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SPECIFIC ALTERATIONS IN THE BIOLOGICAL ACTIVITIES OF C-20′-MODIFIED VINBLASTINE CONGENERS*

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Abstract—Both the anti-tumor and toxic activities of the vinca alkaloid dimers, vinblastine (VBL) and vincristine (VCR), may reside at the level of their known cellular target, the microtubule system. The contributions made by each of the various actions of these alkaloids on this system are unknown. We have used new, complete synthetic methodologies to create a series of eight C-20′ alkyl congeners of VBL and have examined these compounds for their abilities to (1) inhibit microtubule assembly, (2) disassemble preformed microtubules, and (3) induce spiral aggregate formation, using purified brain microtubule protein. By combining turbidimetric and electron microscopic techniques, we discovered that *each* of the various effects of VBL on the microtubule system *in vitro* was amenable to alteration by specific modification at this single molecular site. In addition, we report two new aberrations of VBL action—the induction of spirals by a concentration of congener below 1 μM and the formation of "opened" microtubules polymerized in the presence of congener. The relationship between anti-microtubule action *in vitro* and the cellular activities of growth inhibition and mitotic arrest by the congeners was examined in leukemic and colon cancer cell lines. In general, we found that both cellular perturbations were correlated to the ability of the congeners to inhibit microtubule polymerization rather than to the actions of spiral formation or microtubule disassembly. These results are a breakthrough in the structure/function relationship of the vinca alkaloid dimers and should provide the means to determine the role of specific anti-microtubule activities to the complex biological actions of these natural product drugs.

The indole-indoline dimeric vinca alkaloids, vinblastine (VBL) and vincristine (VCR), are cancer chemotherapeutic agents of proven efficacy against hematologic cancers and certain embryonal tumors such as neuroblastoma and rhabdomyosarcoma [1]. New drug development to overcome the refractory nature of most epithelial cancers to therapy with vinca dimer alkaloids will require an understanding of the drug's mechanism of action in sensitive cell types and the pharmacologic factors that modulate that activity. One apparent therapeutic limitation of many carcinomas is the relatively small proliferative cell fraction in the tumor. VBL and VCR are known spindle poisons, and this anti-mitotic activity can be reasonably evoked as the mechanism for their efficacy against leukemia, lymphomas and other rapidly growing cancers. Indeed, vinca dimer cytotoxicity in various cultured tumor cell lines is correlated to the cell cycle arrest, aberrant cell division, and resulting cell necrosis [2–5]. Also, patients who receive drug therapy accumulate mitotic figures in their tumors [6–9]. However, non-growing tissues are not protected *a priori* from vinca dimer-induced toxicity as neuropathy is a significant obstacle to therapy, being dose-limiting for vincristine [10]. In animals [11, 12] and patients [13] exhibiting neuropathy, there is severe neuronal pathology in various CNS structures including neurotubule dissolution and tubulin paracrystal formation. Although the functional significance of these findings to the observed peripheral neuropathy induced by the drugs is not clear, the fact remains that VBL and VCR can elicit profound damage to cells *both* proliferating and quiescent, and this action appears to be directed at the *same* cellular target. This property is unusual (unique) for cancer chemotherapeutic agents and reflects the non-DNA directed cellular target of these compounds. This fact has profound implications for new drug development, also. Theoretically, manipulation of the anti-microtubule activities of VBL/VCR could eliminate neurotoxicity, retain or enhance anti-tumor activity, and perhaps permit targeting of slow (non-) growing cell populations of carcinomas through suitable perturbation of the microtubule integrity required for motility and secretion. To accomplish these goals, the contribution of each of the various, known, anti-microtubule actions of the vinca drugs [for review see Ref. 14]—inhibition of microtubule assembly, disassembly of preformed microtubules and intracellular paracrystal formation—to cytotoxicity and therapeutic efficacy must be understood.

Over the past several years, we have developed

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new complete synthetic methodologies for the vinca alkaloid dimers [15]. Our earlier studies with a variety of such congeners established several structural and stereochemical constraints for biological activity and reported the first modification of activity against microtubules *in vitro* by structural alteration at the C-20′ position of the cleavamine (upper) moiety of VBL [16].

We have synthesized a series of eight new C-20′ alkyl congeners of VBL and have examined their biological activities with purified microtubule protein *in vitro* and with cultured leukemic and colon cancer cell lines. Our results disclosed unprecedented alterations in the various anti-microtubule activities of the parent molecule by such modifications at this single molecular locus. Preliminary structure/function relationships for these properties and cell growth perturbation are discussed. A preliminary report of these findings has been made [17].

