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DISTRIBUTION AND METABOLISM OF ESTRAMUSTINE IN HeLa CELLS AND THE HUMAN PROSTATIC TUMOUR CELL LINE 1013L

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Abstract—The metabolism of estramustine [estradiol-3-*N*-bis(2-chloroethyl) carbamate] was investigated in the human prostatic tumour cell line 1013L and the human cervix tumour cell line HeLa S3. Uptake studies revealed that estramustine (EM), and its 17-ketoanalogue estromustine (EoM), differed in their nuclear binding pattern in 1013L cells but not in HeLa cells. Most of the nuclear radioactivity from both EM and EoM was found in the fraction containing the majority of the phospholipids. HPLC studies on EM-treated 1013L cells showed the presence of the oxidized metabolite EoM, in the medium, an enrichment of estradiol and estrone in whole cells and EM and EoM bound to the nuclear protein matrix. Similar studies on the HeLa cell line showed a completely different pattern, no metabolites other than EoM were found in the cell medium and whole cells but several very lipophilic metabolites were found bound to the nuclear protein matrix. On investigation of other tumour cell lines these metabolites were found to be unique to HeLa cells. The results extend our knowledge concerning EM and demonstrate that the cell line 1013L is a relevant model system for studying drugs active against human prostatic tumours.

Considerable effort has been expended on possible explanations and discussions regarding the hormonal and/or eventual alkylating effects of estramustine phosphate, Estracyt, without fully being able to explain its clinical efficacy [1–3]. During the last five years new light has been thrown on this problem as numerous studies have shown that estramustine (the dephosphorylated metabolite of Estracyt) has a mode of action different from that of either of its constituents estradiol or nor-nitrogen mustard. Estramustine exerts its cytotoxic effect via inhibition of microtubule functions. This is probably caused by the specific binding of EM to the microtubule associated proteins [4], thereby preventing the assembly and/or causing disassembly of microtubules [5]. This leads to disturbance of the cytoskeleton [6, 7] and mitotic arrest, which results in cell death of human prostatic tumour cells DU145 and PC-3 and HeLa cells *in vitro* [8, 9]. However, HeLa cells exhibited a greater sensitivity to EM than the human prostatic tumour cells, which could not be explained at the cytoskeleton level. Preliminary investigations did, however, demonstrate that more substance was associated with the nuclear protein matrix in HeLa cells compared to that found in human prostate tumour cells [9, 10]. The NPM isolation method of Berezney and Coffey [11] provides an opportunity to determine where nuclear association of a drug occurs, as each fraction has been well characterized. For instance the low salt buffer fraction (LSB) contains the majority of the proteins. The nuclear spheres obtained after this treatment contain a nucleolus and a nuclear envelope, but large areas of condensed chromatin are no longer seen. The following treatment with high salt buffer (2 M NaCl), extracts proteins, most of the total matrix DNA and large amounts of RNA (HSB). The following extraction with Triton X-100 removes the majority of the total nuclear phospholipids (TX-TM). After this treatment only a residual nuclear envelope is left, containing the nuclear pore complexes. The final treatment with DNase and RNase removes the residual matrix DNA and the remaining RNA. The remaining structure, the NPM, consists of non-histone protein and its function has been studied in great detail, e.g. it function as an anchor for DNA/RNA synthesis [12, 13].

Although estramustine is a major metabolite after oral administration of Estracyt, it is not the only metabolite. Estromustine, EoM, the oxidized form, is also present in high concentrations in plasma and in prostatic tumour tissue together with estrone and estradiol [14, 15]. Thus to understand fully the intranuclear distribution of EM, we deemed it necessary to carry out extensive metabolism studies in connection with these experiments.

