Testosterone-induced DNA synthesis in cultured rat ventral prostate: Effects of estracyt and its derivatives

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Summary Testosterone-induced DNA synthesis in cultured rat ventral prostate was used to compare the direct effects of Estracyt® and Emcyt® with that of their metabolite, estramustine, and their carrier-hormone, oestradiol-17β on prostatic growth. In serum-supplemented medium (5–20% FCS), all the compounds were equally effective in suppressing testosterone stimulated DNA synthesis which was reduced by between 40–50%, whereas in serum-free medium the estramustine compounds were consistently less effective than oestradiol-17β. In the presence of 4 x 10⁻⁷ M testosterone in serum-free medium, stimulated DNA synthesis was reduced by 15–30% following incubation with 4 x 10⁻⁷ M of Estracyt®, Emcyt® and estramustine and by 60% with 4 x 10⁻⁷ M oestradiol-17β. Thus, none of the estramustine compounds appear to offer any selective advantage over that of oestradiol-17β in suppressing prostatic DNA synthesis at the target tissue level.

Prostatic cancer is the most common malignancy of the male urogenital tract and a leading cause of death due to cancer in aging men (Chisholm, 1981; Flanders, 1984). As conventional oestrogen therapy for prostatic cancer is not curative, several new drugs have recently been developed in an attempt to enhance tumour selectivity of chemotherapeutic agents and thereby improve the clinical management of prostatic cancer patients. Among these, the hormone-cytotoxic agent Estracyt® (estramustine phosphate; Emcyt®, estramustine phosphate disodium) has been extensively investigated for use in the treatment of prostatic cancer (Jonsson & Hogberg, 1971; Nilsson & Jonsson, 1977; Edsmyr et al., 1982; Hoisaeter & Bakke, 1983; Murphy et al., 1983; Walzer et al., 1984). The rationale for Estracyt therapy is based on the concept that the hormonal moiety of the drug complex, oestradiol-17β, would impart tissue specificity by interacting with steroid hormone receptors in the prostate and thus result in a selective accumulation of the alkylating agent, a non-nitrogen mustard, within the target tissue. Yet, despite both clinical and experimental investigations, the mechanism of action of Estracyt remains unknown (Tew, 1983; Hoisaeter, 1984).

In both the rat (Plym-Forsell & Nilsson, 1974; Hoisaeter, 1976a, 1977; Hoisaeter & Bakke, 1983) and man (Plym-Forsell et al., 1976; Sandberg, 1983), Estracyt is rapidly dephosphorylated to yield estramustine and its dehydrogenated counterpart, estramustine. These metabolites are selectively retained in the prostate by interactions with estramustine binding protein (Forsgren et al., 1979, 1981; Bjork et al., 1982) and subsequent intracellular hydrolysis of these compounds slowly liberates free nitrogen mustard and oestrogen moieties. However, the question remains whether the antiprostatic actions of Estracyt are mediated by the oestrogen moiety, the nitrogen mustard moiety or the intact drug molecule itself.

While in vivo studies have shown that Estracyt is a more potent inhibitor of rat prostatic DNA synthesis than either its hormone or cytostatic parts (Hoisaeter, 1976b, 1977), such comparisons are limited by the extensive metabolism of Estracyt in vivo. Thus, in the present study, an in vitro model of induced DNA synthesis in cultured rat ventral prostate (Mistry et al., 1982; Buchanan & Riches, 1985, 1986) was used to compare the direct effects of Estracyt, Emcyt, estramustine and oestradiol-17β on prostatic growth.

Materials and methods

Organ culture

Ventral prostate from young adult Wistar rats (4–6 months old) was maintained in serum free and serum-supplemented (5, 10 or 20% foetal calf serum) organ culture for 4 days (Riches et al., 1976; Mistry et al., 1982; Buchanan & Riches, 1985, 1986). Explants of approximately 2 mm³ were prepared with 16 explants per treatment group.

