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Growth Inhibiting Effect of Estramustine on Two Prostatic Carcinoma Cell Lines, LNCaP and LNCaP-r

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Summary. The effect of estramustine (EM), estradiol-17β (E_2) or 5α-dihydrotestosterone (DHT) on the growth of two human prostatic carcinoma cell lines, LNCaP and LNCaP-r was investigated. The hormone resistant subline LNCaP-r was derived in our laboratory, from the hormone sensitive LNCaP cell line. E_2, 10^{-8} or 10^{-5} M inhibited the growth of the LNCaP cells, but did not affect the LNCaP-r. DHT, 10^{-8} M, had a stabilizing effect at the stationary phase on the growth of the LNCaP cells whereas at higher concentrations, 10^{-5} M, the growth rate was decreased. The LNCaP-r cell line was previously reported to be unaffected by DHT. EM inhibited the growth of both cell lines but LNCaP was more sensitive than LNCaP-r. E_2 and DHT modulated the effect of EM. When treated with 10^{-7} M EM, addition of E_2 or DHT (10^{-7}–10^{-5} M) further inhibited the growth. When EM was used at a higher concentration (10^{-5} M), the enhanced effect of growth inhibition by hormone addition was lost. Based on these results it is suggested that the presence of endogenous hormones, or estrogens released from EM on hydrolysis, may play a contributory role in the cytotoxicity of estramustine.

Key words: Cancer treatment – Estramustine – Hormone sensitivity – LNCaP Prostatic carcinoma

Introduction

Although prostatic carcinoma is one of the most common neoplastic diseases in the western world, very few models are available for studying the effect of different therapeutic regimens (for review see [1]). A few human prostatic carcinoma cell lines have been characterized, but some of these lack the characteristics typical for primary prostatic carcinomas. The LNCaP cell line was established at Roswell Park Memorial Institute in Buffalo N.Y. and was found to be sensitive to steroid hormones [2, 3]. Using this cell line for developing methods for easy estimation of cell growth, it was shown that ATP can be used for this purpose, instead of enumeration with a hemocytometer [4]. This has also been shown by others [5–8].

We found that the cell line had become resistant to steroid hormones and it was thus renamed LNCaP-r [4, 9]. As estramustine phosphate (Estracyt®) is used for treatment of hormone resistant prostatic carcinomas [10, 11], we found it of interest to study the effect of EM alone or in combination with steroid hormones, on the growth rate of these two cell lines, LNCaP and LNCaP-r. The present paper describes these studies and based on the results obtained, the way in which steroids might affect the mechanism of action of EM is discussed.

Materials and Methods

Cell Lines

The LNCaP cell line at passage 60 was a gift from Dr. Horoszewicz, Roswell Park Memorial Institute, Buffalo, N.Y. The present studies were carried out with cells between 60th and 70th passages. The LNCaP-r cell line was derived from the LNCaP cell line and characterized at our laboratory [4]. This cell line is hormone resistant and has a different chromosomal distribution pattern and morphology, compared to that of the LNCaP cell line.

Cell Culture

The cells were cultured in a constant environment (37 °C, 5.0% CO_2) using RPMI 1640 medium (Flow Lab, Scotland) supplemented with 10% (v/v) inactivated fetal calf serum (Gibco, Scotland), 50 μg/ml penicillin, 50 μg/ml streptomycin, and 2.0 μmol/ml L-glutamine. Final concentrations of endogenous hormones were as previously reported [4]. Culture medium was changed every second day, and the confluent cells subcultured 1:5 in 80 cm^2 or 25 cm^2 flasks (A/S Nunc, Roskilde, Denmark). Usually the cells were cultured approximately 200 h before termination of experiment or next subculture.

