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INHIBITION OF MICROTUBULE ASSEMBLY IS A POSSIBLE MECHANISM OF ACTION OF MITOXANTRONE

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SUMMARY: We have found that mitoxantrone can inhibit the polymerization of brain tubulin in a dose dependent manner. MXT had relatively high affinity for tubulin but had no appreciable effect on tubulin associated guanosinetriphosphatase (GTPase) activity nor could it compete with vinblastine (VB) and colchicine (Col) for tubulin binding sites. Furthermore, MXT (0.1-10 μM) is antiproliferative to cold-treated (0° C) epithelial cells after only brief exposure (30 min). These results indicated that MXT is a microtubule inhibitory agent and can exert its anticellular effect through modulation of microtubule assembly. © 1991 Academic Press, Inc.

Mitoxantrone (MXT) is a new anthracenedione which has proven broad spectrum antineoplastic activity in both experimental models and clinical trials against breast cancer and a variety of other malignant diseases (1-3) with potency equal to or superior than doxorubicin but has much less potential accumulative cardiatoxic effect (4). MXT is designed to be an intercalator of DNA (5) and is able to induce DNA breakage, and inhibit nucleic acid biosynthesis (1,6). However, there is no direct correlation between the potent anticellular effect of MXT and its antitumor effect in vivo (2,3). Therefore, its exact mechanism of action remains to be determined. In the present study we have shown that

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Abbreviations: MXT, mitoxantrone ; TLC, thin layer chromatograpy ; DEAE, diethylaminoethyl ; P-G buffer, phosphate-glutamate buffer ; GTPase, guanosinetriphosphatase ; DMEM, Dubelco minimal essential medium ; VB, vinblastine ; Col, colchicine.

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MXT can inhibit the polymerization of brain tubulin and delay the reattachment of epithelial cells and subsequent cell growth. These results imply that the anti-cellular effect of MXT may be mediated through restriction of microtubule assembly.

MATERIALS AND METHODS

Inhibition of Tubulin assembly : Highly purified bovine brain tubulin was prepared as described by Hamel and Lin (7) using DE-52 ionic exchange chromatography followed by 3 cycles of polymerization and depolymerization and assembly of tubulin was monitored by the viscometric method of Kuriyama & Sakai (8) in the presence and absence of various concentrations of MXT (2-50 µM) or vinblastine (5 or 10 µM) and the effect of MXT on tubulin associated GTPase activity was determined by TLC as described by David-Pfeuty et al (9) .

Electron Microscopy : Highly purified tubulin (1-2 mg/ml) in assembly buffer was incubated at 37°C for 30 min in the presence or absence of MXT (10 µM) and samples were diluted (1:1) with a buffer containing 2 M hexylene glycol (in 0.2 M Mes pH 6.9 + 1.0 mM $MgCl_2$) and microtubule was revealed using a standard negative staining technique (8).

Binding of MXT to tubulin : Tubulin was subjected to 7.5% polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions in Tris-HCl buffer pH 8.6. Following electrophoresis, individual slots were sliced and then treated as described in the figure legend. The binding of ^{3}H-vinblastine (2 µCi/µM) or ^{3}H-colchicine (2 µCi/µM) in the presence or absence of various doses of MXT (1-100 fold excess) to tubulin was determined by the DE-81 paper discs method of Weisenberg et al (10).

Anticellular effect : TL (cytotrophoblastic), KB and WISH cells were resuspended at 2 x 10^5/ml in DMEM plus 10% fetal bovine serum and gentamycin (20 µg/ml) and then incubated on ice for 6 h. The cells were then incubated for an additional 30 min in the presence or absence of MXT (0.1-10 µM), washed 3X and plated onto 60 mm tissue cultures plates and then incubated at 37°C in 5% CO_2. At various times, cells were observed under an inverted microscope for cell attachment and cell number of all cultures was determined at 48 h following incubation by trypan blue stain.

RESULTS

Inhibition of tubulin assembly : As could be seen in Figure 1, polymerization of tubulin was inhibited in the presence of MXT (2 or 10 µM) in proportion to dosage. The kinetics of inhibition is similar to that of VB but is less efficient. The inhibitory effect of MXT could also be observed under the electron microscope. A dense network of tubular structure could be observed when tubulin polymerized (Fig 2A), however, in the presence of MXT the extent of polymerization was greatly reduced (Fig.2B). Unlike VB and Col, over a wide dose range, MXT could neither enhance nor suppress GTPase activity (Fig.4).

