Effect of Microtubule-associated Proteins on the Interaction of Vincristine with Microtubules and Tubulin¹

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INTRODUCTION

The Vinca alkaloids are commonly used as chemotherapeutic agents in the treatment of cancer. Although the mechanism of action of these drugs in vivo has not been clearly elucidated, it appears that the main targets are tubulin and MT.³ The different Vinca alkaloids vary widely in their effectiveness against specific tumors (11, 22) and in their toxic side effects (22), although they are quite similar in their action on brain MT in vitro and as inhibitors of the self-assembly of bovine brain tubulin (10, 17). The binding constants of the drugs to brain tubulin are also similar (12, 18, 26). This suggests that the different biological specificities of the drugs may be due to factors other than the direct interaction with tubulin.

One factor which may influence the interaction of drugs with MT in vivo is the microenvironment in which these organelles exist. For example, it is well known that a filamentous material is associated with MT in vivo (1) and with MT formed in vitro from tubulin prepared by polymerization-depolymerization methods (3, 4, 7). The filamentous material is apparently equivalent to a group of proteins known as MAP’s, which co-purify with tubulin and, under the solution conditions normally used, stimulate the formation of MT in vitro (16, 21, 24). Antibodies against MAP’s have been shown to bind to MT in vivo (2, 15, 20). Possibly, the amount or type of MAP’s influences the manner in which drugs interact with MT. Since tubulin can be assembled with or without MAP’s (9, 13), this possibility can be investigated. The subject of this paper is the difference in interaction of VCR with MAP-free and MAP-containing MT. The results show that when MAP’s are present VCR causes the MT to be converted to spirals, whereas when MAP’s are absent the MT completely disassembles.

MATERIALS AND METHODS

Bovine brain tubulin was purified by the polymerization method of Shelanski et al. (19) with some modification as described previously (14). The homogenization buffer was 20 mM 2-(N-morpholino)ethanesulfonate, 70 mM NaCl, 1 mM EGTA, and 0.5 mM MgCl₂, pH 6.4. GTP (0.5 mM) and glycerol (4 mM) were added for polymerization. The protein was purified through 2 polymerization cycles and was stored at −80°C in the buffer containing 2 mM glycerol. Tubulin lacking associated proteins (6S tubulin) was prepared by polymerizing the twice-cycled protein in 0.4 M PIPES:10% DMSO, pH 6.9, and passing the resuspended pellet through phosphocellulose (8).

MAP’s were prepared from purified tubulin by passing the protein through a phosphocellulose column at room temperature. Twice-cycled tubulin (30 to 50 mg) was first dialyzed against 20 mM PIPES, pH 6.9, for 2 hr in the cold to remove glycerol. The dialyzed protein was loaded onto a 4- x 1.2-cm phosphocellulose column at room temperature, allowed to stand 30 min, and then eluted with 20 mM PIPES, pH 6.9, followed by 20 mM PIPES:0.8 mM NaCl, pH 6.9. MAP’s are eluted with the high-salt buffer. The fractions containing MAP’s were pooled and concentrated about 2-fold by centrifugation at low speed through an Amicon membrane cone to a final concentration of 3 to 4 mg/ml. The concentrated protein was then dialyzed against 20 mM PIPES, pH 6.9, in the cold for 1.5 to 2 hr with fresh changes of buffer every 15 to 20 min. The dialyzed protein could be kept for 2 or 3 days at 4°C but gave maximal activity in the polymerization reaction if used fresh.

Assembly reaction buffer contained 0.1 M PIPES (pH 6.9), 0.5 mM MgCl₂, 1.0 mM EGTA, and 0.5 mM GTP. Tubulin and MAP concentrations are given in the figure and chart legends. The polymerization reaction was started by addition of GTP and was measured by following the increase in absorbance at 350 nm using a Gilford 2000 spectrophotometer. Samples for electron microscopy were negatively stained with 2% uranyl acetate. Ten-μl samples were applied to Formvar- and carbon-coated grids and after 10 to 15 sec were washed with the uranyl acetate solution. One min later, the grids were dried with filter paper.

VCR sulfate (Oncovin) was obtained from Lilly, Indianapolis, Ind.; PIPES, 2-(N-morpholino)ethanesulfonate, GTP, and EGTA were purchased from Sigma Chemical Co., St. Louis, Mo.
RESULTS