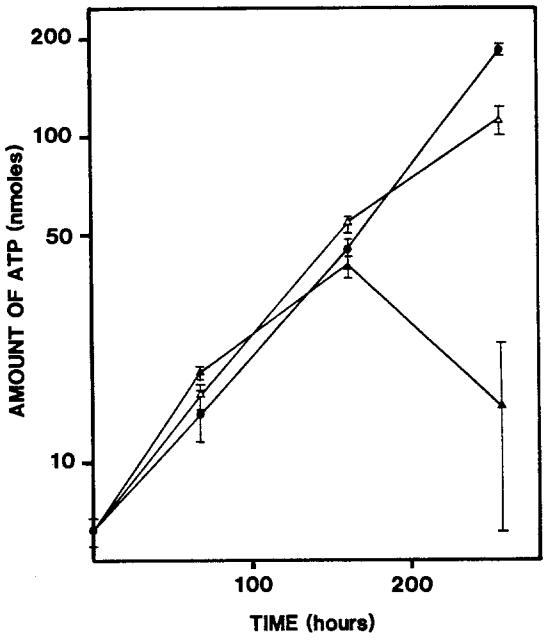

Fig. 1. Effect of different concentrations of E_2 on the LNCaP cell line. ATP determinations were carried out on days 1, 4, 8 and 12. (•) Standard medium with 0.1% ethanol, (△) 10^{-8} M or (▲) 10^{-5} M estradiol. Standard deviation (SD) of each point indicated by bars

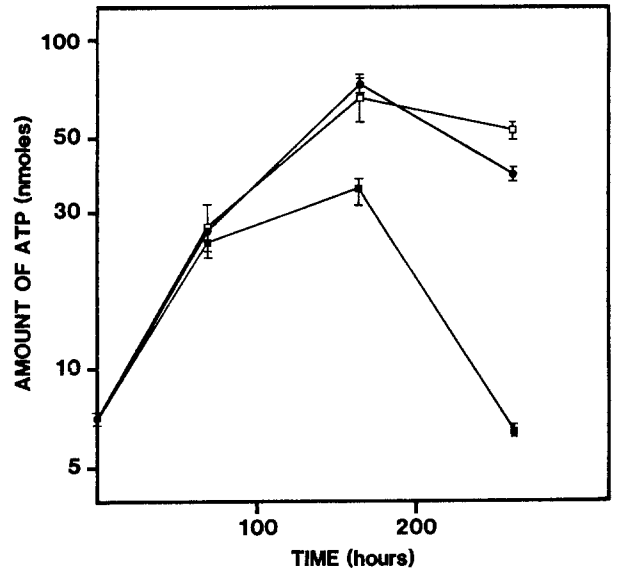

Fig. 2. Growth of LNCaP cell line in the presence of different concentrations of DHT. ATP determinations were done on days 1, 4, 8 and 12. (•) Standard medium with 0.1% ethanol, (□) 10^{-8} M or (■) 10^{-5} M DHT

Substances

Estramustine, estradiol-3-N-bis(2-chloroethyl)carbamate (EM), was synthesized by AB Leo, Helsingborg, Sweden. Estradiol-17β (E_2) and 5α-dihydrotestosterone (DHT) were purchased from Sigma (St. Louis).

Determination of ATP

ATP was determined as previously reported [4, 9], except that the cells were extracted in situ in flasks with 5 ml 5% (w/v) TCA in 10 ml of culture medium. As was previously shown [4, 9], ATP is at least as good a variable as cellcounting for estimation of cell growth. One LNCaP cell contains approximately 20 fmoles ATP.

Studies on Estramustine and Steroid Sensitivy

LNCaP cells in 60–70th passage were used throughout the experiments. These had been stored in liquid nitrogen, since their arrival from Buffalo 1982. On day 1 (hour 0), cells were dispersed with Na-citrate and subcultured 1:5 in 25 cm^2 flasks. On day 3 (approx. 48 h later) the medium was decanted and 10 ml of medium, supplemented with different concentrations of EM or steroids, were added. Steroids and EM were dissolved and added to the medium in 95% ethanol (v/v), giving a final ethanol concentration of 0.1% in the medium (v/v). The same procedure was carried out also on days 5, 7, 9 and 11. On days 4 (approximately 70 h after subculturing), 8 and 12 or days 4, 6 and 8 the ATP content was measured as described previously. Five cultures were routinely used for each assay. The figures presented here, represent typical results confirmed by repeated experiments.

Chromosome Analysis

The modal chromosome number was determined as previously reported [4].

Light Microscopy

The morphology of LNCaP and LNCaP-r cells was determined by routine methods. Cells from the logarithmic growth phase, obtained with a cotton swab, were gently spread on glass slides. Half of the material was air dried and stained according to May-Grünwald-Gimsa. The other half was fixed in absolute ethanol and processed according to Papanicolaou.