MATERIALS AND METHODS

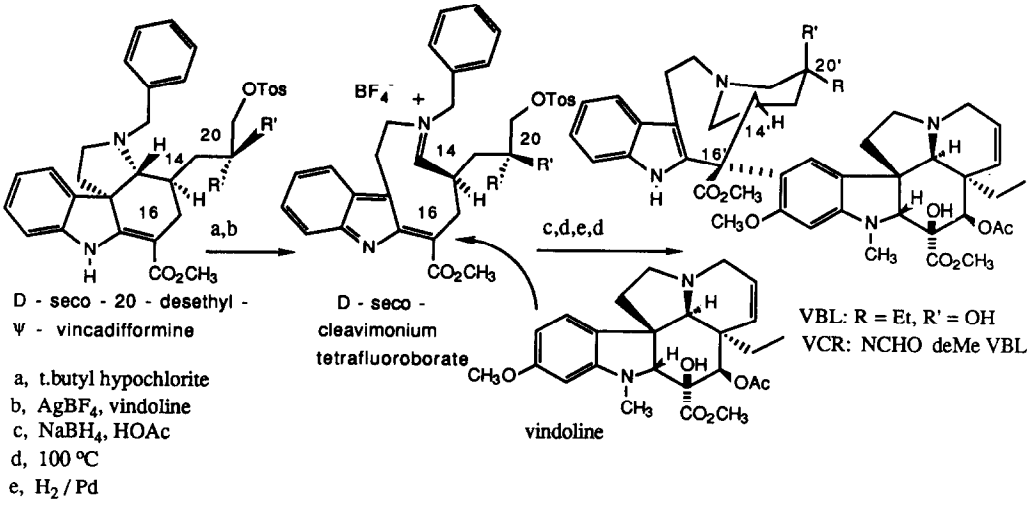
Vinblastine congener synthesis. We have developed new methodologies for the complete practical syntheses of the indole-indoline vinca alkaloids based most effectively on the key stereoselective coupling reactions shown below [see also Ref. 15].

Congeners were prepared as their methanesulfonate salts and were dissolved in 0.9% NaCl just prior to use, with the exception of the free base of 20′-desethyl VBL and its epimer, which were dissolved in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide in the reaction mixture at 10^{-4} M congener was 1%.

Turbidimetric assay. Microtubule protein (MTP) was purified from 100 g of bovine calf brain by two cycles of depolymerization/polymerization as described by Shelanski *et al.* [18]. The final pellet was resuspended in 4 M glycerol in assembly buffer [0.1 M morpholinoethanesulfonate (MES), 1.0 mM ethylene glycol-bis(aminoethyl ether)tetraacetate (EGTA) and 0.5 mM $MgCl_2$, pH 6.4] to a protein concentration of 10–15 mg/ml and stored at −70°. We monitored the effects of VBL and congeners on MTP and assembly into microtubules by the light-scattering procedure of Gaskin *et al.* [19]. MTP (1.0 to 1.2 mg/ml) was exposed to 10^{-7} M–10^{-5} M VBL or congener, and assembly was initiated by the addition of 1 mM GTP and warming to 37° in a recording spectrophotometer (Beckman Instruments) at 350 nm. The IC_{50} for assembly inhibition was determined from plots of the absorbance reached at steady-state with each concentration of compound. The self-association of MTP into spiral aggregates and the disassembly of steady-state microtubules of MTP by a high concentration (10^{-4} M) of VBL or congener were followed also by turbidimetric measurements. It should be noted that the proportionality between turbidity and total polymer mass applies only to rod-like structures (microtubules) under these conditions [19]. Therefore, the degree of light scatter caused by polymers of other shapes may not be comparable directly. All of the congeners presented in this report were examined for turbidimetric profile and polymeric products (see below) in at least two separate MTP preparations with consistent results.

Transmission electron microscopy. An aliquot of each mixture from the turbidimetric assay at the steady-state condition was placed on duplicate Formvar-coated copper grids and processed as described previously [20]. After 1 min, the excess fluid was withdrawn with a piece of filter paper (Whatman No. 1), and 1 mg/ml cytochrome *c* in water was placed on the sample for an additional minute and wicked again. The specimen was rinsed with water and post-stained with 1% uranyl acetate in water for 1.5 min. The grids were air-dried. The specimens were examined using a Philips 201 EM with a cold finger operated at 60 or 80 kV.

Cellular assays. Mouse L1210 leukemia cells were maintained in McCoy's 5A medium (K-C Biologicals), pH 7.2, supplemented with 5% heat-inactivated (56°, 20 min) horse serum (Hazelton Research Products). The population doubling time was 11 hr. The IC_{50} for cell growth was calculated



Key Steps for Syntheses of VBL and its Analogues

from data of cultures enumerated 2 days after seeding 2×10^4 cells/ml from mid-exponential populations in the absence of drug or in the presence of various concentrations of each congener. The mitotic index of L1210 cells was measured following a 6-hr exposure to a growth-arresting concentration of congener (10-fold the IC_{70}). These cells were pelleted, resuspended in 0.075 M KCl, and incubated at 37° for 13 min. After centrifugation, the cell pellets were fixed in fresh 3 : 1 methanol : acetic acid, washed twice with fixative, and then dropped onto wet glass slides. Samples were stained with 2% Giemsa in water for 4 min. At least 400 nuclei were examined for each determination and expressed as the number of metaphase nuclei divided by the total number of nuclei × 100.

The rat colon adenocarcinoma cell line RCC-2 [21] was maintained in monolayer cultures with Ham's F12 medium (Hazelton Research Products) at pH 7.4 and supplemented with 5% heat-inactivated fetal bovine serum (Hyclone Defined Sterile Systems). The IC_{50} of each congener for cell growth and the mitotic index of treated cells were determined as described for the L1210 leukemia cell line with an additional protease treatment (0.25% trypsin/0.02% EDTA) for removal of the cells from the substratum. As the population doubling of the RCC-2 cell line is 22 hr, we incubated cultures for 3 days for the growth studies and for 19–21 hr for the mitotic indices.