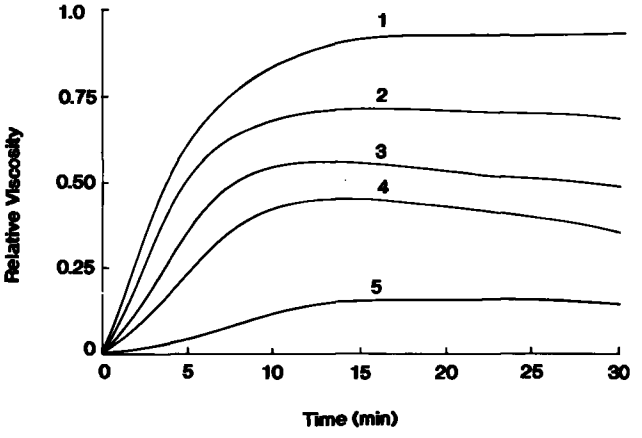

Fig.1. The inhibitory effect of mitoxantrone on the polymerization of tubulin as assayed by the viscometric method. Purified bovine brain tubulin was assembled in Mes buffer at 37°C in the absence (1) and presence of 2,10 and 50 μM (2-4) of MXT respectively or 10 μM of VB (5). Relative viscosity was arbitrarily set at 0-1 unit.

Binding of MXT to tubulin : The physical interaction between MXT and tubulin was assessed by a modified immunoblot assay based on the intense blue color of MXT. Fig 3 indicates that MXT binds to tubulin (in 15-25 min post-incubation) in proportion to concentration but under the same conditions, MXT does not react with albumin or transferrin which are known MXT-binding proteins.

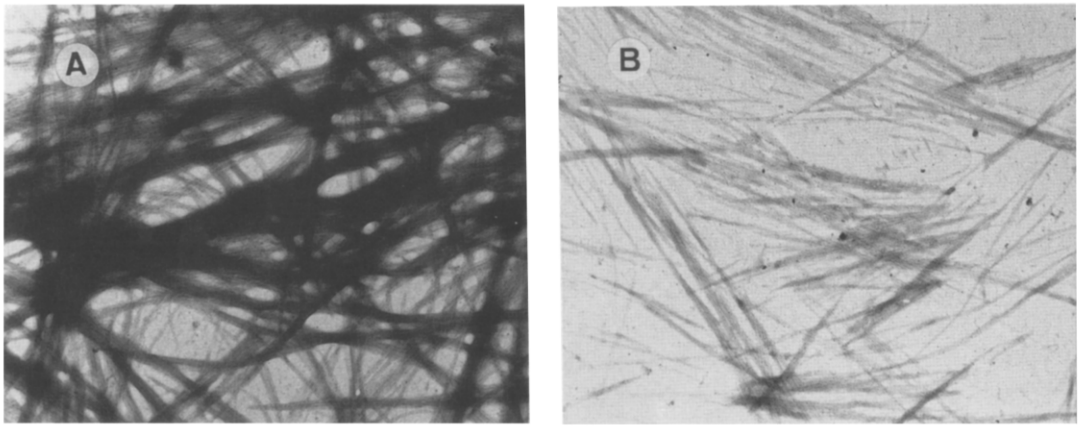

Fig. 2. Electron micrographs of negatively stained specimens of tubulin polymers showing a dense network of microtubule structure following incubation at 37°C for 30 min (A) and relatively few tubular filaments when tubulin was assembled in the presence of 10 μM of MXT (B). x16,000.

