Dissociation of tubulin assembly-inhibiting and aggregation-promoting activities by a vinblastine derivative

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Abstract A fluorescent vinblastine analogue, vinblastine-4′-anthranilate (Antvin), that binds to the vinca site on tubulin, inhibits tubulin assembly but does not lead to spiral or other large aggregate formation at concentrations up to 1.6 mM. As judged by turbidity, 90° light scattering and fluorescence anisotropy, little aggregation could be detected. This is in marked contrast to vinblastine and suggests that inhibition of assembly and aggregate formation can be dissociated from each other by suitable substitution in vinblastine.

Key words: Vinblastine; Vinblastine-4′-anthranilate; Tubulin assembly inhibition; Spiral aggregate

1. Introduction

It is well known that low concentrations of the vinca alkaloids, vinblastine and vincristine, reduce microtubule dynamics, inhibit polymerization at intermediate concentrations, and lead to the formation of spirals, paracrystals and other aggregates at high concentrations [1–3]. Attempts to ascribe these opposing effects to binding at different sites have been rejected on the basis of linkage between binding and self-association of tubulin, yielding a thermodynamic cycle that requires only a single vinblastine binding site [4,5]. Indeed, only one high affinity binding site is readily demonstrable by photocrosslinking with a fluorescent vinblastine derivative, vinblastine-4′-anthranilate (AntVin) [6]. This derivative inhibits tubulin polymerization with an IC_{50} of 4.8 μM, shows an apparent binding constant of ~40 μM, is competitively displaced by vinblastine or maytansine, and is covalently attached to residues 172–213 of β-tubulin upon irradiation at the anthranilate absorption. Because of potential linkage between binding and self-association, it was important to ascertain whether tubulin self-association, as caused by vinblastine, would complicate interpretation of AntVin binding [4,5]. To our surprise a large molar excess of AntVin failed to promote the formation of tubulin aggregates as measured by turbidity. Because this derivative might offer clues to the relation between the polymerization-inhibiting and aggregation-promoting effects of the vinca alkaloids, we present here details of the difference in the interaction between Antvin and tubulin on the one hand, and vinblastine and tubulin on the other.

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Abbreviations: AntVin, vinblastine-4′-anthranilate; MES, 2-(*N*-morpholino) ethanesulfonic acid; EGTA, ethyleneglycol-bis-(β-aminoethyl ether) *N*,*N*′-tetraacetic acid; DMSO, dimethyl sulfoxide

2. Materials and methods

Vinblastine-4′-anthranilate (AntVin) was synthesized from vinblastine and isatoic anhydride as previously described Fig. 1 [6]. Pure rat brain tubulin was prepared from microtubule protein as described [7,8]. Tubulin was labeled with isatoic anhydride at pH 6.9 and a molar ratio of 1/1 according to [9]. Assembly and aggregation reactions were carried out at 37°C in 3 mm light path cuvettes with a working volume of 100 μl in a thermostatted Cary 219 spectrophotometer. Studies were carried out with 1.96–2.24 mg/ml of pure rat brain tubulin in MES buffer (0.1 M MES pH 6.9, 1 mM EGTA) containing 1 mM GTP and 10% dimethyl sulfoxide. Total $MgCl_2$ concentration was either 1.0 or 6.0 mM. Turbidity was measured at 400 nm (slit width = 1 nm) to avoid absorption by AntVin. Light scattering at 90° was determined in a Perkin Elmer MPF66 spectrofluorometer operating in ratio mode (uncorrected, with slits at 5 nm each) at room temperature in MES buffer using cuvettes with a 3 mm light path. To avoid emission from AntVin excitation was at 450 nm. Steady state fluorescence anisotropies were measured at room temperature in MES buffer (no DMSO) with film polarizers inserted into the MPF-66 fluorimeter. Excitation was at 330 nm and emission was measured at 440 nm.

