Urol Res (1987) 15:155–160

Urological Research

Hormone-Independent, Non-Alkylating Mechanism of Cytotoxicity for Estramustine

K. D. Tew and M. E. Stearns

Department of Pharmacology, Fox Chase Cancer Center, Philadelphia, Pennsylvania, USA

Accepted: January 12, 1987

Summary. Over two decades, experience with estramustine has provided limited data which support an estrogenic mechanism of action and no data which indicate the nitrogen mustard involvement in the cytotoxic properties of the drug. Consideration of the carbamate-ester portion of estramustine supports the pharmacokinetic evidence that estramustine has a long half life since enzymatic hydrolysis of the carbamate is an uncommon event. Using a variety of immunocytochemical and cellular morphology procedures, estramustine per se has been found to express anticytoskeletal properties through non-covalent binding to microtubule associated proteins (MAP's). In both fish erythrophores and in dividing human prostatic carcinoma cells, estramustine exerts an antimicrotubule effect at micromolar concentrations. Thus, estramustine possesses unique pharmacology and protein binding specificity. As such, it should not be classified as an alkylating agent. The estrogenic effects, while possibly of relevance to clinical administration, are not the primary mechanism by which the drug exerts cytotoxicity.

Key words: Estracyt, Non-steroidal, Non-alkylating, Antimicrotubule agent, Anti-MAPs drug.

Introduction

Although estramustine was synthesized over two decades ago [4] the mechanism by which it exerts its cytotoxicity both in experimental systems and in the clinical management of cancer patients is poorly understood. The volume of literature produced over the last decade (for review see [1]) reflects the enigma which surrounds the drug. Central to most of the mechanistic theories lies the release of estradiol and estrone and the purported alkylating activity of the residual nor nitrogen mustard. While the release of steroids from the parent drug is documented [12, 16, 18] and is of potential therapeutic consequence, there is no evidence, either experimental or clinical, which implicates the mustard moiety in drug activity. Consideration of drug structure and the potential enzymatic cleavage sites (Fig. 1) support the experimental findings that the parent drug, after dephosphorylation, has an extremely long plasma half-life in humans [6–8]. The major metabolite of estramustine, estromustine loses a proton at the 17β position of the steroid D ring, but has activity in cell culture similar to estramustine [10, 11]. Because of the stability of the parent drug, the relatively limited cardiac and other normal tissue toxicity which can be attributed to estrogen release and the complete lack of any normal tissue toxicity which can be attributed to nitrogen mustard, it would seem critical to ascertain whether estramustine per se has pharmacological effects in cellular systems. Recent evidence suggests that the drug exerts antimicrotubule and antimitotic effects and that these are responsible for cytotoxicity [10, 11, 14, 21, 22, 24, 25, 27]. This report details some of the evidence

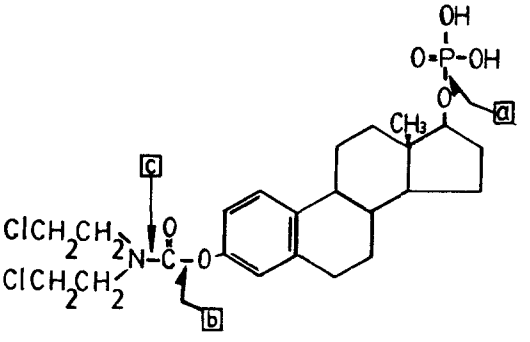


Fig. 1. Structure of estramustine phosphate. *Arrows* indicate bonds which are available for cleavage by (*a*) phosphatases, (*b*) esterases, (*c*) carbaminidases or proteases

Part of this paper was presented at the 5th Congress of the European Society for Urological Oncology and Endocrinology, 18–20 August, 1986, Edinburgh, UK

which supports this concept and suggests that the clinical use of estracyt should perhaps reflect its similarity in mechanism to compounds such as the vinca alkaloids or nocodazole and less on the steroid/mustard components. Such a theory appears iconoclastic with respect to the rationale for estramustine synthesis [4]. However, consideration of drug structure, chemistry, pharmacokinetics and toxicity provide a sound basis for such an assessment of its mechanism of action.