MATERIALS AND METHODS

Substances. ^{3}H-estramustine [2,4,6,7-^{3}H] (90.4 Ci/mmol), estramustine, ^{3}H-estromustine (41.5 Ci/mmol), and estromustine were synthesized at AB Leo (Helsingborg, Sweden). The purity of the radioactive chemicals was at least 98% as determined by high performance liquid chromatography. HPLC (Waters μBondapak C18; acetonitrile : water : acetic

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acid (63:36:1) 0.4 ml/min; injection 7 μl). Stock solutions of ^{3}H-EM, ^{3}H-EoM and EM were made up in 95% ethanol. Unlabelled EoM was dissolved in dimethyl sulfoxide. The final concentration of the solvent in the incubation mixture did not exceed 0.2%. Minimum essential medium for suspension culture (S-MEM), RPMI 1640 medium, foetal calf serum, glutamine, streptomycin and penicillin were bought from Gibco Biocult, Scotland. All chemicals were of analytical grade and high quality distilled water was used. Deoxyribonuclease 1 (DNase) from Beef Pancreas and Ribonuclease-A (RNase) from Bovine Pancreas, type 1-A, were bought from Sigma.

Cell cultivation. HeLa cells were grown in S-MEM, supplemented with 10% foetal calf serum, 5% glutamine, streptomycin and penicillin, in 5% CO_2 humidified atmosphere at 37°. The cells were grown as a suspension culture in a glass culture flask (700 ml, Techne, U.K.) under constant stirring (40 rpm) and were diluted at 1×10^6 cells/ml. The human prostatic cancer cell line 1013L [16] was kindly donated by Dr D. Mickey, Duke University, U.S.A. This was grown as described previously [9].

The human prostatic tumour cell line PC-3 [17] was grown as described by Hartley-Asp [8], the human breast tumour cell line MCF-7 [18] was grown in MEM medium supplemented as for HeLa cells and the rat prostatic tumour cell line Dunning G as described by Isaacs [19].

Cytotoxicity studies. 1013L cells were treated for 24 hr in early and late logarithmic phase growth with various concentrations of EM and EoM. After treatment the cells were washed and allowed to proliferate in drug-free medium for 4 days. The number of cells present was counted in a Coulter Counter and the number found in the treated groups assessed in relation to those found in the control group, i.e. % cell survival.

Uptake and metabolism of EM and EoM in HeLa and 1013L cells. Uptake and cellular distribution of ^{3}H-EM and ^{3}H-EoM in HeLa cells was investigated by incubating the cells for 24 hr at 37°, with either 10 nM ^{3}H-EM or 10 nM ^{3}H-EoM. After the 24 hr treatment the cells were allowed to recover in substance-free medium and samples were collected at 1, 2 and 7 hr. Cells were then washed with fresh medium, without serum, before the nuclear protein matrix (NPM) isolation was carried out. This fraction was isolated from each sample and the amount of radioactivity present in all fractions measured. Similar experiments with 1 nM ^{3}H-EM and ^{3}H-EoM were carried out in 1013L cells. The other cell lines PC-3, MCF-7 and Dunning G were treated for 24 hr with 10 nM ^{3}H-EM followed by nuclear extraction and HPLC-studies as described for HeLa and 1013L cells.

Nuclear extraction and nuclear protein matrix isolation. The modified NPM isolation was carried out as we have described previously [10] (see Introduction for further details).

High performance liquid chromatography (HPLC). The liquid chromatograph consisted of a Model 6000 A pump, a guard column, a U6K injector and a Model 440 UV-detector, 254 nM (Waters Assoc., Milford U.S.A.) The column was a Radial-Pak C18 cartridge. The chromatograms were recorded on a W + W Tarkan recorder 600 and the outlet of the detector was connected to an LKB 7000 Ultrorac fraction collector. Two different HPLC systems were used to separate the metabolites. In system 1 the first mobile phase consisted of acetonitrile–water (60:40) and 5 ml was collected. Then the mobile phase was changed to acetonitrile–water (75:25), about 20 ml was collected.

The mobile phase in system 2 was 100% acetonitrile throughout the program. The flow rate was 0.4 ml/min and the volume of each fraction was 240 μl (system 1) and 200 μl (system 2). The four references used were estramustine (R1), estromustine (R2), estradiol (R3) and estrone (R4). These metabolites were added to the samples before injection (40 μg dissolved in the mobile phase). The localization of the reference compounds was identified by their UV peaks.

