Effect of Estramustine Phosphate on the Assembly of Isolated Bovine Brain Microtubules and Fast Axonal Transport in the Frog Sciatic Nerve

Martin Kanje, Johanna Deinum, Margareta Wallin, Per Ekström, Anders Edström, and Beryl Hartley-Asp

ABSTRACT

Estramustine phosphate (0.01 to 0.5 mM), an estradiol mustard derivative used in the therapy of prostatic carcinoma, inhibited the assembly of brain microtubule proteins in vitro and disassembled preformed microtubules. In the presence of estramustine phosphate, the minimum microtubule-protein concentration sufficient for the assembly of microtubules was increased. Low concentrations of taxol (20 nM) completely reversed the inhibition of assembly by estramustine phosphate. The effects were specific to estramustine phosphate since neither estradiol 17β-phosphate, the hormonal moiety of the drug, nor nonnitrogen mustard, the alkylating moiety, had any effect on assembly.

Estramustine phosphate (0.1 to 0.5 mM) was also found to reversibly inhibit fast axonal transport in the frog sciatic nerve. The nerve content of adenosine triphosphate, adenosine diphosphate (ADP), adenosine monophosphate (AMP), and inorganic phosphate was not significantly affected by estramustine phosphate.

Our results suggest that the cytotoxic action of estramustine phosphate could be dependent partially on an interaction with microtubules, probably via the microtubule-associated proteins.

INTRODUCTION

Estramustine phosphate (estradiol 3-[(N,N-bis[2-chloroethyl]carbamate] 17β-phosphate) is active against advanced prostatic carcinoma (14). The mechanism of action of the drug is not fully understood and cannot solely be ascribed to its antgonadotropic properties (10, 20). It is cytotoxic in human prostatic carcinoma cells in culture (13) and induces mitotic arrest at metaphase (12). These metaphases have contracted chromosomes, which no longer are aligned in the metaphase plane. Other drugs which exhibit this property, such as colchicine, vinblastine, and nocodazole (23), are inhibitors of the assembly of microtubules, the main component of the mitotic spindle. Hitherto, a direct effect of estramustine phosphate on microtubules in vitro or on other microtubule-dependent processes, such as fast axonal transport, has not been studied.

In the present study, the inhibitory effect of estramustine phosphate on the assembly of isolated brain microtubules and on fast axonal transport is reported. The results indicate that the cytotoxicity of estramustine might be caused by an interaction with microtubule proteins.

MATERIALS AND METHODS

Chemicals. Estramustine (estradiol 3-[(N,N-bis[2-chloroethyl]carbamate] 17β-hydroxy), estramustine phosphate (estradiol 3-[(N,N-bis[2-chloroethyl]carbamate] 17β-phosphate), and estradiol 17β-phosphate were synthesized by AB Leo, Helsingborg, Sweden. Estramustine phosphate was dissolved in distilled water, and its concentration was estimated from the absorbance at 274 nm using the extinction coefficient of estramustine, E274 = 17.7.1 In all the experiments with the drug, either Ca2+ and Mg2+-free solutions or solutions containing EDTA were used to avoid precipitation of an insoluble Ca2+ or Mg2+-estramustine phosphate complex. EDTA was used as the chelator because the buffer contained 0.5 mM MgSO4 but no added Ca2+. Estramustine was dissolved in Cremophor EL:ethanol (1:9) before dilution in standard frog Ringer's solution. Cremophor EL was kindly provided by Dr. K.E. Falk, AB Hassle, Mölndal, Sweden.

Taxol was a gift from Dr. M. Sufness at the NIH, Bethesda, MD. L-[4,5-3H]Leucine (130 Ci/mmol, 1 mCi/mL) was obtained from The Radiochemical Centre, Amersham, England. All other chemicals were reagent grade.

Microtubule Proteins. Microtubule proteins were prepared from bovine brain in the absence of glycerol by 2 or 3 cycles of assembly-disassembly in the presence of 0.5 mM MgSO4 (2, 19). In the first cycle, ethyleneglycolbis(2-aminoethyl ether)-N,N'-tetraacetic acid was present to complex free Ca2+. The final pellet, which contains approximately 80% of tubulin (5), was stored in liquid nitrogen. Prior to use, the pellet was resuspended in buffer (100 mM piperazine-N,N'-bis(2-ethanesulfonic acid), 0.5 mM MgSO4, with 1 mM GTP at pH 6.8). After incubation at 4°C for 30 min, the sample was clarified by centrifugation at 35,000 x g for 30 min at 4°C. Protein solutions were stored in liquid nitrogen after drop freezing (5).

Protein Concentration. Microtubule protein concentration was determined by Bio-Rad protein assay using Coomassie Brilliant Blue based on the method of Bradford (3) with tubulin as a standard. The concentration of the standard was determined from E278 = 1.2 mg-1 cm-1 and a molecular weight of 110,000 (23).