Additives

Unless otherwise stated, testosterone was added alone or in combination with Estracyt (LS 299), Emcyt (LS 299Z), estramustine (LS 275) or oestradiol-17β on day 0 of the culture period and replenished following the medium change on day 2. The steroids were first dissolved in absolute alcohol and then diluted in Waymouths medium to the desired final concentration. Control cultures were maintained in medium only or received an equal volume of the alcohol diluent. The estramustine compounds (LS 299, LS 299Z, LS 275) were a generous gift of AKTIEBOLAGET LEO Research Laboratories, Helsingborg, Sweden.

¹²³Iododeoxyuridine (I-UDr) uptake

During the final 24 h of the culture period, each culture was labelled with 37 kBq (1 μCi) of ¹²³Iododeoxyuridine (I-UDr: specific activity 185 GBq mg⁻¹, Amersham) as previously described (Mistry et al., 1982). The explants were then fixed in Bouins fluid, washed in 70% alcohol and weighed (Riches et al., 1976).

I-UDr uptake (cpm mg⁻¹ tissue) was monitored in a Minigamma counter and the results expressed as a Stimulus Index such that the testosterone only supplemented control cultures equaled 1.0. In the cultures supplemented with foetal calf serum and testosterone, explants are expressed relative to 0% FCS and testosterone addition. All the uptakes are plotted as mean ± s.e. and represent the pooled results from 3 replicate experiments. Following the determination of I-UDr uptake, the explants were processed for routine light microscopy.

Data analysis

In each experiment, 16 explants were randomly allocated per treatment group and comprised 4 replicate cultures. Data are presented as the mean ± s.e. from n>3 experiments. Data were analyzed by analysis of variance and Duncan’s multiple range test (P<0.01).

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Results

Effects of serum

I-UdR uptake  Figure 1 illustrates the effects of $4 \times 10^{-5}$M Estracyt, estramustine and oestradiol-17β on the proliferative response of rat ventral prostate to $4 \times 10^{-8}$M testosterone following four days of organ culture in medium containing 0, 5, 10 or 20% foetal calf serum. I-UdR uptakes are normalised to the 0% FCS and testosterone-stimulated measurements. In serum-free medium, only oestradiol-17β significantly ($P<0.01$) inhibited the proliferative response to testosterone. Estracyt was not significantly inhibitory in serum free cultures ($P>0.05$). However, in serum supplemented media, the estramustine compounds and oestradiol-17β all reduced the testosterone response to the level of the unstimulated controls.

![Figure 1](image1.png)

**Figure 1** Effects of $4 \times 10^{-5}$ Estracyt (LS 299), estramustine (LS 275) and oestradiol-17β (E$_2$-17β) on $4 \times 10^{-8}$M testosterone stimulated I-UdR uptake in serum-free and serum-supplemented (5, 10, 20% FCS) cultures of rat ventral prostate. Testosterone-free control cultures are represented by □ and testosterone supplemented control cultures by ○ (mean ± s.e.: 16 explants/treatment group, 3 separate experiments).

Histology  Normal rat ventral prostate consists of tubulo-alveolar glands which are lined by a single layer of columnar epithelial cells (Figure 2A). Cultures maintained in serum-supplemented medium without testosterone underwent epithelial atrophy, whereas the addition of $4 \times 10^{-8}$M testosterone maintained the height and secretory activity of the alveolar epithelium and promoted epithelial cell proliferation (Figure 2B). Similar cultures treated with testosterone and $4 \times 10^{-8}$M Estracyt (Figure 2C) or estramustine exhibited extensive necrosis of the alveolar epithelium, whereas treatment with $4 \times 10^{-8}$M oestradiol-17β caused marked epithelial atrophy (Figure 2D).

In serum-free medium, cultures maintained in the absence of testosterone were atrophic, whereas supplementation with $4 \times 10^{-8}$M testosterone preserved the normal morphology and secretory activity of the epithelium (Figure 3A). Similar cultures treated with $4 \times 10^{-8}$M testosterone and $4 \times 10^{-8}$M Estracyt (Figure 3B) or estramustine also exhibited well-maintained alveolar epithelium. In contrast, treatment with $4 \times 10^{-8}$M oestradiol-17β was cytotoxic causing widespread necrosis of the alveolar epithelium and fibromuscular stroma (Figure 3C).