Statistical Analysis

The statistical analysis was performed on a tabletop Hewlett-Packard 97 calculator using Students *t*-test.

Results

Hormonal Sensitivity

Estradiol in concentrations 10^{-8} M and 10^{-5} M decreased the growth rate of LNCaP cells over an eight day period when measured by the ATP assay (Fig. 1). The difference was significant ($p < 0.001$) at day 8. As reported previously the LNCaP-r cells were unresponsive to E_2 at corresponding concentrations [4].

Dihydrotestosterone also had a significant growth regulating effect on the LNCaP cells. DHT, 10^{-8} M, had a slightly

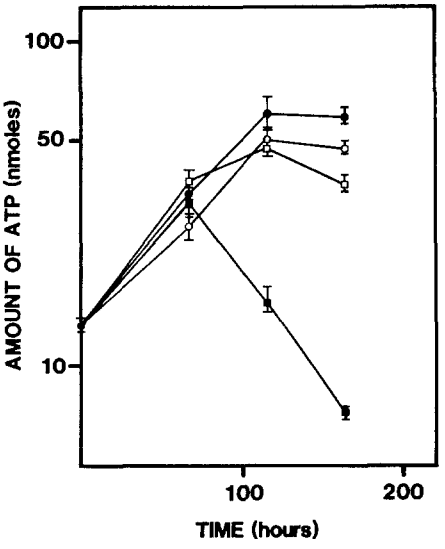

Fig. 3. Effect of different concentrations of EM on the LNCaP cell line. ATP determinations were carried out on days 1, 4, 6 and 8. (•) Standard medium with 0.1% ethanol, (○) 10^{-7} M, (□) 10^{-6} M or (■) 10^{-5} M EM

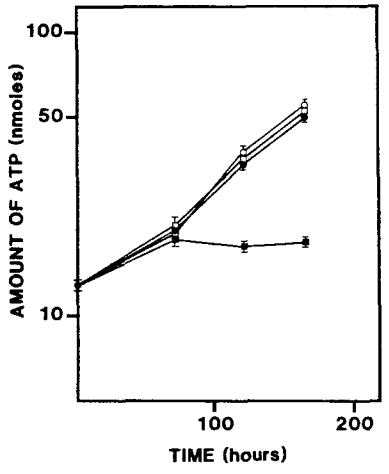

Fig. 4. Effect of different concentrations of EM on the LNCaP-r cell line. ATP determinations were carried out on days 1, 4, 6 and 8. (•) Standard medium with 0.1% ethanol, (○) 10^{-7} M, (□) 10^{-6} M or (■) 10^{-5} M EM

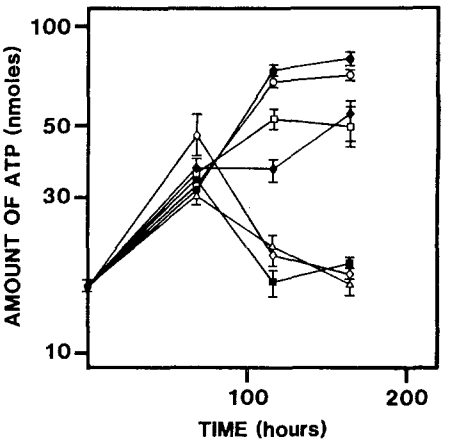

Fig. 5. Growth of the LNCaP cell line in the presence of different combinations of EM and E_2 concentrations in the culture medium. (•) Standard medium with 0.1% ethanol, (○) 10^{-7} M or (■) 10^{-5} M EM (□) 10^{-7} M EM and 10^{-7} M E_2, (♦) 10^{-7} M EM and 10^{-5} M E_2, (◇) 10^{-5} M EM and 10^{-7} M E_2, (△) 10^{-5} M EM and 10^{-5} M E_2

