Interaction of estramustine phosphate with microtubule-associated proteins

Margareta Wallin, Johanna Deinum° and Bo Fridén

Dept. Zoophysiology, University of Göteborg, Box 250 59, S-400 31 Göteborg and °Dept. of Medical Physics, University of Göteborg, Box 330 31, S-400 33 Göteborg, Sweden

We have reported [(1984) Cancer Res., in press] that estramustine phosphate inhibits microtubule assembly and disassembled preformed microtubules. We now present evidence that estramustine phosphate inhibits microtubule assembly by binding to the microtubule-associated proteins. We have found that: (1) additional microtubule-associated proteins relieved the inhibition of assembly by estramustine phosphate; (2) ^{3}H-labelled estramustine phosphate bound predominantly to the microtubule-associated proteins; and (3) the content of the microtubule-associated proteins was reduced in taxol reversed estramustine phosphate-inhibited microtubules.

Microtubule   Tubulin   Taxol   Estramustine phosphate   Microtubule-associated protein

1. INTRODUCTION

Estramustine phosphate, estradiol-3-[N,N-bis(2-chloroethyl)carbamate] 17β-phosphate, is active against advanced prostatic carcinoma [2]. It exhibits several classical mitotic inhibitory characteristics: it is cytotoxic and induces mitotic arrest at metaphase [3,4]. However, its mechanism of action has been unknown. We reported that estramustine phosphate inhibits assembly and disassembles microtubules isolated from bovine brain. These results indicated that the cytotoxicity of estramustine phosphate might be caused by interference with the microtubule proteins.

Microtubule proteins consist of 80% tubulin and 20% microtubule-associated proteins. Mitotic inhibitors such as colchicine, vinblastine and nocodazole [5] inhibit assembly of microtubules by interacting with tubulin, the subunit of the microtubule-backbone. However, the effect of estramustine phosphate on microtubule assembly

was different from that of these tubulin-binding drugs [1]. It was therefore of main interest to further characterize the interaction of estramustine phosphate with the microtubule proteins. Here, additional microtubule-associated proteins were found to counteract the inhibitory effect of estramustine phosphate on microtubules. Furthermore, ^{3}H-labelled estramustine phosphate predominantly bound to the microtubule-associated proteins, but to a lesser extent to tubulin. Moreover, the amount of microtubule-associated proteins was reduced in microtubules reassembled by taxol in the presence of estramustine phosphate. Our results thus suggest that estramustine phosphate belongs to a new class of microtubule inhibitors, which bind to the microtubule-associated proteins and not to tubulin.

2. MATERIALS AND METHODS

2.1. Chemicals

Estramustine phosphate, estradiol-3-[N,N-bis-(2-chloroethyl)carbamate] 17β-phosphate, and estramustine [6,7-^{3}H]phosphate were synthesized

by AB Leo, Helsingborg, Sweden. Estramustine phosphate was dissolved in distilled water and its concentration estimated from the absorbance at 274 nm using the extinction coefficient of estramustine, $E_{1\%} = 17.7$ (AB Leo, personal communication). In all the experiments with the drug the solutions contained 1 mM EDTA to avoid precipitation of an insoluble Ca^{2+} or Mg^{2+} estramustine phosphate complex. No difference in assembly characteristics was seen in the presence of 1 mM EDTA.

Taxol was a gift from Dr M. Sufness at the National Institute of Health, Bethesda, MD 20205, USA. All other chemicals were reagent grade.

2.2. Microtubule proteins

Microtubule proteins were prepared from bovine brain in the absence of glycerol by two or three cycles of assembly-disassembly in the presence of 0.5 mM $MgSO_4$ [6,7]. In the first cycle EGTA was present to complex free Ca^{2+}. The final pellet, which contains approximately 80% tubulin, was stored in liquid nitrogen. Prior to use, the pellet was re-suspended in buffer (0.1 M Pipes, 0.5 mM $MgSO_4$, with 1 mM GTP at pH 6.8). After incubation at 4°C for 30 min, the sample was centrifuged at 35000 × g for 30 min at 4°C.

The microtubule-associated proteins (MAPs) were separated from tubulin by ion-exchange chromatography on a column containing from top to bottom phosphocellulose (Whatman P11) and Sephadex G-25 Fine (Pharmacia) in 20 mM Pipes and 0.5 mM $MgSO_4$ at pH 6.8. The phosphocellulose was pretreated with Mg^{2+} [8]. Tubulin was eluted in the void volume and the MAPs in one fraction by addition of 0.6 M NaCl to the same buffer. The MAPs fraction was further desalted on a Sephadex G-25, Pharmacia column in 0.1 M Pipes, 0.5 mM $MgSO_4$, 1 mM EDTA at pH 6.8.