Radioactivity measurements. The cellular, nuclear and NPM fractions were combusted in a Packard Sample Oxidizer and counted in 10 ml Instagel (Packard) by a liquid scintillation counter (Philips) for 10 min. The soluble cellular fractions and HPLC fractions were counted directly in 5 ml Instagel. Corrections for quenching were made by the external standard ratio method.

RESULTS

Cytotoxicity studies on 1013L cells

In our previous work [10] we discovered a lower uptake of EM in whole cells, nuclei and NPM during early log phase than during late log phase. We therefore continued the investigations in both growth phases, with EM and EoM. As can be seen in Fig. 1, EoM is less effective than EM during both these phases of cell growth. For example the cell survival after treatment in late log phase, with 5 μg/ml EoM and EM was 95% and 65% respectively.

Similar experiments with the steroid hormones estradiol and testosterone (10^{-11}–10^{-9} M) produced no change in cell growth demonstrating that 1013L cells are hormone insensitive. A slight toxic effect was achieved at higher hormone concentrations (10^{-7}–10^{-6} M).

HeLa cell line

Table 1 shows that the uptake and distribution pattern of radioactivity in the nucleus of HeLa cells is almost identical after a 24 hr incubation with either EM or EoM. Under the following recovery period the concentration of radioactivity diminished, and after 7 hr the remaining substance constituted approximately 50% of the original amount found bound to the NPM (Table 2). HPLC was carried out on the 24 hr treatment–7 hr recovery fractions. Using HPLC system 1, it was discovered that although most of the radioactivity was EM, a relatively large amount of radioactivity was eluted later from the column, indicating metabolites of a more lipophilic nature. A more thorough study was therefore necessary and after modification of the HPLC system (system 2) these lipophilic metabolites were clearly seen.

The lipophilic metabolites were found in the whole cell sample (Fig. 2B), but were much clearer in the NPM fraction from HeLa cells (Fig. 2C). Hence,

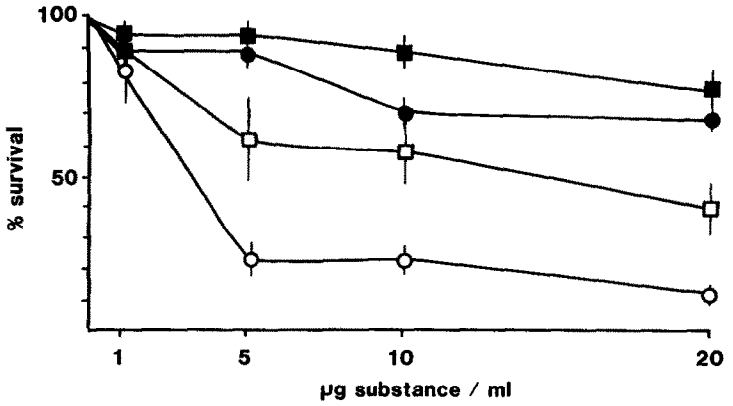

Fig. 1. The effect of 24 hr treatments with various concentrations of estramustine (EM) and estromustine (EoM) in early and late log phase growth of human prostatic 1013L tumour cells. Survival is given as percentage of cells surviving after 4 days in relation to the control. Each point is the mean of 2 experiments: —■—, EoM late log; —●—, EoM early log; —□—, EM late log; —○—, EM early log.

EM is transformed in HeLa cells to at least five very non-polar metabolites. No estrone or estradiol was found in HeLa cells.

The incubation medium contained mainly EM and a small amount of the oxidized metabolite EoM (Fig. 2A). Control experiments, not shown, demonstrated no oxidation of EM to EoM in medium alone.

These results in HeLa cells although very interesting might not be pertinent for prostatic tumour cells. Thus on the establishment of a human prostatic tumour cell line, 1013L, which grew in suspension culture, we carried out similar experiments in these cells.