3. Results

A comparison between normal microtubule assembly and vinca alkaloid-induced aggregate formation as a function of time is depicted in Fig. 2. Because of the absorption of AntVin at 350 nm, all turbidity observations were made at 400 nm; as the scattering coefficient ($OD_{400} \times$ml/mg protein) is different for microtubules and spirals etc., direct comparison of the OD values cannot be made. Microtubule assembly showed the expected lag period and plateau typical of these tubulin preparations. When 35 μM vinblastine was used, there was a rapid increase in the OD_{400} after a short lag. Electron micrographs of these aggregates showed that the bulk of the aggregates were composed of single or double spirals many of which were clumped together (data not shown). In marked contrast to the effects of vinblastine, when a large concentration (1.6 mM) of AntVin was used, there was virtually no increase in the OD_{400} as a function of time for up to 30 min. This suggests that no spirals or large aggregates were formed by AntVin.

The concentration of Mg^{2+} has a profound effect on tubulin polymerization and also on the response to vinca alkaloids. The concentration dependence for VLB and AntVin of the rate of polymerization and aggregate formation is shown in Fig. 3. Both drugs readily prevent microtubule assembly at low micromolar concentrations. With 1 mM Mg^{2+} the IC_{50} values were 0.64 and 4.8 μM, respectively. After polymerization is completely inhibited, there is a region of drug concentration in which there is no change in OD_{400}; its length is a function of the Mg^{2+} concentration. At 1 mM Mg^{2+} and with 10% DMSO (solid lines), the OD_{400} does not increase again until 88 μM vinblastine is present. The greater concentration

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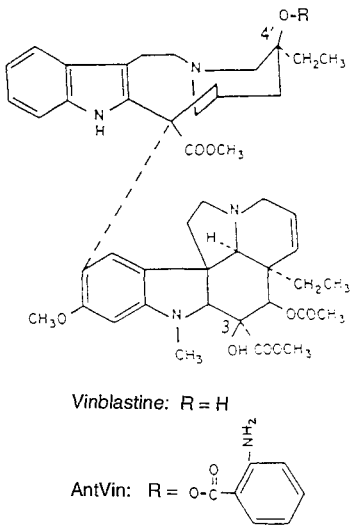

Fig. 1. The structures of vinblastine and AntVin.

of VLB is required because GTP antagonizes the aggregation effect (manuscript submitted). High concentrations of Mg^{2+} ion decrease the sensitivity to the inhibiting action of vinblastine on polymerization, changing the IC_{50} from 0.64 μM to 1.4 μM (dashed line). At the same time 6 mM Mg^{2+} increases the sensitivity to vinblastine for tubulin aggregation, shifting the curve of rising optical density toward lower concentrations (21 μM in our experiments at 6 mM $MgCl_2$ [5,9]). Thus, the zero ΔOD_{400} zone between downward and upward slopes of the rates narrows considerably as the Mg^{2+} concentrations increases. When AntVin was used to inhibit tubulin polymerization, the IC_{50} values were 4.8 μM and 11 μM resp. for 1 and 6 mM Mg^{2+} (Fig. 3). No increase in OD_{400} occurred even with an AntVin concentration of 1.6 mM. This was also true at 6 mM Mg^{2+}. The single substitution at C4′ of the carbomethoxy-velbanamine moiety of vinblastine has thus created an analogue of remarkably different properties suggesting that dissociation between inhibition of polymerization and spiral or aggregate formation can be achieved.