When required, the proteins were concentrated by pressure dialysis with an Amicon ultrafiltration cell and a PM30 membrane at 4°C. Protein solutions were stored in liquid nitrogen after drop freezing.

2.3. Protein concentration

Microtubule protein concentration was determined according to Lowry et al. [9] with bovine serum albumin as a standard.

2.4. Assembly

Assembly of microtubule proteins in assembly buffer, 0.1 M Pipes, 0.5 mM $MgSO_4$, 1 mM EDTA and 1 mM GTP was started by increasing the temperature from 4 to 37°C and was monitored continuously by the change in absorbance at 350 nm. Estramustine phosphate or an equivalent amount of buffer was added from a stock solution to the proteins at 4°C. Taxol was added from a 3 mM stock solution in dimethyl sulfoxide. 300 μl of the solution with the assembled microtubules were pelleted through a 6 ml 50% (w/w) sucrose cushion in the same buffer by ultracentrifugation for 1 h at 100000 × g and the pellet was analysed by SDS-polyacrylamide gel electrophoresis (PAGE).

2.5. SDS-PAGE

SDS-PAGE was performed according to the method of O'Farrell [10] on a linear (5–12%) gradient. Gels were stained with 0.25% Coomassie brilliant blue in methanol:acetic acid:water (5:1:5), and destained in 7% acetic acid and 5% methanol.

2.6. Gel filtration

Microtubule proteins, tubulin and MAPs, respectively, 1 ml, were incubated with estramustine [6,7-^{3}H]phosphate in 0.1 M Pipes, 0.5 mM $MgSO_4$, 1 mM EDTA for 10 min at 4°C and separated from free estramustine phosphate on a Biogel P-6DG column (9 × 1.3 cm). The absorbance at 280 nm was determined in each 1 ml fraction as a measure for the protein content. GTP, which also absorbs in ultraviolet, was therefore not present in the incubation mixture. However, we found that the presence of GTP did not alter the results. Each fraction was thereafter transferred to a scintillation vial with 10 ml scintillation solution: 2 parts toluene, 1 part Triton X-100 and 5 g/l Permablend (Packard Ltd) and counted in a LKB Wallac Rackbeta Liquid Scintillation Counter.

3. RESULTS AND DISCUSSION

We reported that estramustine phosphate inhibits microtubule assembly in vitro [1]. Here, it was found that addition of more MAPs counteracted the inhibition of microtubule

assembly by estramustine phosphate (fig.1). When MAPs were added to a concentration of 0.5 mg/ml to 2 mg/ml microtubule proteins inhibited by 0.2 mM estramustine phosphate, assembly was reinduced and reached the same level as that of the microtubule proteins without any additions. A similar effect has recently been found when the inhibition of microtubule assembly by micromolar concentrations of heparin was counteracted by additional MAPs [11]. Both
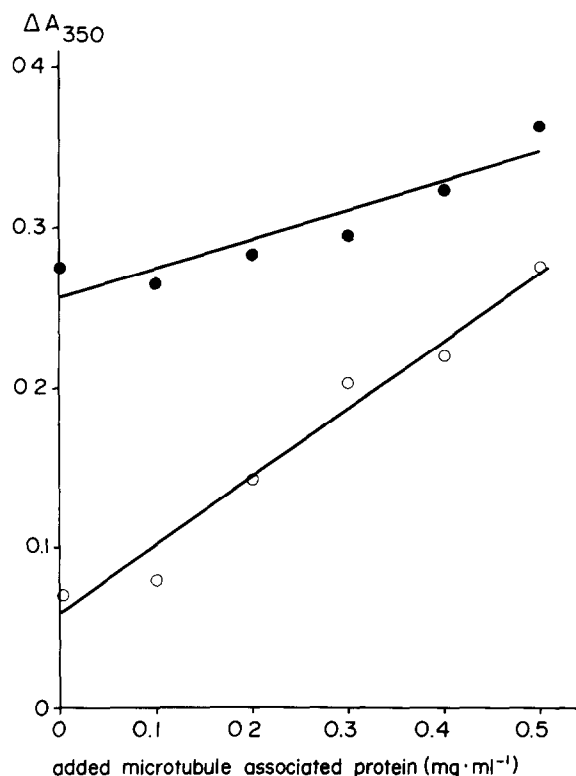