![Figure 2](image2.png)

**Figure 2** Alveoli in a fresh fixed specimen and in explants of young adult rat ventral prostate cultured for 4 days in medium containing 5% foetal calf serum H & E staining. (A) Control (uncultured) section showing the columnar epithelium of rat ventral prostate (×500). (B) Explant treated with $4 \times 10^{-6}$M testosterone showing actively secreting columnar epithelium (×500). (C) Explant treated with $4 \times 10^{-8}$M testosterone and $4 \times 10^{-8}$M Estracyt showing marked degeneration and necrosis of the secretory epithelium (∗×500). (D) Explant treated with $4 \times 10^{-8}$M testosterone and $4 \times 10^{-5}$M oestradiol-17β showing dilated alveoli and epithelial atrophy (∗×500).

Effects of concentration

I-UdR uptake  The effects of $4 \times 10^{-9}$, $4 \times 10^{-7}$ and $4 \times 10^{-5}$M Estracyt, Emcyt, estramustine and oestradiol-17β on the proliferative response of rat ventral prostate to $4 \times 10^{-7}$M testosterone following four days of culture in serum-free medium are shown in Figure 4. At $4 \times 10^{-9}$ and $4 \times 10^{-7}$M, oestradiol-17β significantly ($P<0.01$) reduced the testosterone effect and was markedly inhibitory ($P<0.01$) at $4 \times 10^{-5}$M. In contrast, the estramustine compounds were consistently less effective than oestradiol-17β at all concentrations used.

Using the same procedure, Figure 5 demonstrates the effects of $4 \times 10^{-9}$, $4 \times 10^{-7}$ and $4 \times 10^{-5}$M Estracyt, Emcyt, estramustine and oestradiol-17β on the proliferative response to $4 \times 10^{-8}$M testosterone, which is the minimum dose of testosterone for maximum stimulation of I-UdR uptake in serum-free cultures of rat ventral prostate (Mistry et al., 1982). Treatment with $4 \times 10^{-9}$ and $4 \times 10^{-7}$M oestradiol-17β reduced the testosterone response to the level of the testosterone-free controls ($P<0.01$), whereas equimolar concentrations of the estramustine compounds were less effective. At $4 \times 10^{-5}$M, Estracyt, Emcyt, and estramustine
also reduced the testosterone response to control levels but treatment with $4 \times 10^{-9} \text{M oestradiol-17\beta}$ remained more inhibitory. Pre-treatment with the estramustine compounds ($4 \times 10^{-6} \text{M}$) for 48 h before the addition of $4 \times 10^{-6} \text{M}$ testosterone did not enhance the inhibitory actions of these drugs, and oestradiol-17\beta remained the most potent inhibitor of the testosterone response (Figure 6).

**Histology**

Histologically, cultures treated with $4 \times 10^{-7} \text{M}$ testosterone alone or in combination with $4 \times 10^{-5} \text{M}$ Estracyt, Emcyt or estramustine in serum-free medium all showed well maintained, actively secreting alveolar epithelium. However, similar cultures treated with $4 \times 10^{-7} \text{M}$ testosterone and $4 \times 10^{-5} \text{M}$ oestradiol-17\beta exhibited extensive epithelial and stromal necrosis.

Cultures treated with only $4 \times 10^{-9} \text{M}$ testosterone were well-preserved (Figure 7A), but the addition of $4 \times 10^{-5} \text{M}$ Estracyt (Figure 7B), Emcyt or estramustine caused epithelial atrophy. However, treatment with $4 \times 10^{-7} \text{M}$ oestradiol-17\beta also produced severe epithelial regression (Figure 7C) and $4 \times 10^{-3} \text{M}$ oestradiol-17\beta remained cytotoxic.