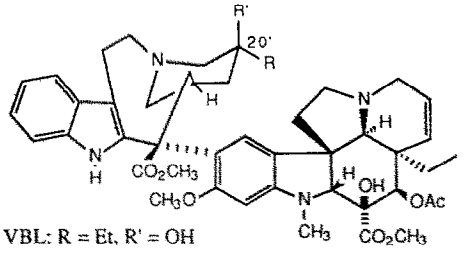
RESULTS

The series of epimeric alkyl modifications at the C-20′ position of vinblastine that were synthesized by us and evaluated in this report are presented in Fig. 1. Most of these congeners are at a lower oxidation level, lacking the OH group at the C-20′ position.

Activity of congeners with MTP. The compounds were examined for their abilities to elicit the two activities typical for the parent compound VBL with purified microtubule protein (MTP): (1) the inhibition of MTP assembly into microtubules at low VBL concentrations (10^{-7} M–10^{-6} M) and (2) the induction of spiral aggregates from soluble MTP at a high concentration (10^{-4} M) of VBL (see Figs 2A and 3A, 3B).

Of the eight C-20′ alkyl congeners examined by turbidimetric analysis, only the two methyl-substituted compounds, **1** and **2**, displayed a concentration-dependent reaction with MTP that was similar to VBL (Fig. 2A). In addition to shortened microtubules at the IC_{50} of 2×10^{-7} M, each congener also elicited *spirals* from MTP that were seen attached to microtubules or free in solution (Fig. 3, C and D). This is the first report of spiral formation by a submicromolar concentration of a vinca alkaloid dimer and MTP/tubulin *in vitro*. Both of these congeners induced spiral aggregates at a 10^{-4} M concentration when added to MTP (data not shown).

Of the six remaining compounds, only one, epidesethyl VBL, **6**, had no detectable effect on MTP assembly over the concentration range of 10^{-7} M–10^{-4} M (data not shown). The other alkyl congeners displayed an approximate 10-fold increase in the IC_{50} for tubulin polymerization and distinctive levels of turbidity at higher concentrations with soluble MTP (Fig. 2B). At the approximate IC_{50} of 2×10^{-6} M, the congeners permitted the formation of short but structurally normal microtubules from MTP (data not shown) in all cases except one. Some of the shortened microtubules formed from MTP incubated with 2×10^{-6} M deoxy-desethyl VBL, **3**, were characterized by the presence of periodic lucent areas along the length of the tubule which in some cases appeared to open fully to reveal the protofilament content of the microtubule (Fig. 3E). This structural phenomenon is a new observation for MTP (tubulin) polymerized in the presence of vinca alkaloid dimers or any other drug.



VBL: R = Et, R' = OH

	R'	R
1. 20'-deoxy-20'-desethyl-20'-α-methyl VBL	H	CH_3
2. 20'-deoxy-20'-desethyl-20'-β-methyl VBL	CH_3	H
3. 20'-deoxy-20'-desethyl VBL	H	H
4. 20'-deoxy-20'-desethyl-20'-dimethyl VBL	CH_3	CH_3
5. 20'-desethyl VBL	OH	H
6. 20'-*epi*-20'-desethyl VBL	H	OH
7. 20'-deoxy-20'-desethyl-20'-α-*n*. propyl VBL	H	C_3H_7
8. 20'-deoxy-20'-desethyl-20'-β-*n*. propyl VBL	C_3H_7	H

Fig. 1. Parent molecule vinblastine and the C-20′ congeners examined in this study.

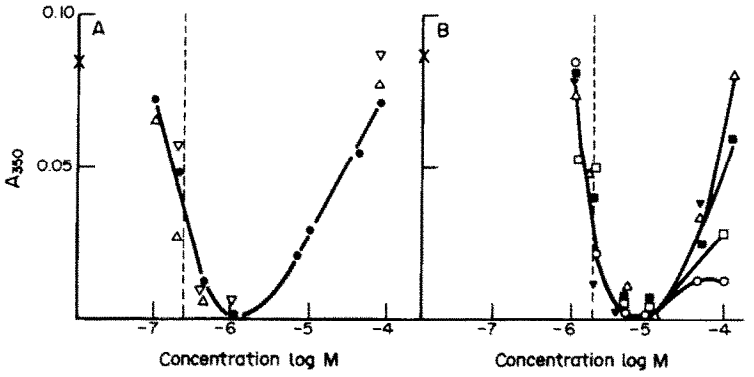


Fig. 2. Dependence of MTP turbidity at the steady-state condition on the concentration of congeners. (A) VBL (●); and methyl derivatives, **1** (△) and **2** (▽); (B) deoxy-desethyl VBL, **3** (○); dimethyl-derivative, **4** (△); desethyl VBL, **5** (□); and propyl derivatives, **7** (▼) and **8** (■). Each compound was added to MTP (8–10 μM) in 1.0 ml assembly buffer (0.1 M MES, 1.0 mM EGTA and 0.5 mM $MgCl_2$, pH 6.4), mixed well, and then 1.0 mM GTP was added prior to incubation at 37°. Turbidity was followed by absorbance at 350 nm. In the absence of VBL or congeners, the steady-state level was 0.075 to 0.085 units (×). Values are from a single, typical experiment for each compound.