Fig.1. Effect of estramustine phosphate on microtubule assembly in the presence of additional microtubule associated proteins. Microtubule proteins were assembled at 37°C in buffer (0.1 M Pipes, 1 mM GTP, 0.5 mM $MgSO_4$ and 1 mM EDTA at pH 6.8) as monitored by the absorbance difference at 350 nm, (A_{350}), against time. Estramustine phosphate was added to the protein samples at 4°C. The level of assembly is given as the absorbance difference at 350 nm vs the additionally added microtubule associated proteins (mg/ml), present initially. (●) No estramustine phosphate added; (○) in the presence of 0.2 mM estramustine phosphate. The microtubule protein concentration was originally 2.1 mg/ml.

estramustine phosphate and heparin increased the critical protein concentration of assembly and furthermore in both cases the inhibition could be relieved by taxol [1,11]. An increase in the critical concentration has also been seen in the presence of DNA [12], which binds to MAPs and inhibits the MAPs-dependent nucleation phase of assembly. These effects are quite different from those of the typical tubulin-binding drugs such as colchicine, vinblastine and nocodazole, which neither increase the critical protein concentration nor can be counteracted by MAPs (unpublished) or taxol [13].

In order to further determine whether estramustine phosphate binds to MAPs, ^{3}H-labelled estramustine phosphate was incubated with either the microtubule proteins, consisting of 80% tubulin and 20% MAPs or purified tubulin, or the MAPs fraction. It was found by gel filtration that estramustine phosphate binds to MAPs (fig.2C) and to a comparable lower extent to microtubule proteins (fig.2A), but only to a small degree to tubulin (fig.2B). The chromatograph shows that protein-bound estramustine phosphate is relatively well separated from free estramustine phosphate without extensive tailing, indicating that estramustine phosphate has a rather high affinity for MAPs. Preliminary results have also shown that the pellet of taxol induced microtubule protein assembled in the presence of ^{3}H-labelled estramustine phosphate was not enriched in [3]H. As the pellet mainly consists of tubulin this supports the hypothesis that estramustine phosphate does not associate with tubulin but only with the MAPs which are left in the supernatant.

A reduced content of all the MAPs was found in the taxol-reversed estramustine phosphate-inhibited microtubules, as judged from the SDS−PAGE (fig.3). The results suggest that estramustine phosphate inhibits assembly by binding to MAPs a complex, which cannot bind to tubulin and induce assembly. Taxol is able to reverse the inhibition by estramustine phosphate or heparin [1,11] as taxol can induce tubulin to assemble into microtubule in the absence of MAPs [13]. However, we cannot yet decide if estramustine phosphate binds preferentially to a specific MAP or more or less unspecifically to all MAPs. A study with purified MAPs fractions is in progress. This question is of main interest in view of the recent findings that the composition of
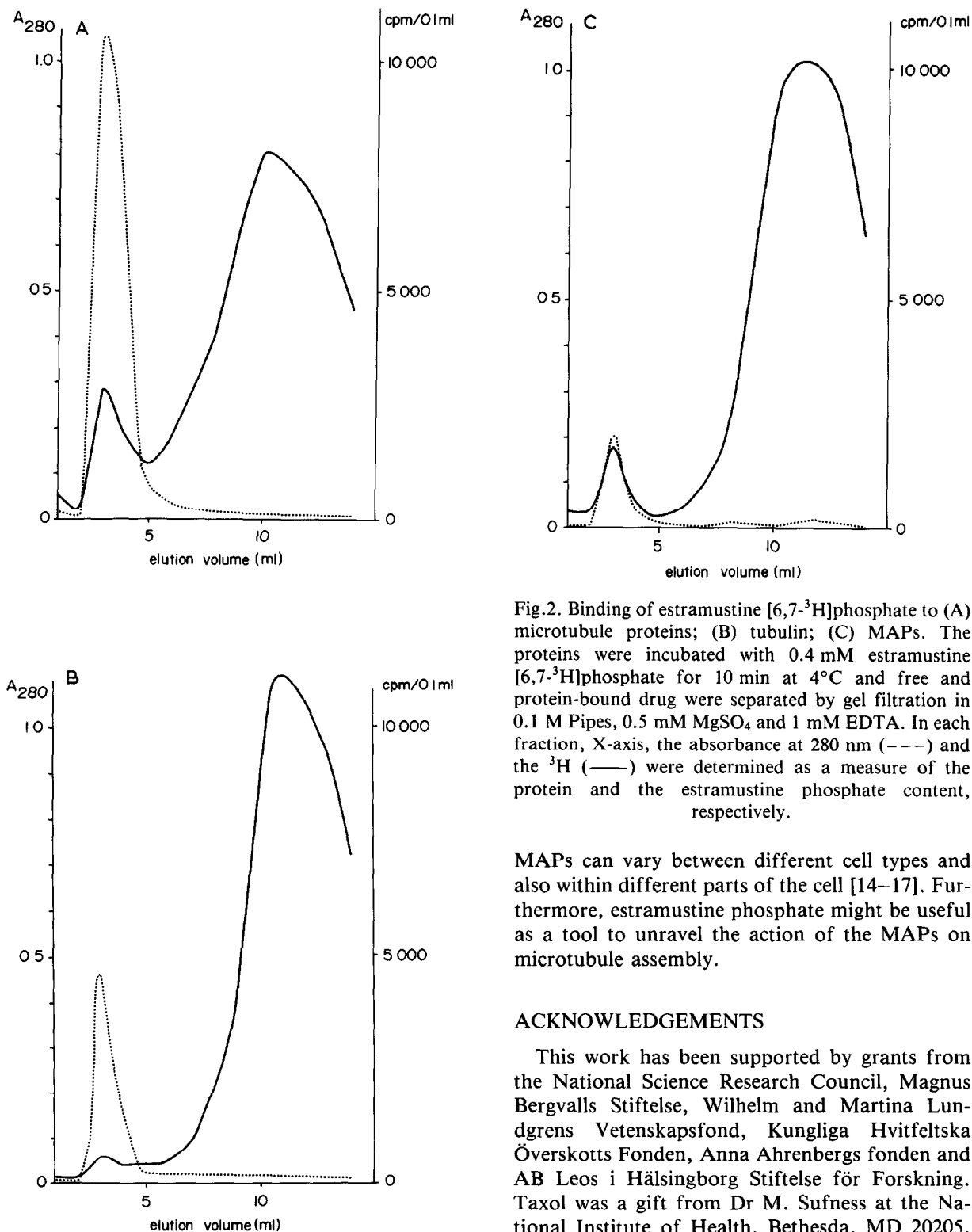
Fig.2. Binding of estramustine [6,7-^{3}H]phosphate to (A) microtubule proteins; (B) tubulin; (C) MAPs. The proteins were incubated with 0.4 mM estramustine [6,7-^{3}H]phosphate for 10 min at 4°C and free and protein-bound drug were separated by gel filtration in 0.1 M Pipes, 0.5 mM MgSO$_4$ and 1 mM EDTA. In each fraction, X-axis, the absorbance at 280 nm (– – –) and the ^{3}H (——) were determined as a measure of the protein and the estramustine phosphate content, respectively.