Materials and Methods

Cell Culture

Erythrophores were cultured from the scales of squirrelfish (*Holocentrus ascensionus*) using procedures as outlined previously [20]. Human prostatic DU145 cells were maintained in culture as described [21]. For microscopy and fluorescent studies both cell types were grown on carbon-coated, glow discharged coverslips. Drug effects were recorded by phase contrast microscopy (for live cells) or after glutaraldehyde fixation and immunolabelling for epifluorescent microscopy.

Fluorescent Studies

The synthesis of dansylated estramustine has been described [19]. Beta-tubulin monoclonal antibodies made against pig brain tubulin were provided by Dr. Lester I. Binder, University of Virginia, Charlottesville, VA [3].

Glutathione Studies

Spectrophotometric analyses of glutathione, glutathione reductase and glutathione-S-transferase have been published previously [25].

Mitotic Index Determinations

Use of a differential staining technique has been described [25]. Mitotic index was determined by expressing the percentage of total cells which were traversing prophase, metaphase, anaphase or telophase.

Results

Erythrophores cultured from the scales of squirrelfish (*Holocentrus ascensionus*) provide ideal model cells for studying antimicrotubule agents since (i) they contain thousands of radially aligned microtubules which extend in uniform arrays from a centrally located organizing center, (ii) the cells are filled with red pigment granules which are arranged in rows along the microtubules in erythrophores with their pigment dispersed and (iii) the pigment granules are rapidly transported along the microtubule to and from the cell center at rates of 10 to 20 microns/s. Also, many individual granules move by saltations along the microtubules in dispersed cells. Thus, both the effects of estramustine on a microtubule and the visible function of transport along a microtubule can be monitored in living cells. Figures 2a and 2b show such a cell either with pigment dispersed and contracted, a process which occurs in response to humoral factors. Exposure to estramustine (120 μM for 20 min) caused a cessation of pigment transport and the eventual collapse of the microtubule system (Fig. 2c) wherein the granules stopped moving and remained at the cell center (Fig. 2d). If erythrophores were fixed and immunofluorescently stained with antibody to β-tubulin, the antimicrotubule effects of estramustine were apparent. Figure 3a shows an erythrophore prior to estramustine treatment

---

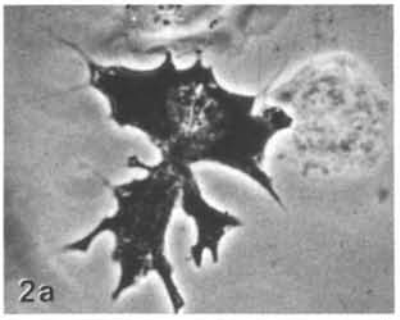
Fig. 2a–d. Effect of estramustine (120 μM at 23 °C) on caffeine stimulated squirrelfish erythrophores. Panels **a** and **b** are cells which are contracting (**b**) and dispersing (**a**) the pigment in a rhythmic fashion. This saltatory pigment pulsing can occur approximately once every few seconds. Panel **c** shows the erythrophore 5 min after drug exposure where pigment has condensed at the cell epicentre and the cellular margins have begun to "round up". Panel **d** shows 20 min after drug exposure where complete aggregation has occurred. Effects are reversible if drug is removed. (Magnification ×430) ▶

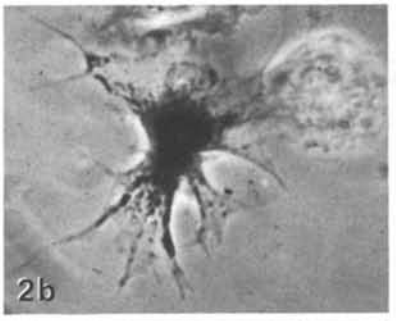
Fig. 3a–c. Squirrelfish erythrophores fixed with glutheraldehyde and stained with β-tubulin antibody and fluorescein isothiocyanate : IgG after estramustine exposure. **a** control; **b** 20 min, 120 μM; **c** drug removed at 15 min followed by 1 h recovery. (Magnification ×430)