The congener-specific turbidity of MTP solutions incubated with a 10^{-4} M concentration of the congeners of Fig. 2B correlated with distinctive polymeric products. The dimethyl derivative of VBL, **4**, induced the greatest degree of turbidity with MTP and formed spiral aggregates similar to those produced with VBL (data not shown). In contrast, the lower turbidity levels of MTP solutions containing desethyl VBL, **5**, and the propyl-substituted compounds, **7** and **8**, were due solely to amorphous aggregates (see Fig. 4A). Deoxy-desethyl VBL, **3**, promoted a minimal increase in the turbidity of MTP which resulted from numerous normal-appearing spirals that failed to associate into spiral *aggregates* (Fig. 4B). This property is unique among the congeners examined by us and represents the first such modulation of VBL action through structural alteration of the molecule. Others have reported that high concentrations of VCR produced amorphous, globular material rather than spiral aggregates from microtubules polymerized from purified tubulin in the *absence* of microtubule-associated proteins [22]. This MAP-dependency for spiral aggregate formation was found to reside with the tau proteins, whereas MAP-2 protein permitted the formation of circles, semicircles and single spirals of irregular appearance [23].

Activity of the congeners with preformed microtubules. VBL at 10^{-4} M caused a time-dependent decline in the turbidity of steady-state microtubules (Fig. 5) that was due to the disassembly of microtubules and spiral aggregate formation (data not shown). Three congeners, deoxy-desethyl VBL, **3**, and both of the methyl-substituted congeners, **1** and **2**, exhibited similar turbidimetric profiles (Fig. 5) that were associated also with spiral aggregate formation (data not shown). However, desethyl VBL, **5**, the dimethyl-substituted compound, **4**, and a propyl-derivative of VBL, **7**, caused a transient increase in mixture turbidity followed by a slight decline in optical density (Fig. 5). The precipitation of congeners in the absence of MTP was observed only for the propyl derivative, and this modest value (0.02 units) was subtracted from those plotted in Fig. 5. When the reaction products were examined 10 min after the addition of these congeners, we observed intact microtubules and some amorphous aggregates in all three cases. The microtubules in mixtures containing desethyl VBL, **5**, appeared normal in structure (data not shown). However, the microtubules remaining after the addition of the dimethyl or propyl derivatives of VBL displayed variable tubule widths and staining properties (Fig. 4C).

A summary of the low and high concentration-dependent effects of our C-20′ alkyl congeners with MTP and microtubules *in vitro* is contained in Table 1. Because most of the alkyl congeners lack the OH group at the C-20′ position as well, the properties of C-20′-deoxy VBL and its epimer were examined and found to be indistinguishable from the parental activity profile.

Activities of the congeners in cultured cells. The activities of the various C-20′ alkyl congeners in the cellular assays of growth inhibition and mitotic arrest are presented in Table 2. Only the two methyl derivatives of deoxy-desethyl VBL, **1** and **2**, which exhibited a profile of activity and product formation at low and high concentrations similar to VBL, were potent inhibitors of leukemic and colon cancer cell growth *in vitro*. All of the other alkyl congeners, characterized by a 10-fold reduction in potency as inhibitors of MTP polymerization and by diverse high concentration effects on MTP and microtubules, were significantly less cytotoxic to both leukemic and colon cancer cells than VBL. We observed that the rat colon cancer cells were more resistant to VBL and its congeners than L1210 leukemia cells, with a rather consistent increase of 10:1 in the IC_{50} value relative to L1210 cells.

The assay of mitotic index was carried out with equipotent levels of each congener at 10-fold the IC_{70}. It is apparent from the data that the induction of mitotic arrest by these vinca dimer congeners can be correlated with their abilities to inhibit microtubule assembly *in vitro* but not to either or both of the high concentration-dependent actions of microtubule disassembly and induction of spiral

