrophyllic substances such as dextran and monosaccharides do not interact with vinblastine to modify its heat precipitation pattern. Various proteins tested, including bovine serum albumin, prevented this occurrence when they were present in a relatively large volume excess over that of vinblastine. We have observed that a solution of 4×10^{-4} M vinblastine (pH = 7·4) will not precipitate when heated. Therefore, removal of some of the vinblastine molecules by binding may reduce the effective concentration of the free compound. On the other hand, a local concentration within the cell by some active metabolic process or due to a gradient may cause dilute solutions to reach the concentration required for intracytoplasmic precipitation.

The co-precipitation of vinblastine with structural proteins other than those of microtubular origin has recently been reported.[26] Since these proteins were also found to precipitate with Ca^{2+}, these investigators suggested the possibility of common combining sites. Findings of a similar nature, measured by changes in the sedimentation rate, were made on the role of divalent cations in the presence and absence of mitotic inhibitors on the self-association of calf brain microtubular protein.[27]

Vinblastine has been shown to cause the aggregation of polysomes in eucaryotic cells[28] and in procaryotic organisms, such as *Escherichia coli*, where microtubules do not seem to be present.[29]

The interaction of vinblastine with SDS is quite interesting. At a 1:1 M ratio, they co-precipitate in a form which is insoluble at all temperatures studied. This may reflect the ionic interaction with charge neutralization between the protonated amino group of vinblastine and the sulfuric acid group of the detergent. The extremely hydrophobic complex formed will therefore come out of solution. Increasing the number of molecules of SDS, which then begin to interact hydrophobically with the vinblastine–SDS complex, successively increases the stability of the micellar structure and progressively retards its heat aggregation. A 5:1 M excess of SDS completely prevents heat precipitation.

It has recently been shown that colchicine's activity is significantly decreased in the presence of a tris buffer, tris(hydroxymethyl) amino ethane.[30] Tris-colchicine crystals were isolated which were markedly soluble in water but were insoluble in benzene, thus reflecting the significance of solubility in organic solvents as a criteria for antimitotic activity.

The experiments discussed, particularly the endothermic aggregation, the favorable partition towards octanol, and the ability to interact with sodium dodecyl sulfate in such a specific fashion, reflect the great tendency of vinblastine to participate in hydrophobic bonding. Since the transfer of hydrophobic residues from water to a

nonpolar environment is an endothermic reaction, such processes are favored by an increase in temperature.[31–34]

It is of interest that all mitotic inhibitors are endowed with a similar lipophilic tendency, including the recently described cytochalasins,[35,36] biologically active mould metabolites, which reversibly inhibit the contractile processes of many cells.

Because the assembly and the dynamics of subunits involved in the formation of macromolecular structures seem to be so dependent on hydrophobic interactions, further investigation of compounds which exhibit physico-chemical properties similar to those of vinblastine, with varied steric and solubility characteristics, offers a promising approach toward further regulating and understanding these fundamental cellular processes.

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