**Discussion**

While several studies have demonstrated inhibitory effects of Estracyt on prostatic DNA synthesis (Forsberg & Holsaeter, 1975; Holsaeter, 1975, 19766, 1977), others have been unable to distinguish the effect of Estracyt from that of oestradiol-17\beta (Yamanaka et al., 1977; Wakisaka et al., 1979; Mistry et
In the present study, treatment with $4 \times 10^{-5}$ M Estracyt, estramustine and oestradiol-17β in serum supplemented medium all had equally inhibitory effects on $4 \times 10^{-6}$ M testosterone induced I-UdR uptake, whereas in serum-free medium only oestradiol-17β was effective in suppressing the response to testosterone. The histological results further demonstrated that the estramustine compounds had no antiprostatic effect in serum-free medium, but were cytotoxic in the presence of serum. In contrast, treatment with oestradiol-17β was markedly cytotoxic in serum free medium, whereas it had an antiandrogenic effect causing epithelial atrophy in the presence of serum. Thus, serum appears to potentiate the antiprostatic activity of the estramustine compounds, while it reduces the inhibitory action of oestradiol-17β. The presence of steroid hormone binding proteins in the serum supplement may be responsible for eliminating the cytotoxic effect of oestradiol-17β and reducing the magnitude of the inhibitory effect on I-UdR uptake by decreasing the concentration of free oestrogen in the medium. Similarly, the presence of steroid binding proteins may account for slight reductions in the stimulatory effect of testosterone on I-UdR uptake in serum supplemented cultures. However, the results did not indicate that increasing serum concentrations further affected the actions of either testosterone or oestradiol-17β.

In contrast to the present study, Hoisaeter (1975) found that in organ cultures of rat ventral prostate maintained in serum-supplemented medium (5% FCS) containing $4 \times 10^{-6}$ M testosterone and either $4 \times 10^{-5}$ M Estracyt or oestradiol-17β, only Estracyt had a significantly inhibitory effect on $^3$H-TdR uptake. Nevertheless, the histological results were remarkably similar to the present study, indicating that Estracyt had a pronounced cytotoxic effect on rat ventral prostate cultured in serum-supplemented medium while the retrogressive changes associated with oestradiol-17β were less severe. Unlike the present study, however, the morphology of cultures maintained in the absence of exogenous testosterone was comparable to intact tissue and did not demonstrate epithelial atrophy typical of androgen deprivation (Hoisaeter, 1975). Moreover, Hoisaeter (1975) was unable to demonstrate any stimulation of $^3$H-TdR uptake in cultures treated with only $4 \times 10^{-6}$ M testosterone and this was attributed to a masking effect caused by endogenous hormones present in the serum supplement. While variations in the hormone content of different batches of foetal calf serum (Esber et al., 1973) may be responsible for these contrasting results, the present study further suggests that serum supplementation obscures differences between the effect of oestradiol-17β and its alkylated analogues.

Subsequent investigations using only serum-free medium showed that oestradiol-17β remained consistently more effective than any of the estramustine compounds in suppressing testosterone induced I-UdR uptake. Oestradiol-17β exhibited dose-related inhibitory effects on both $4 \times 10^{-7}$ and $4 \times 10^{-8}$ M testosterone stimulated I-UdR uptake, whereas the estramustine compounds were only inhibitory at $4 \times 10^{-5}$ M in cultures treated with physiological concentrations of testosterone (i.e. $4 \times 10^{-9}$ M). Pre-treatment (48 h) with the estramustine compounds did not enhance their ability to suppress the testosterone response, and their addition 48 h after testosterone reduced the magnitude of the inhibitory response. The histological results further demonstrated that treatment with $4 \times 10^{-9}$ M testosterone and any of the estramustine compounds at $4 \times 10^{-5}$ M caused epithelial atrophy, however lower concentrations of oestradiol-17β produced comparable inhibition of I-UdR uptake and similar histological changes. Thus, the anti-androgenic effects of the estramustine compounds may be
due to the release of some oestradiol-17β from the hormone cytosolic complex. Although Hoisæter (1975) did not detect any hydrolysis of Estracyt by rat ventral prostate in organ culture, more recently Symes & Milroy (1982) found that the cleavage rate of estramustine by prostatic tissue in vitro is low but it is nevertheless, dose dependent, being greater at $10^{-5}$ than $10^{-6}$ M. The inhibitory effects described with compounds in the range of $10^{-9}$ to $10^{-8}$ M would be in the physiological range of concentrations. Steady state concentrations of estramustine in the plasma are of a similar range to those used in these experiments ($10^{-9}$ to $10^{-7}$ M) (Hartley-Asp & Gunnarsson, 1982).