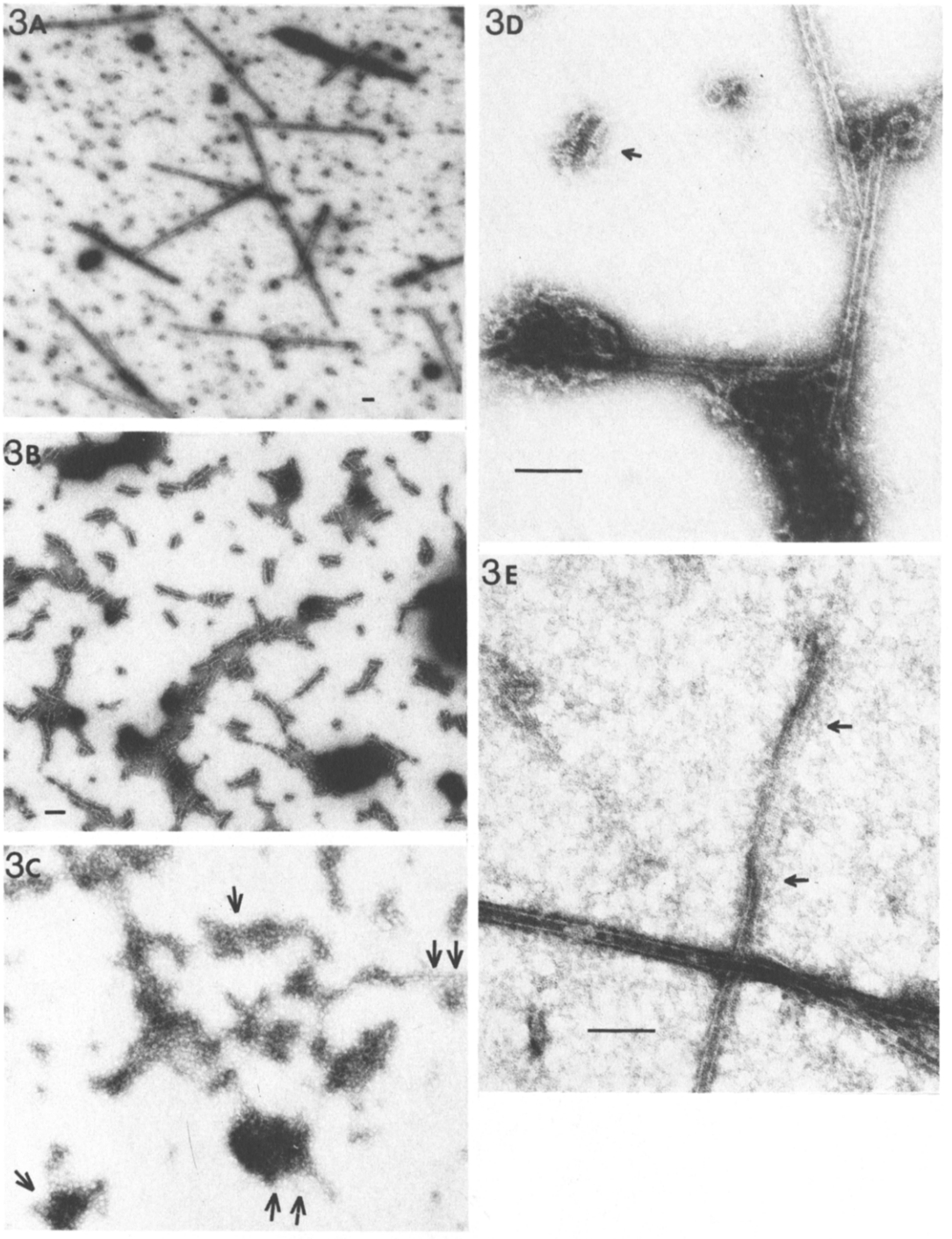


Fig. 3. Transmission electron microscopy of MTP reaction mixtures incubated as described in the legend of Fig. 2. (A) The microtubules formed in the presence of the IC_{50} of VBL (2×10^{-7} M) were structurally normal but shorter (1–3 μm) than the 8–10 μm length typical for control (drug-free) mixtures. (B) The addition of 10^{-4} M VBL to MTP resulted in the formation of numerous aggregates containing protofilament spirals with a diameter of approximately 350 nm. Microtubules were not observed. (C) MTP incubated with the IC_{50} (2×10^{-7} M) of methyl-deoxy-desethyl VBL, **2**, formed spiral aggregates both free in solution (single arrows) and associated with microtubules (double arrows). (D) A higher magnification picture of MTP incubated with the IC_{50} (2×10^{-7} M) of methyl-deoxy-desethyl VBL, **1**, displays a free spiral (arrow) and spiralized material on the microtubules. (E) An opened area (denoted by arrows) is seen in a microtubule polymerized in the presence of the IC_{50} (3×10^{-6} M) of deoxy-desethyl VBL, **3**. Bar = 0.1 μm.