Assembly. Assembly of microtubule proteins in 100 mM piperazine-N,N'-bis(2-ethanesulfonic acid), 0.5 mM MgSO4, 1 mM EDTA, and 1 mM GTP was started by addition of 50 μl microtubule proteins at 4°C to 300 μl buffer at 37°C, and the increase in turbidity was monitored continuously by the change in absorbance at 350 nm (5). Estramustine phosphate or an equivalent amount of water was added from a stock solution either to the buffer or to the protein. It was found that the addition of 1 μM EDTA did not inhibit microtubule assembly. Taxol was added from a 3 μM stock solution in dimethyl sulfoxide.

Axonal Transport. The sciatic nerve together with the eighth and ninth dorsal ganglia were dissected from frogs (Rana temporaria). A ligature was placed on the nerve 30 mm from the ganglia. Treated and control nerve preparations were from the same frog. The nerves were incubated in an apparatus where the ganglia could be separated from the nerve by silicon grease barriers (8). Frog Ringer's solution devoid of Ca2+ and Mg2+ with or without estramustine phosphate was added to the nerve compartment. The absence of these cations in the Ringer's solution led to the conclusion that the observed effects were specifically caused by estramustine phosphate.

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2 To whom requests for reprints should be addressed.

3 AB Leo, personal communication.

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solutions does not affect axonal transport (6) in sheathed frog sciatic nerves. The ganglia were exposed to [3H]leucine containing standard frog Ringer's solution. After incubation for 17 h at 18°C, the nerve was cut into 3-mm segments and analyzed for protein-incorporated radioactivity as described previously (6). The transport rate was determined using pulse-labeling of the ganglia for 2 h with Ringer's solution containing [3H]leucine followed by incubation for 10 h in Ca2+-, Mg2+-free Ringer's solution with or without the drug.

Adenosine Nucleotide Determination. Nerves were incubated for 17 h at 18°C, blotted on filter paper, weighed, dropped into liquid nitrogen, and crushed. Nucleotides were extracted with 10% trichloroacetic acid. The extract was neutralized by extracting the trichloroacetic acid by trioctylamine in 1,1,2-trichlorotrifluoroethane (21) prior to injection into a Polyanyon SI column (Pharmacia) attached to a Varian 5000 high-pressure liquid chromatograph. Nucleotides were eluted by a linear phosphate gradient, 10 to 850 mM, pH 7.0, at a flow rate of 1 ml/min during 15 min. ATP, ADP, and AMP were detected by their absorbance at 254 nm and quantified using a nucleotide standard.

Electron Microscopy. Negatively stained microtubule specimens for electron microscopy were prepared from 5-μl protein samples. Fixation was performed with 1 drop of Karnovsky's solution (17), after which the specimen was washed with distilled water and stained with 1% uranyl acetate. Embedded microtubule specimens were prepared after centrifugation at 35,000 x g for 30 min at 35°C. The pellets were fixed with Karnovsky's solution followed by 1% osmium tetroxide in 0.1 M cacodylate buffer (17). The pellets were dehydrated in a graded series of acetone and embedded in Epon. Thin sections were made on a LKB ultramicrotome and were double stained with uranyl acetate and lead citrate. The specimens were viewed in a Zeiss 109 electron microscope.

Nerves exposed to estramustine phosphate were fixed in 2.5% glutaraldehyde (16) followed by 1% osmium tetroxide. Subsequently, the nerves were dehydrated in a graded series of ethanol, block stained in 1% phosphotungstic acid and 0.5% uranyl acetate, and embedded in Epon:Araldite.

RESULTS

Effects of Estramustine Phosphate. Estramustine phosphate inhibited the assembly of microtubule proteins in a concentration-dependent manner (Charts 1 and 2). The rate and extent of assembly was less in the presence of estramustine phosphate. Furthermore, estramustine phosphate induced rapid disassembly of microtubules at steady state (Chart 1). No difference in the level of assembly was found whether estramustine phosphate was added initially or at steady state (see Chart 2) or if the microtubule proteins were preincubated with the drug for 30 min at 4°C. The electron micrographs (not shown) of microtubules disassembled to approximately 50%, taken directly after the addition of estramustine phosphate, showed normal but fewer microtubules with extending projections.

The extent of microtubule assembly in the presence of increasing amounts of estramustine phosphate showed a nonlinear dose-response relationship (Chart 2). The same curve was obtained if the disassembly level was plotted.