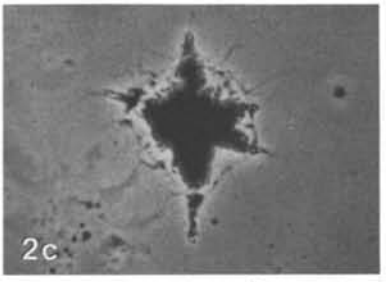
Fig. 4a–d. Gluteraldehyde fixed human prostate carcinoma cells (DU145) stained with β-tubulin antibodies and fluorescein isothiocyanate : IgG. **a** before estramustine showing the bright microtubule array from the nucleus (*N*) to the cell periphery. **b** 60 μM estramustine (37 °C) for 20 min causes the microtubule arrays to break down and collapse onto the nucleus (*N*) in interphase cells. **c** similar treatment results in the removal of pole to chromosome spindle fibres (*small arrow*) in a metaphase mitotic figure. **d** the midbody between two daughter cells at cytokinesis (*small arrow*) shows that the microtubules which form this structure are relatively resistant to the drug. (Magnification **a** and **b** ×800; **c** and **d** ×1,600)

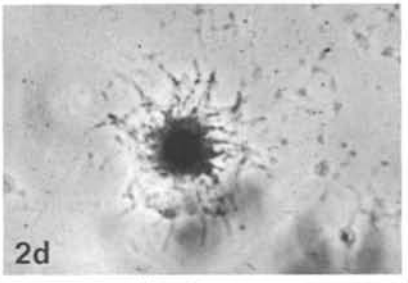
Fig. 5a–f. Fluorescent micrographs (490 nm) of erythrophores (**a, d**), DU145 cells (**b, e**) and rat sperm (**c, f**) stained with 120 μM dansylated estramustine following detergent lysis (**a, b, c**) and lysis plus extraction with 0.3 M KCl (**d, e, f**). The microtubule structures were stained in lysed cells and the 0.3 M KCl removed this material. (Magnification ×800)

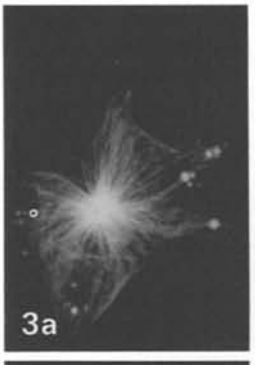
Fig. 6a–c. Uptake of dansylated extramustine in live DU145 cells. **a** shows a cluster of DU145 cells by phase contrast. **b** 5 min exposure to 60 μM dansylated estramustine. **c** 20 min after drug. **b** and **c** were photographed using fluorescent optics at 490 nM (*n* = nucleus). (Magnification ×430)