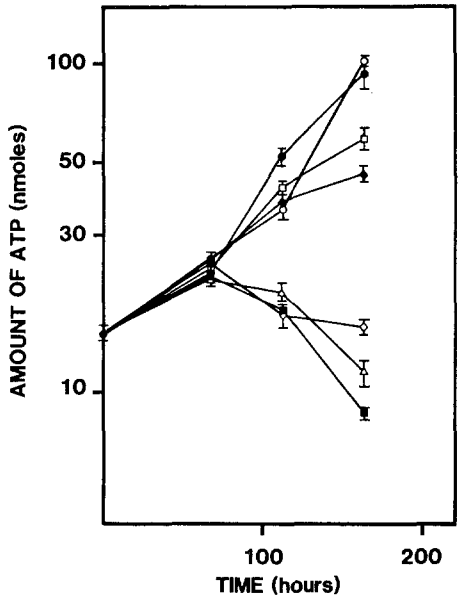

Fig. 6. Growth of the LNCaP cell line in the presence of different combinations of EM and DHT concentrations in the culture medium. (•) Standard medium with 0.1% ethanol, (○) 10^{-7} M or (■) 10^{-5} M EM (□) 10^{-7} M EM and 10^{-7} M DHT, (♦) 10^{-7} EM and 10^{-5} M DHT, (◇) 10^{-5} M EM and 10^{-7} M DHT, (△) 10^{-5} M EM and 10^{-5} M DHT

stabilizing effect at confluence between days 8 and 12, whilst higher concentrations (10^{-5} M), significantly inhibited cell growth when measured days 8 and 12 ($p < 0.001$), and compared to cells in standard medium (Fig. 2). A growth inhibitory effect of DHT has previously been reported by Horoszewicz et al. [2]. As previously described, DHT did not affect the growth of the LNCaP-r cell line [4].

On treatment with EM, (10^{-7}, 10^{-6} and 10^{-5} M), the growth rate of LNCaP cells was significantly ($p < 0.05$) decreased days 6 and 8, when treated with the two latter concentrations (Fig. 3). Also the LNCaP-r cells, showed a significant ($p < 0.001$) decrease in growth rate at day 6 and 8, when treated with high concentrations of EM (10^{-5} M) (Fig. 4). The LNCaP cells were however more sensitive to EM than the LNCaP-r cells (Fig. 3 and 4), since no effect was found with lower concentrations of EM on LNCaP-r cells.

Interestingly, combination treatment of the LNCaP cells with EM and E_2 or DHT, gave some unexpected results. Both of the steroids at 10^{-7} and 10^{-5} M in combination with 10^{-7} M EM slightly increased the inhibitory effect of EM (Figs. 5 and 6) day 8. In contrast, when E_2 or DHT

were administered in combination with 10^{-5} M EM, the modulating effect of the steroids tended to be lost i.e. addition of steroids did not affect the inhibitory effect of EM.

Chromosome Analysis

The modal chromosome number in the two cell lines was clearly different. The LNCaP cell population consists mainly of cells with 46 chromosomes as reported by Horoszewicz [2]. The LNCaP-r cells are however more aneuploid [4].

Light Microscopy

The LNCaP cell line was poorly differentiated, but clearly of epithelial origin. The cell population was moderately pleomorphic and mainly appeared in rather tight clusters with occasional acinar formation. The cytological findings indicated a poorly differentiated adenocarcinoma.
The LNCaP-r cell line was pleomorphic with bizarre, often multinuclear cell forms. The nucleoli varied in number, size and shape. Many nuclei appeared naked, but when present, the cytoplasm was rather abundant, sometimes vacuolized. The cells tended to dissociate or formed loose aggregates without organoid structures. Cytologically the dominating picture was consistent with an anaplastic neoplasm showing no definite epithelial features.

Discussion

The original LNCaP prostatic carcinoma cell line from Roswell Park Memorial Institute, was reported to be hormone sensitive [2]. Cultured in fetal serum a decrease in growth rate, when given DHT in increasing concentrations, was reported. These experiments were performed at passages 30–55. We later tested the hormone sensitivity of the cell line we had obtained in 1982. These experiments were carried out at passages 80–88. We found that during the culture the cell line had changed and become hormone resistant. In conjunction with this, an increase in the chromosome number was found. This biological dedifferentiation was also expressed in the cytological picture, changing from poorly differentiated adenocarcinoma to an anaplastic cancer. This cell line was called LNCaP-r due to its resistance to hormonal treatment and was used for establishing methods for estimation of cell growth and energy charge [4, 9]. In these studies, ATP and energy charge were shown to be good tools for measuring cell growth (ATP) and the overall physiological status of the cells (energy charge). We related the amount of ATP to cell enumeration using a hemocytometer (Bürker type) and found the ATP method to be more accurate, with regard to the intra-assay variations and sensitivity. The amount of ATP/cell was also found to be stable during the logarithmic growth phase, while in the lag- and stationary phases the ATP/cell ratio was slightly lower [4].