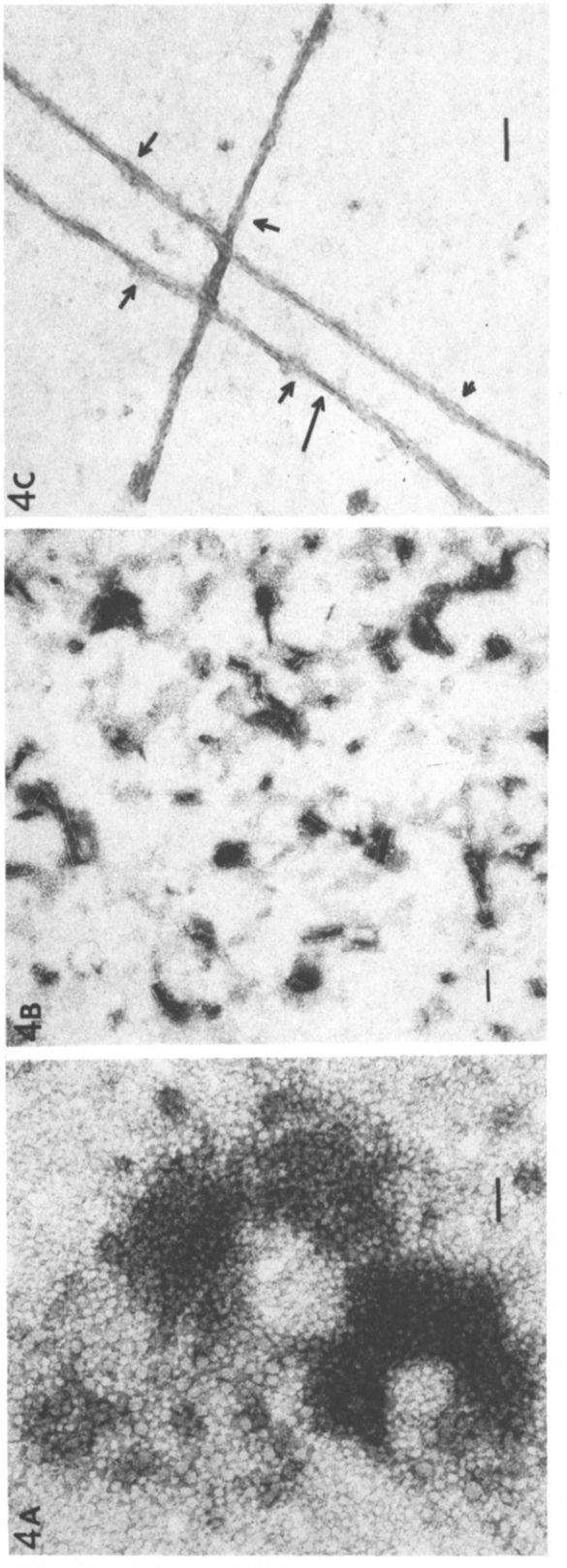


Fig. 4. Ultrastructure of MTP or microtubules exposed to a high concentration of the congeners. (A) MTP incubated in the presence of the propyl derivative, **7**, at 10^{-4} M induced amorphous aggregate formation. (B) Single spirals were formed from MTP and 10^{-4} M deoxy-desethyl VBL, **3**. (C) The addition of 10^{-4} M propyl derivative, **8**, to steady-state microtubules failed to disassemble the microtubules but resulted in variable tubule widths and staining regions (short arrows). A normal staining region is denoted by the long arrow. Bar = 0.1 μm.

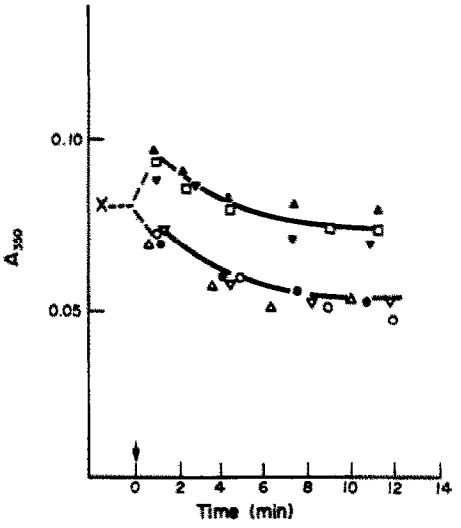


Fig. 5. Time course of mixture turbidity upon the addition of VBL or congener to steady-state microtubules. Key: VBL (●); methyl derivatives, **1** (△) and **2** (▽); deoxy-desethyl VBL, **3** (○); dimethyl derivative, **4** (▲); desethyl VBL, **5** (□); propyl derivative, **7** (▼). MTP (8–10 μM) was assembled into microtubules as described in the legend of Fig. 2. At the time indicated (arrow) a 10^{-4} M concentration of each compound was added to the reaction mixture, and the absorbance at 350 nm was followed. Data are plotted from a single, typical experiment for each compound.

aggregates. A possible exception to this conclusion was epi-desethyl VBL, **6**, which did not inhibit MTP assembly. However, the low mitotic index of this congener compared to the other congeners and the exceedingly high concentration used for the mitotic index determination (7×10^{-5} M) may reflect a general perturbation (delay) of all phases of the cell cycle rather than specific mitotic phase arrest.

DISCUSSION

The vinca alkaloid indole-indoline dimers, VBL and VCR, perturb microtubule structures that are dynamic (mitotic spindle) or stable (neurotubules). In addition, these drugs induce the formation of a unique biophysical entity, the tubulin paracrystal [24]. Investigations into the mechanistic bases for these various activities of these two vinca alkaloids have concentrated predominantly on the target protein, as the dimer subunit tubulin, or as microtubules. The results of such work often have been equivocal with varied binding stoichiometries and affinity constants for the tubulin dimer [25–27] that have been attributed to differences in purity, age or source of the tubulin. However, a recent treatment of the multiple equilibria of the ligand (VBL)-induced self-association of tubulin has disputed the findings of the previous literature and, based on thermodynamic considerations, has proposed the existence of a

Table 1. Tubulin/microtubule activities of C-20′ vinblastine congeners

Compound	MTP polymerization IC_{50} (M) ($\times 10^7$)	Product of 10^{-4} M compound added to MTP	Product of 10^{-4} M compound added to Microtubules
Vinblastine (VBL)	2.0, 2.4	SA	SA
Deoxy VBL (R′-H)	2.3, 2.7	SA	SA
Epi-deoxy VBL (R-H)	3.5, 5.5	SA	SA
Alkyl congeners			
Methyl deoxy-desethyl VBL (R-methyl)	3.2, 1.5 [S]	SA	SA
Epi-methyl deoxy-desethyl VBL (R′-methyl)	3.2, 2.8 [S]	SA	SA
Deoxy-desethyl VBL	26, 25	S	SA
Dimethyl deoxy-desethyl VBL (R = R′-methyl)	29, 36	SA	MT
Desethyl VBL (R′-OH)	32, 30	Am	MT
Epi-desethyl VBL (R-OH)	No effect	MT	MT
Propyl deoxy-desethyl VBL (R-propyl)	25, 17	Am	N.D.
Epi-propyl deoxy-desethyl VBL (R′-propyl)	25, 21	Am	MT