2a

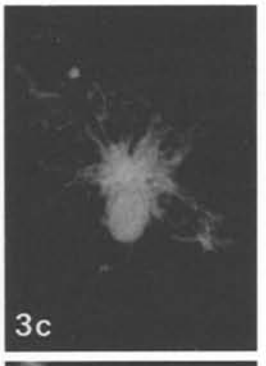

2b

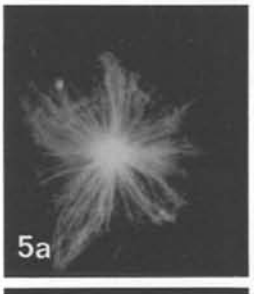

2c

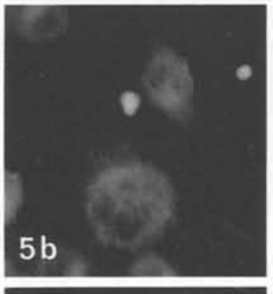

2d

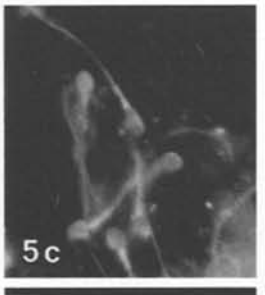

3a

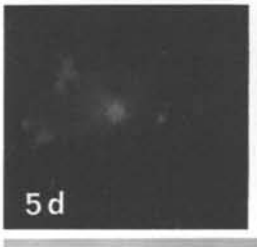

3b

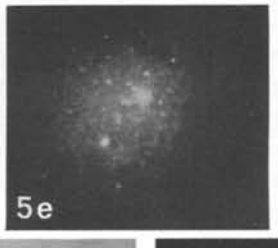

3c


5a

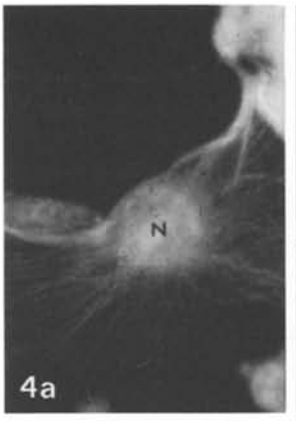

5b


5c

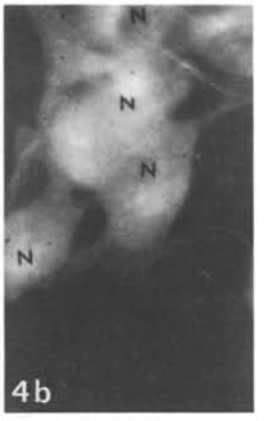

5d


5e

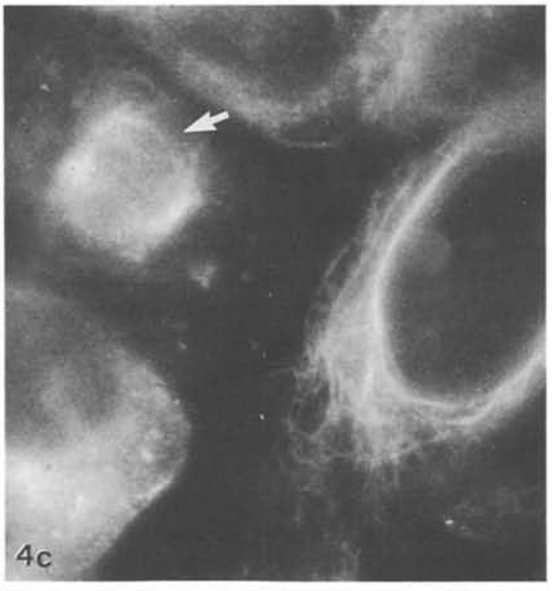

5f

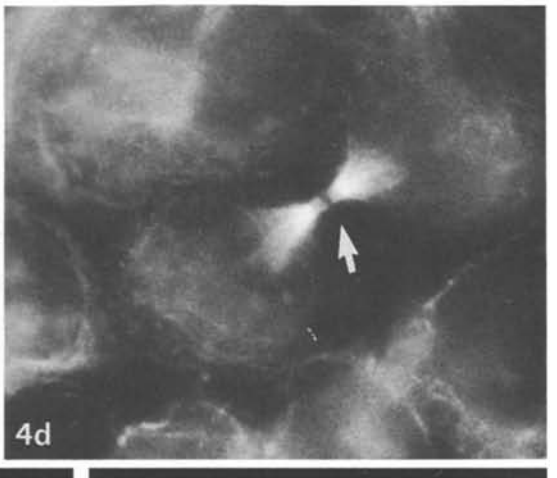


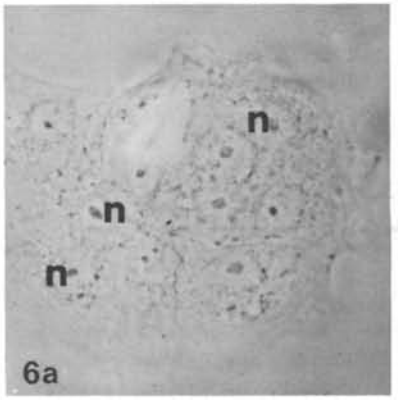
4a



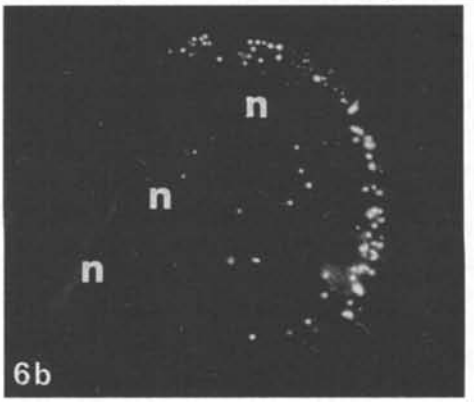
4b


4c

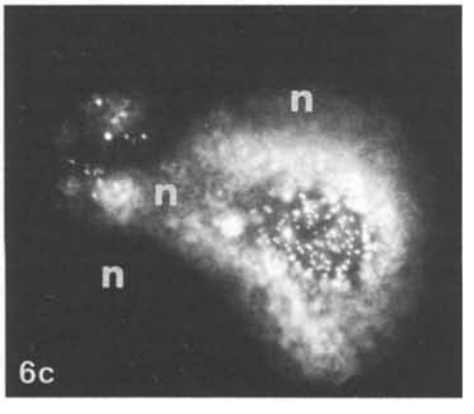

4d



6a



6b



6c

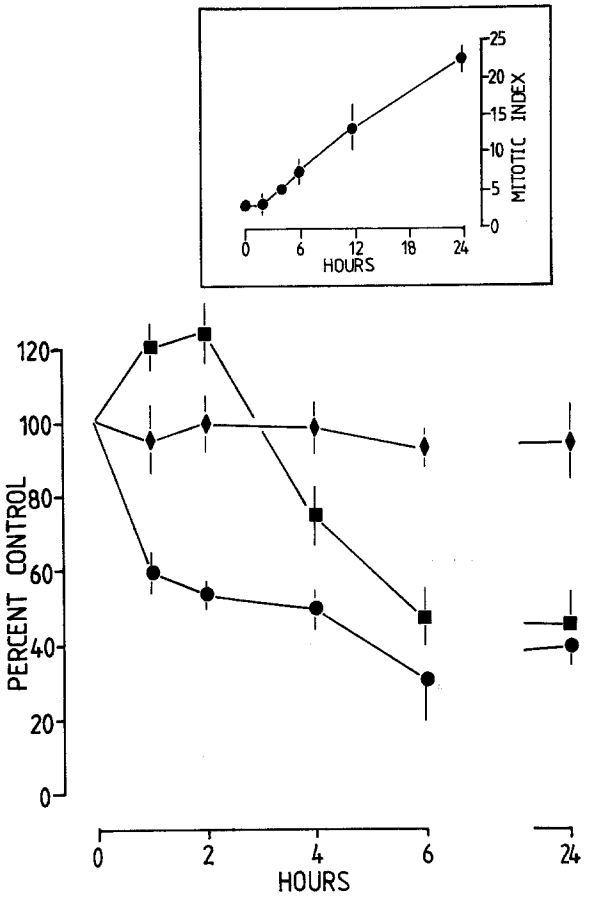


Fig. 7. The effect of 10 μM estramustine on intracellular glutathione (•), glutathione-S-transferase activity (▪) and glutathione reductase activity (♦) in DU145 cells. The *insert* shows the mitotic index increase which is concomitant with 10 μM estramustine exposure

and Fig. 3b shows a cell after exposure to drug (120 μM for 20 min). The results demonstrate that the ordered, radial microtubule array was destroyed as a result of drug treatment. If drug was removed by washing cells with fresh medium, a slow recovery of the network occurred (Fig. 3c). The reassembled microtubules exhibited a "wavy" appearance (compare Fig. 3a, c).

Similar studies using DU145 human prostatic carcinoma cells are shown in Fig. 4. In untreated cells, a radial array of microtubules extends from the cell nucleus to the cell margins (Fig. 4a). Exposure to estramustine (120 μM for 20 min) resulted in removal of the ordered microtubule arrays, with the concomitant "rounding up" of cells (Fig. 4b) Figures 5a and 5b show interphase cells where residual microtubules remain in the perinuclear region following drug treatment. Figure 4c also shows that estramustine removes the spindle fibres of metaphase DU145 cells, a response typical of the anti-mitotic effects of estramustine. Interestingly, at later stages of mitosis, when daughter cells separate at cytokinesis (Fig. 4d) the microtubules which form the midbody are highly resistant to the effects of estramustine.

Taken together, the micrographs of Figs. 2–4 suggested that estramustine exerts antimicrotubule effects in cells of diverse evolutionary development. Cytotoxicity occurred in cells capable of division and in committed, nondividing cells; in both interphase and in mitotic cells; in cells which contain estramustine binding protein (DU145) and cells which do not (erythrophores).