In recent experiments performed on LNCaP cells at the 60–70th passage we found that these cells exhibit more stability at confluency, when treated with DHT at 10^{-8} M and a growth inhibitory response when treated with higher doses of DHT or varying concentrations of E_2.

Experiments with the two cell lines during the same period of time clearly demonstrated different sensitivities to hormonal stimulation. It is therefore tempting to use these cell lines as models for prostatic carcinoma. If so, the LNCaP cell line should be used as a model for a hormone sensitive tumour and LNCaP-r as a model for hormone resistant prostatic carcinoma. These cell lines could then be used for studies on the effects of drugs used in chemotherapy and also to clarify how different drugs affect the further development of the cells. In this work the short time effect of estramustine, estradiol or dihydrotestosterone have been studied.

The most dramatic effect on growth was seen when EM was administered. The mechanism of action of EM is still only partially revealed. The specific uptake of EM into the prostatic cell is most likely due to the binding of EM to a specific protein, the estramustine binding protein (EMBP), which is present in high concentrations in the rat ventral prostate [12, 13] and is also found in the human prostate [14, 15]. This protein is probably the reason for the tissue specific effect, as the uptake of EM in the prostate is much higher than in other tissues and correlated to the subcellular distribution of EMBP in rats [16, 17]. The cytotoxic effect of EM is however probably caused by the interaction of the substance with the microtubule associated proteins [18] via which it inhibits microtubule functions both during cell division and interphase [19–21]. Whether this interaction is tissue specific or the tissue specificity is due only to its accumulation is not known at the present time. For further optimization of therapy it is important to further clarify the total mechanism of action of EM.

The combination of steroids together with EM gave somewhat surprising results. Low EM concentrations on addition of steroids resulted in an increase in the potency of estramustine. Conversely, high EM concentrations on combination with steroids showed no statistically significant additional effect ($p > 0.5$).

The modification of the effect on EM caused by addition of steroids is at present a subject of speculation. The steroids may influence uptake, tissue distribution or the binding of EM to the microtubule associated proteins. Also the higher growth inhibiting effect of EM on the steroid sensitive cells (LNCaP), than on the hormone resistant cells (LNCaP-r), indicates that the steroid control mechanism of the cells is of importance for the mechanism of action of estramustine.

The modulating effect of steroid hormones on EM might however be of clinical importance. The tissue concentration of EM is approximately 10^{-7} M or more [22]. On oral

treatment with estramustine phosphate (Estracyt®) four major metabolites are rapidly formed – estramustine, estromustine, estradiol and estrone. These metabolites are present in the prostatic tumour tissue at 10^{-7}–10^{-6} M [22]. In tissue culture, EM is always oxidised to estromustine, but further metabolism varies from cell type to cell type [23]. Thus the cytotoxic effect of EM is a combination of the effect of estra- and estromustine, both of which probably have the same mode of action.

In the experiments presented here, we have shown that both E_2 and DHT at 10^{-7} and 10^{-5} M cause an enhancement of estramustine induced cell death. Therefore it is possible that estrogens formed from EM, could play a contributory role for the cytotoxic effects of estramustine in man.