Microtubule protein (MTP) was purified from bovine calf brain by two cycles of depolymerization and polymerization [18]. The effect of low concentrations (10^{-7} M–10^{-5} M) of the vinca dimer alkaloids on the polymerization of MTP (8–10 μM) in assembly buffer (0.1 M MES, 1 mM EGTA 0.5 mM $MgCl_2$ and 1.0 mM GTP, pH 6.4) was measured by turbidimetric assay [17], and the IC_{50} was calculated from the steady-state turbidity levels obtained at each concentration. Values are from two separate experiments. The products induced by a high concentration (10^{-4} M) of each compound with soluble MTP or microtubules assembled from MTP in the same buffer were determined by transmission electron microscopy of steady-state solutions from at least two separate preparations of MTP with similar results. Congeners were used as their methanesulfonate salts and dissolved in 0.9% NaCl just prior to use with the exception of the free base of 20′-desethyl VBL and its epimer which were dissolved in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide at 10^{-4} M congener was 1% in the reaction mixture. Abbreviations: SA, spiral aggregates; S, single spirals: Am, amorphous aggregate; MT, microtubules; and N.D., not done.

Table 2. Activities of C-20′-vinblastine congeners with cultured cells

Compound	Cell growth inhibition* IC_{50} M ($\times 10^9$) L1210	RCC-2	Mitotic index† % L1210	RCC-2
None			3.0	1.0
Vinblastine (VBL)	0.49 ± 0.2	4.84 ± 2.0	29.9	65.9
Deoxy VBL (R′-H)	2.77 ± 0.9	34.0 ± 5.0	23.2	71.3
Epi-deoxy VBL (R-H)	2.9‡	36.0‡	25.9	N.D.
C-20′ alkyl congeners				
Methyl deoxy-desethyl VBL (R-methyl)	7.5 ± 2.2	36.0‡	28.7	N.D.
Epi-methyl deoxy-desethyl VBL (R′-methyl)	3.1 ± 1.5	48.0‡	20.1	N.D.
Deoxy-desethyl VBL	104 ± 1.1	593 ± 83	24.0	70.5
Dimethyl deoxy-desethyl VBL (R = R′-methyl)	26.0 ± 5.2	716 ± 164	28.9	75.9
Desethyl VBL (R′-OH)	1150 ± 340	1000	26.2	N.D.
Epi-desethyl VBL (R-OH)	6800‡	1000	14.3	N.D.
Propyl deoxy-desethyl VBL (R-propyl)	50.0‡	580‡	19.0	N.D.
Epi-propyl deoxy-desethyl VBL (R′-propyl)	80.5 ± 1.5	630‡	20.0	N.D.

Murine leukemia cell line L1210 was maintained in McCoy's 5A medium supplemented with 5% heat-inactivated horse serum with a population doubling time of 11 hr. Rat colon adenocarcinoma cell line RCC-2 was maintained in Ham's F12 medium supplemented with 5% heat-inactivated fetal bovine serum with a population doubling time of 23 hr. Stock cultures in the midexponential phase of population growth were used to seed all experimental cultures.

* Duplicate samples of 1×10^4 cells/ml (L1210 cells) or 2×10^4 cells/well of a 24-well cluster dish (RCC-2 cells) received various concentrations of VBL or congener, and 48 hr (L1210 cells) or 72 hr (RCC-2 cells) later the cell population number was enumerated by an electronic particle counter, Coulter Counter model ZF. The IC_{50} was determined from probability plots of concentration versus percent control cell number; values (mean ± SD) were averaged from at least three separate experiments except where noted.

† The mitotic index of the populations was measured after a 6-hr (L1210 cells) or a 21-hr (RCC-2 cells) incubation with VBL or congener at a concentration of 10-fold the IC_{70} value. The L1210 cell suspension or trypsinized RCC-2 cells (and washes) were centrifuged at 175 *g*, 4°, for 5 min, and the pellets were resuspended in 0.75 M KCl prior to incubation for 13 min at 37°. After centrifugation, the cell pellet was resuspended in fresh fixative of 3:1 methanol:acetic acid, washed twice in the fixative, and then dropped onto wet glass slides. The specimens were stained with 2% Giemsa in water for 4 min and then scored. At least 400 nuclei were counted and the percent mitotic index was calculated as the number of metaphases divided by the total number of nuclei counted × 100. Values are from a single, typical experiment.

‡ Denotes a value averaged from the duplicate samples of a single experiment.

single, specific VBL binding site per tubulin dimer with an intrinsic binding affinity $K_I = 4 \times 10^4\ M^{-1}$ [28, 29].

Our work, from the vantage point of the *molecule* itself, is compatible with the latter conclusion based on the fact that subtle, single-site alterations of VBL can affect *all* three activities typical for the parent molecule. However, the selective nature of these effects extends the hypothesis to include the notion that the VBL binding site is modified when tubulin exists in the polymeric tubule form, because the reactions of VBL with soluble MTP and steady-state microtubules are clearly dissociated one from the other in the activity profiles of our C-20′ congeners.