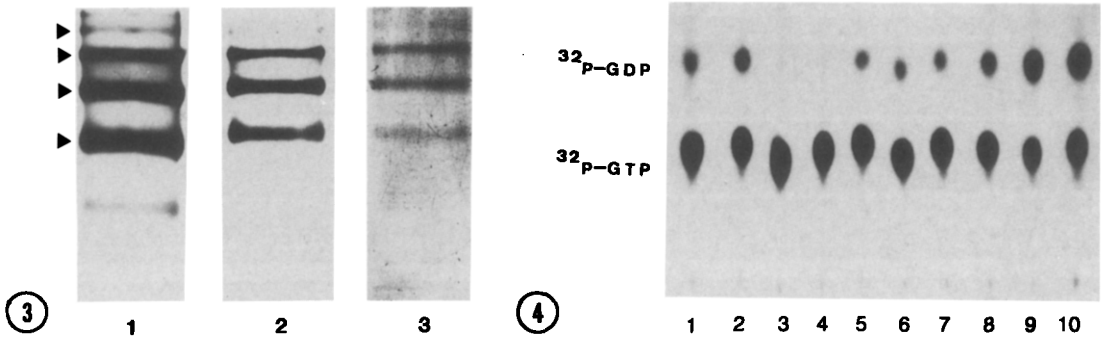

Fig. 3. The binding of MXT to tubulin as determined by a modified immunoblot assay. Tubulin in semi-preparative 7.5% polyacrylamide gel was electrophoresed under non-denatured conditions in Tris-HCl buffer pH 8.6. Individual slots of the same gel were treated separately as follows: Lane 1, stained with coomassie blue stain; Lane 2, immunoblotted with a mouse anti-rat tubulin monoclonal antibody using EIA and incubated with MXT (100 µg/ml) after transfer onto nitro-cellulose membrane (Lane 3). Three major and one minor MXT-reactive protein bands as indicated by arrowheads were consistently observed.

Fig. 4. Assay for polymerization-dependent tubulin-associated GTPase activity by TLC based on the conversion of ^{32}P-GTP to ^{32}P-GDP. Bovine tubulin (2-5 mg/ml) was incubated with assembly buffer only (Lane 1) and in the presence of 2, 10 and 50 µM of VB (Lanes 2-4), MXT (Lanes 5-7) or Col (Lanes 8-10) respectively. Individual spots were revealed by autoradiography.

Furthermore, protein-bound MXT could be eluted with P-G buffer indicating that the binding is reversible. Our tubulin preparation is homogenous in SDS-PAGE (a single anti-tubulin reactive band), but the same preparation consistently reveals 4 protein bands (3 major and 1 minor ; arrowheads) in non-denatured gel (Fig.3, lane 1) which may represent heterodimers and concatamers. All of these bands can bind MXT (Fig.3 lane 3) and are reactive to anti-tubulin monoclonal antibody (Fig.3, lane 2). However, based on the tracing of radioactive drugs, MXT does not compete with either VB or Col for tubulin binding sites even at a 100-fold excess (Not shown).

Effect on cell growth : Following cold treatment, the cytoskeletal structures of all cells were totally disrupted as revealed by indirect immunofluorescence using acetone-fixed cytospin cell smears and anti-tubulin antibody. Cells in cultures without MXT became adherent in about 2 h after incubation at 37°C however most MXT-treated cells remained in suspension for at least 8 h at all the cytotoxic doses tested. In addition, no appreciable increase in cell number or cell death was observed in these cultures after 48 h (Not shown).

DISCUSSION

Despite the intense interest in MXT, its mechanism of action is not completely understood. Accumulated evidence thus far suggests that MXT is an anti-topoisomerase II (11) drug and can inhibit nuclei acid synthesis (6,12). However, these activities do not correlate well with its in vivo antitumor effect (2,3) suggesting that other mechanisms may be involved.

We have shown in the present study that MXT can suppress the polymerization of purified bovine brain tubulin (Figs 1&2). However, unlike VB or Col, MXT has no effect on tubulin-associated GTPase activity (Fig 4), nor does it compete with the two tubulin drugs for binding sites (Not shown). In addition, MXT has high affinity of MXT for tubulin comparing to other known MXT binding proteins such as albumin and transferrin (Fig 3 and not shown) and this may be the probable cause of its ability to interfere with tubulin assembly.

The microtubule system plays a key role in mitosis and is the supporting network for cell surface receptors as well as the conductor for cellular communication (13). Therefore it is speculative that MXT can exert its anticellular effect through restriction of microtubule formation and modulation of plasma membrane activity. This hypothesis is supported by our recent observation that MXT is preferentially encapsulated by negatively-charged liposomes which then become positively charged (14). In addition, it has been reported that a MXT-resistent gastric carcinoma cell line has structural changes in its microtubule system (15).

Taken together, results of the present study and those of others strongly suggest that apart from its established ability to perturb nucleic acid biosynthesis, MXT can also target on membranous structures and the associated cytoskeleton. A previous observation that doxorubicin can exert its cytotoxic action solely by interaction with the cell surface (16) is in accord with our suggestion since the two drugs are closely related. Our findings offer a more detailed explanation to account for the diversity of the biological effects of MXT and provide some clues which may be useful for the design of more effective treatment protocals against various neoplastic diseases.

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