Action of VCR on MT Containing MAP's. The action of VCR on MT formed *in vitro* from brain tubulin has been examined previously (5, 6, 10, 23). In the studies of the effect of VCR on MAP-free MT to be described later, DMSO is present. Therefore, it is necessary to reexamine the interaction of the Vinca compound with MAP-containing MT in the presence of DMSO. When an amount of VCR approximately equal to the amount of tubulin present was added after the formation of MT (after the absorbance reached a plateau value), a small and reproducible decrease in absorbance was observed (Chart 1A). The new absorbance value remained constant up to 60 min. Samples of this solution were taken before VCR addition and at different times after VCR exposure, negatively stained, and examined with the electron microscope. Samples prior to VCR exposure showed a large number of MT, 25 nm in diameter, and usually 6 protofilaments could be identified (Fig. 1A). In samples obtained shortly after VCR addition, we observed a large number of apparently normal MT, some of which contained curved protofilaments at their ends (Fig. 1B). No spirals were seen in this preparation or in the sample taken 5 min after VCR addition, although short, curly filamentous material was observed at the microtubular extremes and at other parts of the MT. The most striking feature observed in the samples taken 15 to 20 min after VCR addition was the presence of spiral-like structures which appeared to originate from different parts of the MT. Those that originate from the ends of the MT are easily detected (Fig. 1C; see also Fig. 1D). They also appear from the middle of the MT (Fig. 1C, inset). This feature occurs less frequently and is more difficult to detect. Eighteen plates from several samples showing MT and spirals together were examined. In these, 73 of 85 observable MT ends showed spirals continuous with protofilaments. In many instances, the spiral structures seem to be formed from one protofilament (Fig. 1C, inset), which can be seen when a turn of the spiral is viewed edge-on or at the origin point. However, spirals containing 2 protofilaments are also observed. Sometimes 2 turns of the filament lay together giving the impression that 2 protofilaments form the spiral. The filament has a thickness of about 5 nm, and the spiral has a length which varies from 100 to 800 nm. The number of turns varies from 2 to 4/100 nm.

Further addition of VCR (sufficient to double the concentration) after 12 min caused a slow increase in the absorbance value (Chart 1A). Negatively stained samples from this solution showed aggregation of the spirals, forming clusters around the MT (Fig. 1D). If a higher concentration of VCR were used initially, spiral formation occurred more rapidly.

The addition of VCR to MT in the absence of DMSO produced a drop in the absorbance greater than that seen in the presence of DMSO (Chart 1B). A constant value was reached after 15 min. Negatively stained samples obtained immediately after VCR addition showed a large number of spirals and few MT. Addition of a second dose of VCR did not affect the absorbance value, but if DMSO was added the absorbance increased rapidly. Negatively stained samples of this solution showed an extreme aggregation of the spirals and few MT. The structures of MT and spirals in this experiment were similar to that described above, with the exception that fewer MT and a larger number of spirals were observed.

Action of VCR on MT Lacking MAP's. The effects of VCR on the self-assembly of tubulin and on MT formed *in vitro* from brain tubulin in the absence of MAP's but in the presence of DMSO were studied. The self-assembly of 6S tubulin was inhibited by low concentrations of VCR. In these experiments, 6S tubulin was preincubated with different concentrations of VCR for varied periods (1 to 10 min) before polymerization was initiated by the addition of GTP. Using 15 μM tubulin, it was found that 0.9 μM VCR completely inhibited the formation of MT. At 0.45 μM VCR, an inhibition of approximately 50% was observed. The action of VCR was not affected by the time of preincubation. These results, which have been termed "stoichiometric poisoning," have been observed previously using MAP-containing tubulin (25). Ultra centrifugal analysis of samples treated with such low levels of VCR showed a spreading of the 6S peak, indicative of the formation of aggregates of
the dimer. Such results have been observed previously (12).

The addition of VCR after the formation of MT from 6S tubulin induced a rapid and almost complete decrease in the absorbance (Chart 1C). Electron microscopic examination immediately after VCR addition showed few MT to be present; some of them which remained had short sections of curled protofilaments emanating from different parts, although this was most clearly seen at the ends of MT. What appeared to be fragments of the curled material was observed scattered around MT (Fig. 2A). Complete disappearance of MT occurred within 15 min, at which time only granular material was observed (Fig. 2B). At no time were spirals of the type described above observed. Further addition of VCR caused the absorbance to increase immediately (Chart 1C). Samples from this solution observed with the electron microscope showed aggregation of globular material; no MT or any type of protofilamentous material was seen.

Effect of VCR on Reconstituted MAP-containing MT. In the experiments described above, VCR was added to MAP-free MT or MT formed from tubulin preparations which contained MAP's. As a further verification that the presence of MAP's is responsible for spiral formation, we prepared a MAP fraction by phosphocellulose chromatography and examined its effect on the interaction of MT with VCR.

Assembly of MT was observed under 4 sets of conditions: (1) 6S tubulin in the presence of DMSO; (2) 6S tubulin and MAP's in the absence of DMSO; (3) 6S tubulin in the presence of MAP's with DMSO added after the completion of assembly; and (4) 6S tubulin in the presence of DMSO with MAP's added after assembly was complete. VCR was then added in each case. The results, summarized in Table 1, were the complete disassembly of MAP-free MT, a 50% reduction in absorbance with MT formed from 6S and MAP's, and only a slight reduction in the absorbance value when MT was formed under Conditions 3 and 4. Negatively stained samples from these experiments were similar to those reported above: no MT or spirals were found after treatment of MAP-free MT with VCR; spirals with few MT remaining were found after VCR treatment of MT formed from 6S and MAP's, reconstituted MAP-containing MT (Fig. 2C); some spirals with more MT remaining were found when DMSO was added before VCR to MAP-containing MT or when MAP's were added before VCR to MT assembled in DMSO (Fig. 2D).