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References

1. Murphy GP (1980) In: Models for prostate cancer. Liss, New York
2. Horoszewicz JS, Leong SS, Chu TM, Wajsman ZL, Friedman M, Papsidero L, Kim U, Chai LS, Kakati S, Arya SK, Sandberg AA (1980) The LNCaP cell line – a new model for studies on human prostatic carcinoma. In: Murphy GP (ed) Models for prostate cancer. Liss, New York, pp 115–132
3. Horoszewicz JS, Leong SS, Kawinski E, Karr JP, Rosenthal H, Chu TM, Mirand EA, Murphy GP (1983) LNCaP model of human prostatic carcinoma. Cancer Res 43:1809–1818
4. Hasenson M, Hartley-Asp B, Kihlfors C, Lundin A, Gustafsson J-A, Pousette A (1985) Effect of hormones on growth and ATP content of a human prostatic carcinoma cell line, LNCaP-r. Prostate 7:183–194
5. Garewal HS, Ahmann FR, Woo L (1984) ATP levels provide a useful assay for the quantitation of growth and drug effects in malignant cells. Clin Res 32:415A
6. Garewal HS, Ahmann FR, Celniker A (1985) The ATP assay: Anticancer drug effects on malignant cell growth. Proc Am Ass Cancer Res 26:1317A
7. Garewal HS, Ahmann FR, Schifman RB, Celniker A (1986) ATP assay: Ability to distinguish cytostatic from cytocidal anticancer drug effects. J Natl Cancer Inst 77:1039–1045
8. Kangas L, Grönroos M, Nieminen AL (1984) Bioluminescence of cellular ATP: a new method for evaluating cytotoxic agents in vitro. Med Biol 62:338–343
9. Lundin A, Hasenson M, Persson J, Pousette Å (1986) Estimation of biomass in growing cell lines by ATP assay. In: Colowick SP, Kaplan NO (eds) Methods in enzymology, vol 133. Part B: Bioluminescence and chemiluminescence. Academic Press, Orlando, pp 27–42
10. Jönsson G, Högberg B, Nilsson T (1977) Treatment of advanced prostatic carcinoma with estramustine phosphate. Scand Urol Nephrol 11:231–238
11. Jönsson G, Högberg B (1971) Treatment of advanced prostatic carcinoma with Estracyt. Scand J Urol Nephrol 5:103–107
12. Forsgren B, Björk P, Carlström K, Gustafsson J-A, Pousette A, Högberg B (1979) Purification and distribution of a major protein in rat prostate that binds estramustine, a nitrogen mustard derivative of estradiol-17β. Proc Nat Acad Sci USA 76:3149–3153
13. Forsgren B, Gustafsson J-A, Pousette A, Högberg B (1979) Binding characteristics of a major protein in rat ventral prostate cytosol that interacts with estramustine, a nitrogen mustard derivative of estradiol-17ß. Cancer Res 39:5155–5164
14. Pousette A, Björk P, Carlström K, Forsgren B, Gustafsson J-A, Högberg B (1980) On the presence of "prostatic secretion protein" in different species. Acta Chem Scand [B] 34:155–156
15. Björk P, Forsgren B, Gustafsson J-A, Pousette A, Högberg B (1982) Partial characterization and quantitation of a human prostatic estramustine binding protein. Cancer Res 42:1935–1942
16. Appelgren L-E, Forsgren B, Gustafsson J-A, Pousette A, Högberg B (1978) Autoradiographic studies of ^{3}H-estramustine in the rat ventral prostate. Acta Pharamcol Toxicol 43:368–374
17. Högberg B, Björk P, Carlström K, Forsgren B, Gustafsson J-A, Hökfelt T, Pousette Å (1979) The interaction of steroidal alkylating agents with binding component in the soluble fraction of the prostate. In: Murphy GP, Sandberg AA (eds) Prostate cancer and hormone receptors. Liss, New York, pp 181–199
18. Hartley-Asp B (1984) Estramustine induced mitotic arrest in two human prostatic carcinoma cell lines, DU 145 and PC-3. Prostate 5:93–100
19. Wallin M, Deinum J, Fridén B (1985) Interaction of estramustine phosphate with microtubule associated proteins. FEBS Lett 179:289–293
20. Kanje M, Deinum J, Wallin M, Ekström P, Edström 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
21. Stearns ME, Tew KD (1985) Antimicrotubule effects of estramustine, an antiprostatic tumor drug. Cancer Res 45: 3891–3897
22. Fritjofsson A, Björk P, Gunnarsson PO, Norlén BS (1983) Binding and accumulation of active metabolites in tumours from prostatic cancer patients treated with Estracyt. Preliminary report. 13th International Congress of Chemotherapy, Vienna
23. Kruse E, Johanson SA, Hartley-Asp B, Gunnarsson PO (1988) Distribution and metabolism of estramustine in Heta cells and the human prostatic tumour cell line 1013L. Biochem Pharmacol 37:3161–3167

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