Addition of estramustine phosphate at a constant concentration to preformed microtubules induced the same change in turbidity independent of the protein concentration. If the turbidity was plotted against different microtubule protein concentrations, it could be seen that with a constant concentration of estramustine phosphate a linear relationship remained with the same slope as for the control but shifted to the right (Chart 3). Thus, in the presence of estramustine phosphate the critical protein concentration, i.e., the minimum microtubule protein concentration sufficient for assembly of microtubules, was increased.

Low concentrations (20 μM) of the microtubule-promoting drug, taxol, were able to reinduce assembly of completely inhibited microtubule proteins to the same or higher levels than that of the control (Chart 1). The electron micrographs of the pellet of the taxol-induced microtubules showed the presence of many aberrant forms of microtubules as compared to the microtubules formed in the absence of the drug (Fig. 1).

The effect on microtubule assembly was specific for estramustine phosphate since neither estradiol 17β-phosphate nor nomitrogen mustard had any effect on microtubule assembly at comparable concentrations (see Chart 2). Furthermore, 1 mM estradiol 17β-phosphate did not compete with estramustine.

EFFECT OF ESTRAMUSTINE PHOSPHATE ON MICROTUBULES

Chart 1. Time course of assembly of brain microtubules. Estramustine phosphate (EMP) (0.42 mM) was added initially (Trace A) or at steady state level of assembly (Trace B at arrow). After complete disassembly, 20 μM taxol was added (Trace C at arrow). Microtubule assembly (1.8 mg/ml protein) was started by raising the temperature from 10 to 37°C and was monitored by the increase in absorbance at 350 nm against time. The assembly buffer contained 0.1 mM piperazine-N,N'-bis(2-ethanesulfonic) acid at pH 6.8, 0.5 mM MgSO4, 1 mM GTP, and 1 mM EDTA.

Chart 2. Microtubule assembly at different concentrations of estramustine phosphate (EMP). The steady state level of assembly was measured after preincubation for 10 min at 10°C with estramustine phosphate, nomitrogen mustard (nor-HW2), or estradiol 17β-phosphate (E2), and furthermore after addition of estramustine phosphate to preformed microtubules (Δ). The level was expressed in percentage of the ΔA280 of the control, since different protein preparations were used. The microtubule protein concentration was 2.1 mg/ml, and the conditions were as described in the legend to Chart 1

The transport rate was determined using pulse-labeling of the ganglia for 2 h with Ringer's solution containing [3H]leucine followed by incubation for 10 h in Ca2+-, Mg2+-free Ringer's solution with or without the drug.
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Chart 3. Microtubule assembly at different protein concentrations in the presence of estramustine phosphate. The steady state level of microtubule assembly was measured after addition of estramustine phosphate (●, 0 μM; ●, 84 μM; ○, 151 μM) to preformed microtubules at 37°C. The conditions were as described in the legend to Chart 1.

Table 1
Effects of estramustine phosphate and estramustine on the accumulation of 3H-labeled protein in front of a ligature

<table>
<thead>
<tr>
<th>Accumulation in drug-treated nerve (%)</th>
<th>0.1 mM</th>
<th>0.5 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estramustine phosphate</td>
<td>57 ± 5%</td>
<td>25 ± 6%</td>
</tr>
<tr>
<td>Estramustine</td>
<td>59 ± 6%</td>
<td>34 ± 6%</td>
</tr>
<tr>
<td>Estradiol 17β-phosphate</td>
<td>82 ± 23 (NS)</td>
<td></td>
</tr>
<tr>
<td>Normitrogen mustard</td>
<td>87 ± 23 (NS)</td>
<td></td>
</tr>
</tbody>
</table>

*Values are expressed as the accumulation in the drug-treated nerve in percentage of that in the control nerve.
* Mean ± standard average.
* P = 0.001.
* NS, not significant.

phosphate for the same binding site, because in its presence the inhibitory action of the drug on microtubule assembly was unchanged. Furthermore, electron micrographs of microtubules assembled in the presence of 0.5 mM concentrations of either estradiol 17β-phosphate or normitrogen mustard showed perfect microtubules (not shown).

Axonal Transport. Estramustine phosphate at 0.5 and 0.1 mM reduced the accumulation of labeled material in front of a ligature to 25 and 57%, respectively, of the control values. Estramustine was equally effective as an inhibitor of axonal transport (Table 1). Furthermore, the effect was specific because neither 0.5 mM estradiol 17β-phosphate nor 0.5 mM normitrogen mustard had any effect on axonal transport (Table 1).

The accumulation of radioactive material in the presence of 0.1 mM estramustine phosphate was more suppressed [31 ± 5% (SD), P < 0.05, N = 8] in nerves preincubated at 1°C for 3 h than in nerves which had not been pretreated by cooling.

The decrease in axonal transport by 0.5 mM estramustine phosphate was reversible. Axonal transport did not differ from the control after 5 h preincubation at 4°C in the presence of 0.5 mM estramustine phosphate, followed by washing with standard Ringer’s solution and subsequent assay. In electron micrographs of the sciatic nerves treated with the drug, microtubules could still be observed (not shown).