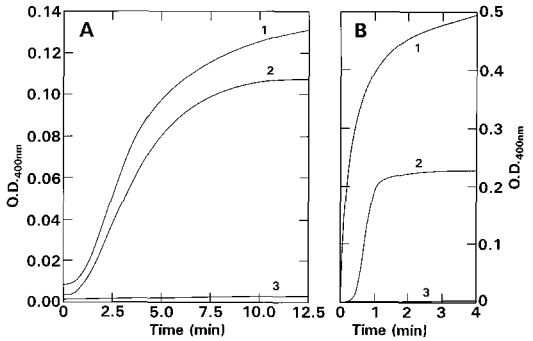

Fig. 2. Effect of AntVin and vinblastine on the polymerization of tubulin at low and high $MgCl_2$ concentrations. A: Tubulin was polymerized at 37°C in MES assembly buffer (0.1 M MES pH 6.9, 1 mM EGTA, 1 mM $MgCl_2$) containing 10% DMSO. Curve 1, 1.96 mg/ml tubulin and 35 μM VLB; curve 2, microtubule assembly with 2.24 mg/ml tubulin and 1 mM GTP; curve 3, 2.24 mg/ml tubulin and 1.6 mM AntVin. B: Polymerization as above but with 6 mM $MgCl_2$. Curve 1, 1.96 mg/ml tubulin and 15 μM VLB; curve 2, microtubule assembly with 1 mM GTP and 2.24 mg/ml tubulin; curve 3, 2.24 mg/ml tubulin and 1.6 mM AntVin. Turbidity was measured at 400 nm in cells with 0.3 mm path length.

Because optical density measurements are not very sensitive to the presence of smaller oligomers, we measured light scattering at 90° in a fluorimeter. Measurements were carried out at 450 nm to avoid emission of AntVin at lower wavelengths. Fig. 4 shows that at 25°C, with both 1 mM Mg^{2+} (left panel) and 6 mM Mg^{2+} (right panel) and 1.35 mg/ml tubulin, but in the absence of added GTP, 30 μM vinblastine produced abundant (90°) light scattering as expected, whereas 146 or 292 μM AntVin (left panel, lower two curves) produced a small increase in scattering (2.3 and 4.7% resp. of VLB scattering). At 6 mM Mg^{2+} the two AntVin curves were superimposed (right panel, lower curve) and showed 1.2% as much scattering as with VLB. Thus, under optimal conditions for spiral formation, AntVin produces very little increase in light scattering. Similar results were obtained at 37°C (data not shown).

As an additional test for the presence of oligomers we compared the fluorescence anisotropies of tubulin with those of AntVin bound to a 4–5-fold molar excess of tubulin such that the bulk of the analogue would be bound at 1 mM Mg^{2+}. Free AntVin had an anisotropy (at 330 nm) of 0.087, tubulin-bound AntVin had a value of 0.174 (at 330 nm), a value comparable to tubulin dimer labeled with anthranilate (0.182). This indicates that the AntVin was bound and that no significant amounts of larger species were present.

4. Discussion

Vinca alkaloid-induced inhibition of tubulin polymerization and the promotion of aggregate formation at higher concentrations have been linked to occupancy of a single, specific, binding site on the tubulin dimer coupled to a drug-induced self-association. Although this model does not account for high affinity binding with stoichiometries of 2 [11,12] or the extremely sensitive (< 0.1 μM) vinblastine effects upon microtubule dynamics [3], it can account for the wide spread in published affinity constants on the basis of different solvent conditions etc. and self association [1,2]. The present data suggest, however, that the linkage of vinca site occupancy and aggregation can be separated; i.e. aggregate formation

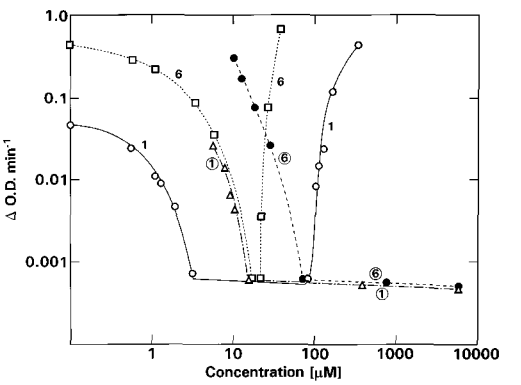

Fig. 3. Maximal rate of polymerization data (ΔOD_{400}/min) of tubulin (2.2 mg/ml) with increasing concentrations of AntVin and VLB at 1 or 6 mM $MgCl_2$ in MES buffer pH 6.9 with 10% DMSO, 1 mM EGTA and 1 mM GTP. The numbers 1 and 6 refer to the $MgCl_2$ concentrations in the presence of VLB, whereas encircled numbers are $MgCl_2$ concentrations in the presence of AntVin. Because GTP affects the response to VLB (to be published), the results of Fig. 2 cannot be directly compared with those presented here. ○, VLB+1 mM $MgCl_2$; □, VLB+6 mM $MgCl_2$; △, AntVin+1 mM $MgCl_2$; ● = AntVin+6 mM $MgCl_2$.