While the oestradiol-17β released upon hydrolysis of Estracyt probably contributes to the antiprostastic activity of this drug (Sandberg, 1983), a purely oestrogenic mode of action for Estracyt and its derivative, should be excluded.

References

BJÖRK, P., FORSGREN, N., GUSTAFSSON, J.-A., POUSSETTE, A. & HÖGBERG, B. (1982). Partial characterization and ‘quantitation’ of human prostatic estramustine binding protein. Cancer Res., 42, 1935.

BUCHANAN, L. J. & RICHES, A.C. (1985). Proliferative responses of normal rat ventral prostate in organ culture: effects of testosterone and its metabolites in chemically defined medium. Prostate, 7, 419.

BUCHANAN, L. J. & RICHES, A.C. (1986). Proliferative responses of rat ventral prostate: effects of variations in organ culture media and methodology. Prostate, 6, 13.

CHRISTOLM, G.D. (1981). Perspectives and prospects. Recent Results Cancer Res., 78, 178.

EDSMYR, F., ANDERSON, L. & KONYVES, I. (1982). Estramustine phosphate (Estracyt): Experimental studies and clinical experience. In Prostate Cancer, G.H. Jacob & R. Hohenfellner (Eds.), 51, William and Wilkins, Baltimore, Maryland.

ESBER, J.J., PAYNE, J.J., BOGDEN, A.E. (1973). Variability of hormone concentration and ratios in commercial sera used for tissue culture. J. Natl Cancer Inst., 50, 559.

FLANDERS, D.W. (1984). Review: Prostate Cancer Epidemiology. Prostate, 5, 621.

FORSGREN, B., BJÖRK, P., CARLSTROM, K., GUSTAFSSON, J.-A., POUSSETTE, A. & HÖGBERG, B. (1979). Purification and distribution of major protein in rat prostatic tissue that binds estramustine, a nitrogen mustard derivative of estradiol-17β. Proc. Nat. Acad. Sci. USA, 76, 3149.

FORSGREN, B., BJÖRK, P., CARLSTROM, K., GUSTAFSSON, J.-A., POUSSETTE, A. & HÖGBERG, B. (1981). The presence in rat and human prostate of proteins that bind steroid-cytostatic complexes. In The Prostatic Cell: Structure and Function, p. 391. Alan R. Liss, Inc., New York.

HARTLEY-ASP, B. (1984). Estramustine induced mitotic arrest in two human prostatic carcinoma cell lines DU 145 and PC-3. Prostate, 5, 93.

HARTLEY-ASP, B. & GUNNARSSON, P.O. (1982). Growth and cell survival following treatment with estramustine, nor-nitrogen mustard, estradiol and testosterone of a human prostatic cancer cell line (DU 145). J. Urol., 127, 818.

HOISÆTER, P.A. (1975). Incorporation of $^{14}C$-thymidine into rat ventral prostate in organ culture. Influence of hormone-cytostatic complexes. Invest. Urol., 12, 479.

HOISÆTER, P.A. (1976a). Studies on the conversion of estradiol linked to a cytostatic agent (Estracyt$^7$) in various rat tissues. Acta Endocrinol., 82, 161.

HOISÆTER, P.A. (1976b). Incorporation of $^{14}C$-thymidine and $^{14}C$-aminos into the ventral prostate after in vivo treatment with estradiol-3N-bis (2 cholethyl) carbonate-17β-phosphate (Estracyt) and its estrogen and cytostatic parts. Invest. Urol., 14, 85.

HOISÆTER, P.A. (1977). Effect of steroid alkylating agent (Estramustine phosphate) in rat ventral prostate. Scand. J. Urol. Nephrol., Suppl. 46, 62.

HOISÆTER, P.A. (1984). Mode of action of Estracyt. Urology, XII (Suppl.), 46.