From the above experiments, in which ligatures were used, differentiation of the effect of the drug on the rate of transport or on the amount of transported materials is not possible. However, the pulse-labeling experiment (Chart 4) showed that there was little difference in the total amount of transported material but a reduced transport rate in the presence of 0.5 mM estramustine phosphate.

Energy Metabolism. In order to exclude a metabolic effect of estramustine phosphate as a cause of the inhibited axonal transport, the levels of ATP, ADP, and AMP were measured in nerves which had been incubated with the drug. As can be seen in Table 2, the adenosine nucleotide composition was not significantly influenced by a 17-h incubation with either estramustine phosphate or estramustine.

DISCUSSION

The mechanism of action of the anticancer agent estramustine phosphate is not known. In humans, the drug is rapidly dephos-
phorylated releasing estramustine, which is readily oxidized to estramustine (11). Thus, on hydrolysis, both estradiol and estrone are released to exert their hormonal effect on the prostatic tumor (10). However, estramustine itself does not compete with estradiol for the estrogen receptor site (9, 20), indicating a lack of classical hormonal effect. Estramustine does not induce DNA strand breaks either, indicating a lack of alkylating activity, in spite of the nonnitrogen mustard moiety of estramustine (22). However, the observation that estramustine inhibits mitosis in prostate tumor cells in culture (12) implicates an involvement of microtubules in its mode of action.

In the present study, we studied the effect of estramustine phosphate on the assembly of cold-labile microtubules from brain. Brain tissue contains relatively large amounts of microtubules. Cold-labile microtubules from different tissues have been shown to have the same general properties with respect to different assembly inhibitors. We found that estramustine phosphate inhibited brain microtubule assembly in vitro at physiologically relevant concentrations and induced rapid disassembly of preformed microtubules. The effect on microtubules in vitro was specific for estramustine phosphate because neither nonnitrogen mustard nor estradiol 17β-phosphate, the 2 parent moieties, affected microtubule assembly or disassembly. Neither could the 2 parent moieties potentiate the effect of estramustine phosphate on microtubule assembly.

Although estramustine has a colchicine-like effect on mitosis, preliminary results indicate that the drug does not interfere with the binding of colchicine to tubulin. Our experiments suggest that estramustine phosphate interacts with MAPs.\(^4\) Hence, estramustine phosphate increased the critical protein concentration required for the assembly of microtubules, in contrast to colchicine, vinblastine, and nocodazole (23), which specifically bind to the tubulin dimer. This effect is also exhibited by drugs which have a direct effect on the MAPs-dependent nucleation phase of assembly and which bind to MAPs, such as DNA (1) and heparin (4). The reversal of the estramustine phosphate-induced inhibition of assembly by taxol is not possible in the presence of tubulin-binding drugs such as colchicine (18), which is further support for an interaction of estramustine phosphate with MAPs. Furthermore, we have found (24) that \(^3\)H-labeled estramustine phosphate bound predominantly to MAPs and moreover that addition of MAPs reversed the inhibition of assembly induced by estramustine phosphate. However, the relatively high molar ratio (compared with the concentration of MAPs) of estramustine phosphate needed for complete inhibition of assembly and the nonlinear dose-response curve (Chart 2) suggest a weak interaction, which is also indicated by the reversibility of the effect on axonal transport.

Axonal transport, a microtubule-dependent process, was used to assess the effect of estramustine phosphate and estramustine in an intact cellular system. In agreement with the inhibition of the drug on microtubule assembly is the finding that estramustine phosphate and estramustine inhibited axonal transport in the sciatic nerve. This also demonstrates that the phospho group contributes only by increasing the solubility of the drug. The inhibition occurred without significant alterations of the nerve content of ATP, ADP, and AMP. The inhibition was reversible, and low temperature intensified the effect of the drug, which is comparable to the effects of colchicine on the sciatic nerve (7).

Together with the observed inhibition of mitosis (12) and the recent observation that estramustine inhibits microtubule-dependent pigment granule movement in squirrel fish erythrocytes, the results suggest that estramustine phosphate exerts its cytotoxic effect by interaction with microtubules in the intact cell also.

**ACKNOWLEDGMENTS**

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**REFERENCES**


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\(^4\) The abbreviation used is: MAPs, microtubule-associated proteins.

\(^6\) M. E. Steams and K. D. Tew, personal communication.
Fig. 1. Electron micrograph of embedded microtubules. A, microtubules; B, microtubules in the presence of 20 μM taxol; C, microtubules in the presence of 0.42 mM estramustine phosphate induced by 20 μM taxol. The conditions were as described in the legend to Chart 1. × 90,000.
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