By attaching a fluorescent probe (dansyl chloride) onto the 17β-OH group of estramustine [19] it was possible to follow drug uptake into living cells and subsequently to investigate intracellular binding sites. Figure 5 shows the fluorescent drug binds to microtubules in 0.190 triton x 100 lysed, gluteraldehyde-fixed squirrelfish erythrophores (Fig. 5a), DU145 cells (Fig. 5b) or to axonemal microtubules of rat sperm (Fig. 5c). In each case, there was obvious staining of the microtubules and in DU145 cells the golgi network and vesicles were also stained (Fig. 5b). By washing the cells with 0.3 M KCl prior to glutaraldehyde fixation, the microtubule associated proteins were removed from microtubules which were preserved intact with the use of 1 μM taxol in the lysis buffer. In Fig. 5d, 5e and 5f of the same cells shown in Fig. 5a, 5b and 5c respectively, it was shown that drug binding was reduced markedly for each cell. These experiments demonstrate that estramustine can bind a microtubule associated component (MAPs). Figure 6a showed a cluster of live DU145 cells attached to a coverslip and photographed by phase microscopy. Following exposure of these cells to dansylated estramustine, drug uptake occurred in discrete vesicles (Fig. 6b) which increased in number with time (Fig. 6c). With time (30 min) the cells became diffusely stained as drug was released intracellularly to bind to other cellular components (Fig. 6c). In comparison, dansylated estramustine diffused into erythrophores and stained the cell uniformly so that noticeable vesicle uptake was not apparent.

The thiol reactivity of estramustine was monitored by following its effects on glutathione and associated enzymes in DU145 cells (Fig. 7). The drug-induced increase in mitotic index (insert) was accompanied by a depletion of intracellular glutathione and delayed inhibition of glutathione-S-transferase activity. This appeared to be a relatively specific effect, since glutathione reductase activity was not affected by drug treatment. The glutathione-S-transferases are known to have steroid-binding properties and to be involved in detoxification, both mechanisms involving reactive thiol groups.

Discussion

The metabolism of estracyt in cell culture, in animals or in humans will depend upon potential enzymatic cleavage of the bonds shown in Fig. 1. Dephosphorylation is readily achieved in most systems and the primary metabolites are estramustine or the oxidised metabolite estromustine [6–8]. Because esterases are relatively common enzymes, the cleavage of the ester bond (Fig. 1, arrow b) can occur resulting in the liberation of estradiol and its oxidised analogue estrone. In fact, clinical pharmacokinetic studies suggest

Table 1. Summary of evidence that estramustine induces cytotoxicity without alkylating activity

	Reference
1. In vitro alkylation of 4-(p-nitrobenzyl) pyridine does not occur	[23]
2. Drug effects are readily reversible if drug is removed prior to cytolysis	[19, 21]
3. Nitrogen mustard resistant cells are sensitive to estramustine	[24]
4. Drug binding is non-covalent	[23]
5. Estramustine causes no direct DNA damage	[23]
6. Estramustine has no clastogenic activity	[24]
7. Accumulates cells in metaphase	[10, 24]
8. Kills cells in any phase of the cell cycle, including non-dividing	[19, 21]
9. The in vitro and in vivo half-life of estramustine is extremely long	[6–8, 18]
10. Dose limiting normal tissues are atypical of mustard therapy toxicity	[1, 15]

Table 2. Antimitotic agents which incorporate a-N(H)-C(=O)-linkage into their structure

1. Isopropyl-N-phenylcarbamate	6. Colcemid
2. Nocodazole[a]	7. Taxol
3. Benzimidazole carbamate(s)[a]	8. Ergocryptine
4. Dihydropyrido pyrazine(s)	9. Maytansine
5. Colchicine	10. Cytochalasin B

[a] Structure activity studies with a series of these compounds have shown the -N(H)-C(=O)-R group is critical to the tubulin binding properties [9]

that compared to estramustine and estromustine, these products constitute a relatively small proportion of the metabolites following oral estracyt treatment [18]. The release of a mustard moiety is dependent upon the cleavage of the carbamate bond (Fig. 1, arrow c). In general, carbamates demonstrate a high degree of stability [26], possibly because the nitrogen atom conveys resonance stabilization. As discussed previously [22], the pi electrons of the nitrogen can demonstrate an affinity for the double bond oxygen, thereby preventing electron withdrawal and subsequent formation of electrophilic chloroethylating species. It has been suggested that the carbamate could be susceptible to cleavage by carbaminidases [18] or proteases [22], but in neither case has this been demonstrated experimentally. In fact, the experimental evidence which is summarized in Table 1 supports the conclusion that a mustard species is not formed during estramustine metabolism.