1013*L Cell line*

On treatment of 1013L cells (0.5×10^6 cells/ml) with 1 nM ^{3}H-EM for 24 hr, a different pattern of uptake was found (Table 1). More radioactivity was present in the TX-TM fraction and less in the LSB-fraction than in the HeLa cells. Less activity was found in nucleus—0.42% of total cellular uptake, and the NPM—15.3% of total nuclear uptake, than in HeLa cells—0.88% and 31.3% respectively. Table 1 also shows that the uptake of ^{3}H-EoM after a similar incubation, varies from that of ^{3}H-EM. More substance is found in the TX-TM fraction and less in the NPM.

We have previously shown that relative uptake into 1013L nucleus and NPM is consistent in the

Table 2. Time course of efflux of ^{3}H-estramustine from HeLa and human prostatic tumour 1013L nuclear protein matrix

Recovery (hr)	HeLa	1013L
0	100	100
1	80.3 ± 3.4	70.7 ± 4.0
2	73.7 ± 1.6	—
7	51.6 ± 3.5	45.5 ± 5.0

Amount of retained radioactivity in the nuclear protein matrix fractions of HeLa and 1013L cells obtained after a 24 hr 1 nM ^{3}H-EM (1013L) and 10 nM 3H-EM (HeLa) treatment followed by different recovery periods in drug-free medium. The figures represent the percentage of radioactivity bound to the nuclear protein matrix when uptake after 24 hr treatment is normalized to 100%. Each point is the mean of two experiments.

Table 1. Nuclear uptake and distribution of estramustine and estromustine in HeLa and the human prostatic tumour 1013L cell line. (The figures represent the total amount of radioactivity in DPM/100 × 10^6 cells)

	HeLa		1013L	
Fraction	^{3}H-EM	^{3}H-EoM	^{3}H-EM	^{3}H-EoM
Nucleus	125375 (0.88[a])	93550 (0.82[a])	24360 (0.42[a])	12000 (0.60[a])
LSB	21314 (17.0)	18710 (20.0)	1291 (5.3)	288 (2.4)
HSB	17803 (14.2)	6361 (6.8)	511 (2.1)	192 (1.6)
TX-TM	43881 (35.0)	39572 (42.3)	14957 (61.4)	9840 (82.0)
DNA	3134 (2.5)	2432 (2.6)	3873 (15.9)	780 (6.5)
NPM	39242 (31.3)	26474 (28.3)	3727 (15.3)	900 (7.5)

[a] Percentage of radioactivity in relation to whole cell uptake. The values are the mean of three separate experiments.

LSB = low salt treated nuclear fraction; HSB = high salt treated nuclear fraction; TX-TM = detergent treated nuclear fraction; DNA = RNase and DNase treated nuclear fraction; NPM = nuclear protein matrix fraction.