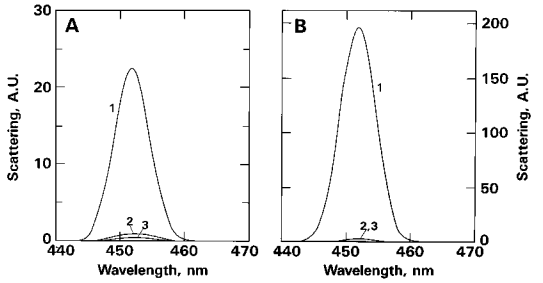

Fig. 4. 90° light scattering measurements of tubulin in the presence of either VLB or AntVin. 1.35 mg/ml tubulin in MES buffer containing (A) 1 mM $MgCl_2$ and (B) 6 mM $MgCl_2$ was measured at 25°C and 450 nm for both excitation and emission (uncorrected, low gain). All curves were corrected by subtraction of the small amount of scatter produced by tubulin alone. Curve 1, 30 μM VLB; curve 2, 292 μM AntVin; curve 3, 146 μM AntVin. The uncorrected values with 1 mM $MgCl_2$ yielded 16.6, 18.5 and 20.5% as much scatter as with VLB for tubulin alone, 146 μM and 292 μM AntVin, respectively. With 6 mM $MgCl_2$ the respective values were 3.1, 4.2 and 4.2%.

is not an obligatory consequence of site occupancy even at drug concentrations >1 mM and with high Mg^{2+}. It also suggests that the measured value of the binding constant of this derivative [6] is not compromised by significant self-association. Such dissociation can also be found for certain other congeners of vinblastine in that the ratio of the half-inhibitory concentrations and half-stimulatory concentrations for aggregate formation of some analogues shows considerable variation under otherwise identical conditions [10,13,14]. Borman and coworkers [10,14] have shown dissociation of inhibition of polymerization and spiral formation by several C4′ vinblastine alkyl derivatives (C20′ in their terminology). This suggested a mechanistic difference between low and high concentration activities for these derivatives. Thus, changes in the potency for inhibiting assembly are not reflected in the potency for spiral aggregate formation. The results with the anthranilate substitution reported here show a marked enhancement in the distinction between these two properties of VLB. Finally, the 16′-*t*-butyl-maleyl vinblastine analogue has good affinity for tubulin but does not induce spiral formation [13]. This behavior is like that of AntVin. Additional evidence that microtubule assembly inhibition and aggregation can be separated comes from studies with the macrolide, maytansine. It has a single, high affinity binding site on tubulin, is a competitive inhibitor of vinblastine binding, is a potent inhibitor of tubulin polymerization, but does not promote the formation of spirals or other aggregates [15–17]. It can, in fact, potently inhibit spiral formation by vinblastine, and displace VLB from its site. Similar results have been obtained with rhizoxin [18], another macrolide that interacts at the vinca site.

The most parsimonious conclusion, therefore, is that the two different responses of tubulin to vinca alkaloids could result from different sensitivities of the assembly and aggregation processes to the different congeners. Two different binding sites are not necessarily required, although two different loci within the single binding site would have to be postulated to remain consistent with competitive binding on the one hand, and different morphological effects on the other. Several mechanistic interpretations can be considered. Vinblastine analogues produce conformational changes in tubulin (as does colchicine) and prevent microtubule assembly, but differ from conformational changes that promote aggregation to spirals etc. Alternatively, certain substitutions on vinblastine sterically inhibit spiral formation or prevent interaction with the domains required for spiral formation, but do not prevent changes required for inhibition of assembly. Finally, a portion of the binding site leading to assembly inhibition is distinct from that portion promoting spiral formation; AntVin and maytansine occupy only the former portion of the binding site. The present data cannot distinguish between these possibilities. Nevertheless, under the appropriate conditions and with the appropriate congener mere occupancy of the vinca site does not obligate spiral formation, i.e. binding and aggregation are not necessarily linked.

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