The long clinical half-life and the relative lack of metabolism of estramustine raises the issue of whether the parent molecule is responsible for the drug's pharmacological properties. The data outlined in this paper, together with a number of previous reports [10, 12, 18, 19, 21, 23, 24, 27] indicate that estramustine exhibits cytotoxic properties per se. It is interesting to note that the carbamate ester bridge found in estramustine is also present in a number of other antimitotic agents, some of which are listed in Table 2. In structure activity studies of a series of benzimidazole carbamates this group was considered to be the most crucial in determining antimicrotubule properties [9]. Furthermore, these drugs also possess a ring structure (of various complexity) and a hydrophilic side chain on either side of the carbamate ester bond. Estramustine differs from these agents in two important aspects: (i) in most of the tubulin binding drugs, the nitrogen is protonated; (ii) in estramustine, the nitrogen is attached to a hydrophilic side chain as opposed to the ring system for the other drugs. Presumably these differences can influence the target specificity of each drug and may be the reason estramustine binds to MAPs, thereby disassociating them from, or interfering with, their normal interaction with tubulin. This produces an antimicrotubule effect, but is distinct from other tubulin binding drugs. In theory, the hydrophobic interaction of the ring system, together with potential hydrogen bonding of the carbamate ester group with amino acids in the target protein could stabilize the drug interaction with MAPs.

At present, estramustine is known to bind to at least three intracellular proteins, MAPs [14, 21, 27], estramustine binding protein (EBP; [5]), and glutathione-S-transferases [25]. The binding affinities are unknown for the latter two groups of proteins. The drug binds non-covalently to EBP which has been shown to be present in a distinct intracellular compartment. For example, EBP has been localized in prostatic vesicles [2] and may be responsible for drug uptake and accumulation. The release of drug from these vesicles with time (cf. Fig. 7) would provide drug to bind with cytoskeletal MAPs, and thereby destroy microtubules. Glutathione-S-transferases which have been recognised as steroid binding proteins, may act as competitive binding proteins in their additional role of detoxification [25].

Finally, it may be worth considering the clinical use of estracyt with respect to its antimicrotubule properties. The relative lack of specificity of this drug places an emphasis on the ability of EBP to concentrate it in the prostate. Increased therapeutic efficacy will result primarily from this concentrating effect, although the presence of tumor specific MAPs may prove to be important, since MAP-1, a 330 Kd protein, is the only MAP detected in cultured human prostatic DU145 cells (unpublished data). For

estramustine to be effective in vitro, it is required that micromolar intracellular concentrations of drug be maintained for some time. Whether this occurs in patients who receive estracyt is not clear and might encourage a reassessment of the treatment regimes which employ the drug. It is interesting to note that estracyt is frequently compared both preclinically [13] and clinically [1] with diethylstibestrol, a steroid which has also been documented to have antimitotic efficacy [17].