MAPs can vary between different cell types and also within different parts of the cell [14–17]. Furthermore, estramustine phosphate might be useful as a tool to unravel the action of the MAPs on microtubule assembly.
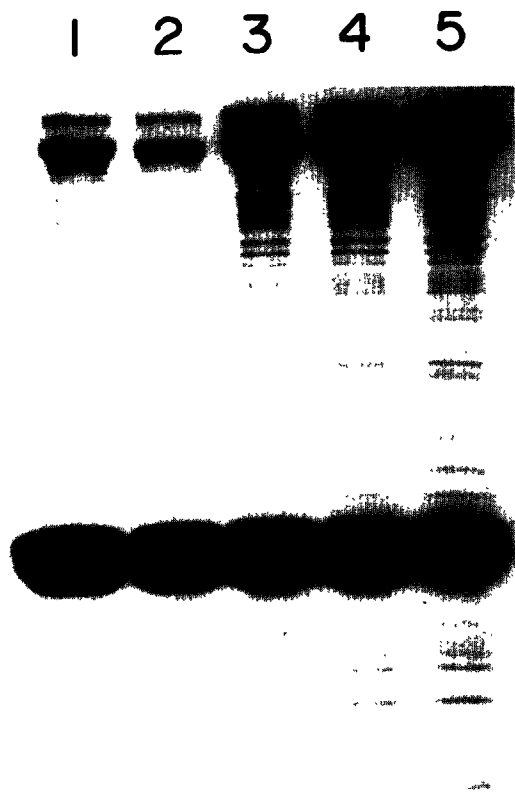
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Fig.3. SDS–PAGE of the pellet of microtubule proteins assembled in the presence of: 0.25 mM estramustine phosphate and 20 μM taxol (lane 1); 0.5 mM estramustine phosphate and 20 μM taxol (lane 2); 20 μM taxol (lane 3); 0.5% dimethyl sulfoxide (lane 4) and at standard conditions. The assembly conditions were as described in the legend to fig.1. 300 μl microtubule solution were pelleted through 6 ml 50% sucrose in assembly buffer at 37°C and the pellet was dissolved in the SDS–PAGE sample buffer immediately after centrifugation.

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