Interaction of VCR with 6S Tubulin in the Presence or Absence of MAP's at 0°. Incubation of 6S tubulin at 0° with GTP and VCR in the absence of MAP's resulted in an absorbance increase. However, electron microscopic examination showed only amorphous aggregates and a complete absence of spirals (Fig. 3A and B).

Incubation of 15 μM 6S tubulin with MAP's in the presence of GTP at 0° caused the formation of ring-shaped structures in a variety of combinations (Fig. 3C). When this solution was exposed to 9 μM VCR at 0°, a significant increase in the absorbance was observed, and electron micrographs showed spiral formation (Fig. 3D). Some of these spirals appeared to be composed of short spirals linked together. In general, they appeared shorter and less compact than those formed from MT at 37°. Spirals are also produced when tubulin preparations containing MAP's, but not subjected to purification by phosphocellulose, are incubated with VCR at 0° (5, 23).

**DISCUSSION**

The results clearly show that proteins found associated with MT play a major role in determining the effect of VCR interaction on MT structure. The interaction of VCR with MT containing MAP's always produces spirals. This occurs whether the MT are formed from tubulin preparations which contain MAP's or whether isolated MAP's are added to MT formed from 6S tubulin. MT which lack MAP's, on the other hand, completely disassemble upon treatment with VCR. This information indicates that MAP's in concert with VCR are responsible for spiral formation.

These studies may provide information concerning the binding sites of MAP's and VCR in MT. The results presented here and by others (5, 6, 23) suggest that spirals can be produced directly from MT. Spirals are not seen when MAP's are absent, but in the early stages of depolymerization by VCR we observe what appear to be very small sections of spirals which rapidly disappear. One possible mechanism to explain these observations is shown in Chart 2. VCR could destabilize the lateral interactions between protofilaments while MAP's stabilize the longitudinal interactions between dimers in a single protofilament. Thus, when VCR binds MAP-containing MT, single protofilament spirals begin to form. These then coil into the spirals which, perhaps, are more thermodynamically stable structures. In the absence of MAP's, the single protofilaments dissociate into smaller pieces and eventually completely depolymerize.

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**Table 1**

**Effect of VCR on reconstituted MAP-containing MT**

<table>
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<tr>
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because of their instability. The fact that in electron micrographs spirals are seen emanating directly from protofilaments supports this mechanism.

An alternative explanation involves the production of rings and very short spirals from the MT, which then assemble into the longer spirals. Support for this comes from the experiment which shows spiral formation at 0° when rings but not MT are present. This process also depends on the presence of MAP’s. It is quite possible that both mechanisms occur simultaneously.

It has been known for some time that the stability of MT to chemical agents and physical parameters varies considerably from cell to cell. This seems not to be a property of the tubulin molecule itself since this protein is very similar from one species to another. The microenvironment appears to be the major factor in determining the stability of MT, and MAP’s may be an important aspect of the microenvironment.

REFERENCES


Fig. 1. Electron micrographs of MT containing MAP's treated with VCR. MT were formed and treated with VCR as described in Chart 1A. A, MT before VCR. B, immediately after the addition of 5 μl of VCR. Arrows point to curled protofilaments at the end of MT. C, 15 min after VCR addition. Inset, spirals formed by one protofilament originating from the middle of the MT. D, 15 min after a second addition of VCR. Arrows in C and D, spirals which are continuous with protofilaments of MT. Bar line for all electron micrographs, 0.1 μm except the inset. Inset, × 210,000.
Fig. 2. Effect of VCR on MAP-free MT and reconstituted MAP-containing MT. A, MT were formed and treated with VCR as described in Chart 1C. The electron micrograph is of a sample taken immediately after VCR addition. Inset and large arrow, curvy material at the end of MT. Small arrow, microtubular fragments. B, 15 min after VCR addition. C, MT were formed from 6S tubulin (1.5 mg/ml) and MAP's (0.9 mg/ml) in the absence of DMSO. VCR (5 µl of a 900 µM solution) was added, and 20 min later a sample was taken for negative staining. D, MT were formed from 6S tubulin as described in Chart 1C. MAP's (final concentration, 0.9 mg/ml) were added after assembly was complete. After 10 min, VCR was added, and 20 min later a sample was removed for negative staining. Bar line for all electron micrographs, 0.1 µm except the inset. Inset, × 210,000.
Fig. 3. Effect of VCR on 6S tubulin containing or lacking MAP's at 0°. A, tubulin lacking MAP's (6S tubulin) was incubated at a concentration of 1.5 mg/ml in assembly buffer for 15 min at 0°. B, same as A, except that 9 μM VCR was included. C, tubulin was incubated as in A, except MAP's (0.9 mg/ml) were included. D, after incubation of 6S tubulin with MAP's for 5 min, VCR (final concentration, 9 μM) was added, and the incubation was continued for 15 min. Bar line for all electron micrographs, 0.1 μm.
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