References

1. Ahlgren JD, Schein PS (1983) Seminars in oncology, Vol X, No 3, Suppl 3
2. Aumuller G, Seitz J, Heyns W, Flickinger CJ (1984) Intracellular localization of prostatic binding protein in rat prostate by light and electron microscopic immunocytochemistry. Histochemistry 76:497–516
3. Cacares A, Payne MR, Binder LI, Oswald S (1983) Immunocytochemical localization of actin and microtubule associated protein MAP 2 in dendritic spines. Proc Natl Acad Sci USA 80:1738–1742
4. Fex H, Hogberg B, Konyves I (1984) Estramustine phosphate-historical overview. Urology 23:4–5
5. Forsgren B, Bjork P, Carlstrom K, Gustafsson JA, Pusette A (1979) Purification and distribution of a major protein in rat prostate that binds estramustine, a nitrogen mustard derivative of estradiol-17β. Proc Natl Acad Sci USA 76:3149–3153
6. Gunnarsson PO, Andersson SB, Johansson SA, Nilsson T, Plym-Forshell G (1984) Pharmacokinetics of estramustine phosphate (Estracyt) in prostatic cancer patients. Eur J Clin Pharmacol 26:113–119
7. Gunnarsson PO, Forshell GP (1984) Clinical pharmacokinetics of estramustine phosphate. Urology 23:22–27
8. Gunnarsson PO, Forshell GP, Fritjofsson A, Norlen BJ (1981) Plasma concentrations of estramustine phosphate and its major metabolites in patients with prostatic carcinoma treated with different doses of estramustine phosphate (Estracyt). Scand J Urol Nephrol 15:201–205
9. Gupta RS (1986) Cross resistance of nocodazole-resistant mutants of CHO cells toward other microtubule inhibitors. Similar mode of action of benzimidazole carbamate derivatives and NSC 181928 and TN16. Mol Pharmacol 30:142–148
10. Hartley-Asp B (1984) Estramustine-induced mitotic arrest in two human prostatic carcinoma cell lines, DU145 and PC-3. Prostate 5:93–100
11. Hartley-Asp B, Gunnarsson PO (1982) Growth and cell survival following treatment with estramustine, nor-nitrogen mustard, estradiol and testosterone of a human prostatic cancer cell line (DU145). J Urol 127:818–22
12. Hoisaeter PA, Bakke A (1983) Estramustine phosphate (Estracyt): experimental and clinical studies in Europe. Semin Oncol 10:27–33
13. Kalland T, Haukaas SA (1984) Effects of diethylstilbestrol and estramustine phosphate (Estracyt) on natural killer cell activity and tumor susceptibility in male mice. Prostate 5:649–660
14. Kanje M, Deinum J, Wallin M, Ekstrom P, Edstrom A, Hartley Asp B (1985) Effect of estramustine phosphate on the assembly of isolated bovine brain microtubules and fast axonal transport in the frog sciatic nerve. Cancer Res 45:2234–2239
15. Lopez R, Karakousis CP, Didolkar MS, Holyoke ED (1978) Estramustine phosphate in the treatment of advanced malignant melanoma. Cancer Treat Rep 62:1329–1332
16. Muntzing J, Gunnarsson K (1984) Preclinical pharmacology and toxicology of estramustine phosphate. Urology 23:6–10
17. Parry EM, Danford N, Parry JM (1982) Differential staining of chromosomes and spindle and its use as an assay for determining the effect of diethylstilbestrol on cultured mammalian cells. Mutat Res 105:243–252
18. Sandberg AA (1983) Metabolic aspects and actions unique to Estracyt. Semin Oncol 10:3–15
19. Stearns ME, Jenkins DP, Tew KD (1985) Dansylated estramustine, a fluorescent probe for studies of estramustine uptake and identification of intracellular targets. Proc Natl Acad Sci USA 82:8483–8487
20. Stearns ME, Ochs RL (1982) A functional in vitro model for studies of intracellular motility in digitonin permeabilized erythrophores. J Cell Biol 94:727–739
21. Stearns ME, Tew KD (1985) Antimicrotubule effects of estramustine, an antiprostatic tumor drug. Cancer Res 45:3891–3897
22. Tew KD (1983) The mechanism of action of estramustine. Semin Oncol 10:21–26
23. Tew KD, Erickson LC, White G, Wang AL, Schein PS, Hartley-Asp B (1983) Cytotoxicity of estramustine, a steroid-nitrogen mustard derivative, through non-DNA targets. Mol Pharmacol 24:324–328
24. Tew KD, Hartley-Asp B (1984) Cytotoxic properties of estramustine unrelated to alkylating and steroid constituents. Urology 23:28–33
25. Tew KD, Woodworth A, Stearns ME (1986) Relationship of glutathione depletion and inhibition of glutathione-S-transferase activity to the antimitotic properties of estramustine. Cancer Treat Rep 70:715–720
26. Verbiscar AJ, Abbod LG (1970) Carbamate ester latentiation of physiologically active amines. J Med Chem 13:1176–1179
27. Wallin M, Deinum J, Friden B (1985) Interaction of estramustine phosphate with microtubule-associated proteins. FEBS Lett 179:289–293

K. D. Tew, Ph.D.
Department of Pharmacology
Fox Chase Cancer Center
7701 Burholme Avenue
Philadelphia, PA 19111
USA