Certain structure/function relationships between the anti-tubulin/microtubule actions of VBL and the nature of the C-20′ position of the cleavamine moiety, are suggested by our work. Clearly, this site on the complex dimeric vinca alkaloid molecule is function-sensitive for *all* of the typical, concentration-dependent reactions of VBL with tubulin (MTP)/microtubules *in vitro*, and the reaction modifications exhibited by the congeners comment on their complexity. First, the replacement of the ethyl substituent with a methyl group appears to *potentiate* markedly the reactivity of the molecule to permit spiral formation at the low concentration of 2×10^{-7} M. We observed only a few microtubules at the steady-state condition, and these tubules were associated with spiralized material. Although this fact is consistent with a reaction process of peeling and coiling of protofilaments from microtubules to form spirals as described for VBL at high concentrations (10^{-5} M–10^{-4} M) [30, 31], we cannot rule out at this time the direct formation of spirals from MTP with secondary inhibition of microtubule assembly. A time–course determination of polymer ultrastructure upon addition of the methyl-substituted congeners to soluble MTP should permit us to distinguish between these possibilities. These congeners are the first VBL derivatives reported to display *enhanced* activity with MTP/microtubules *in vitro*, and they contrast markedly with the rest of our alkyl congeners which harbor various types of

impaired function. This distinction among the congeners was reiterated in their abilities to inhibit the growth of cultured tumor cells, as only the methyl derivatives of VBL exhibited activity below 10^{-8} M. We did observe a difference in cytotoxic activity between VBL and its congeners that share identical activity profiles with MTP and microtubules—deoxy VBL and epi-deoxy VBL. The 6- to 8-fold higher IC_{50} values of the congeners compared to VBL may result from impaired net transport of the C-20′ deoxy derivative. The possible effect of C-20′ modifications on vinca alkaloid dimer transport is a subject worthy of investigation.

The absence of any alkyl function at the C-20′ position, found in the congener deoxy-desethyl VBL, **3**, modified both the high and low concentration-dependent reactions of VBL with soluble MTP, namely assembly inhibition and spiral aggregate formation respectively. There was a 10-fold reduction in congener potency as an inhibitor of MTP assembly, that was associated with the presence of opened areas along the length of some microtubules at the steady-state condition. This was a unique finding for microtubules polymerized in the presence of a drug. However, similar structures have been described as intermediate polymers formed during microtubule assembly *in vitro* from the incorporation of protofilament sheets into the growing microtubule [32]. The persistence of such structures into the steady-state condition of assembly suggests aberrant closure of the protofilament sheet in the presence of this VBL congener. The coupling of this phenomenon to the inability of the spirals formed from MTP and 10^{-4} M deoxy-desethyl VBL to associate into aggregates may signal a common defect in the lateral associations of protofilaments formed in the presence of this compound, which are required for both seam closure and spiral–spiral association. The ability of this congener to induce normal spiral aggregates from preformed microtubules distinguishes its reaction with the polymer from the former reaction with soluble MTP tubulin. Because deoxy-desethyl VBL, **3**, lacks both of the groups present in VBL at the C-20′ position, we propose that this congener may bind in an altered configuration or at additional sites on the tubulin subunit to cause these aberrant reaction products with the dimer, whereas its binding to tubulin contained in microtubules is specifically analogous to VBL. This supposition is not contradictory to the conclusion reached by Jordan *et al.* [33] that the inhibition of assembly results from the binding of the drug to microtubule ends with or without a complex to tubulin. The 10-fold increase in IC_{50} for assembly inhibition with deoxy-desethyl VBL (and most of our other congeners) renders it a stoichiometric inhibitor of polymerization, unlike VBL, and permits a mechanism of direct inactivation of tubulin for assembly through congener binding. Of interest is the fact that the 10-fold increase in IC_{50} for MTP assembly inhibition by this congener was reflected in a similar reduction in activity compared to VBL as an inhibitor of murine leukemic cell growth *in vivo* (data not presented).

Finally, three different types of C-20′ congeners—desethyl VBL (**5**) and the propyl- (**7**) or dimethyl- (**4**) derivatives of VBL—exhibited a loss of the activity of microtubule disassembly at high congener concentrations. All of these compounds retained the ability to inhibit MTP polymerization and one, the dimethyl-substituted compound, elicited spiral aggregates from MTP at 10^{-4} M congener. In these cases there are clear distinctions in congener action between the target protein as monomer or as assembled microtubules.

In conclusion, we have discovered that subtle modifications of the C-20′ position of the cleavamine moiety can affect each and all of the typical concentration-dependent reactions of VBL with MTP/microtubules *in vitro*. The unique profiles of most of our C-20′ alkyl congeners distinguish each of the reactions at the mechanistic level and establish the dissociation of vinca dimeric alkaloid action at this cellular target through structural alteration of the ligand. This accomplishment was made possible by our newly developed complete synthetic methodologies for generation of the indole-indoline vinca dimers [15]. Of the numerous semisynthetic derivatives of VBL made and evaluated over the last 25 years, most have behaved similarly to the parent molecule in biochemical, cellular or *in vivo* assays, or they lacked any biological activity [see Ref. 34 for review]. Only one compound, C-16′-*t*-butylcarboxy VBL, displayed a significant modification of an MTP/microtubule activity, this being an 18-fold increase in the IC_{50} for MTP assembly [35]. It is our hope that further evaluation of the novel congeners described in this report and new congeners created in future synthetic efforts will break open the shell that has kept the structure/function relationship of this complex molecule in obscurity.

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