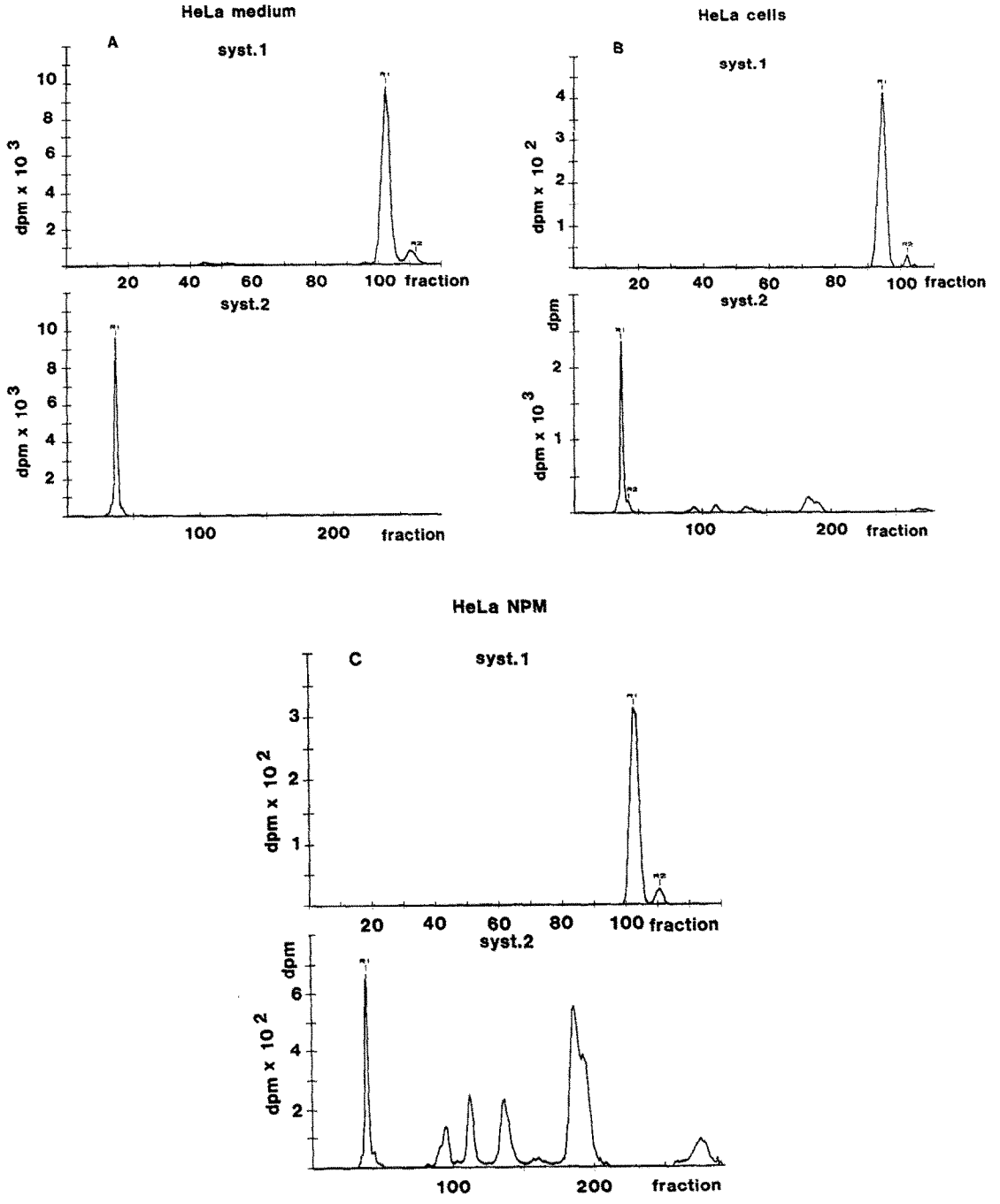

Fig. 2. Metabolism of estramustine by HeLa cells. HPLC diagrams of extracts obtained from HeLa cells treated for 24 hr with 10 nM ^{3}H-estramustine plus a 7 hr recovery period. (A) MEM medium; (B) whole cells; (C) nuclear protein matrix. References are: R1-estramustine, R2-estromustine, R3-estradiol, R4-estrone.

range 1 nM to 2 μM [10]. We also found identical distribution at 1 nM in comparison with cytotoxic concentrations. However, we preferred the further security of a higher amount of DPMs in the HeLa uptake study and in the HPLC studies on other cell lines. That is why we have used a higher ^{3}H-EM concentration in the later experiments.

Metabolism studies were carried out on fractions obtained from 1013L cells treated for 24 hr followed by the 7 hr recovery period. Oxidation of EM to EoM in 1013L cells and leakage into the medium was found (Fig. 3A). Estradiol and estrone were found in the cellular fraction (Fig. 3B) but not in the whole nuclear fraction (data not shown) nor in the NPM (Fig. 3C). No lipophilic metabolites were found in this cell line. After a recovery period of 7 hr, similar to that carried out with HeLa cells, approximately the same relative amount of radioactivity was

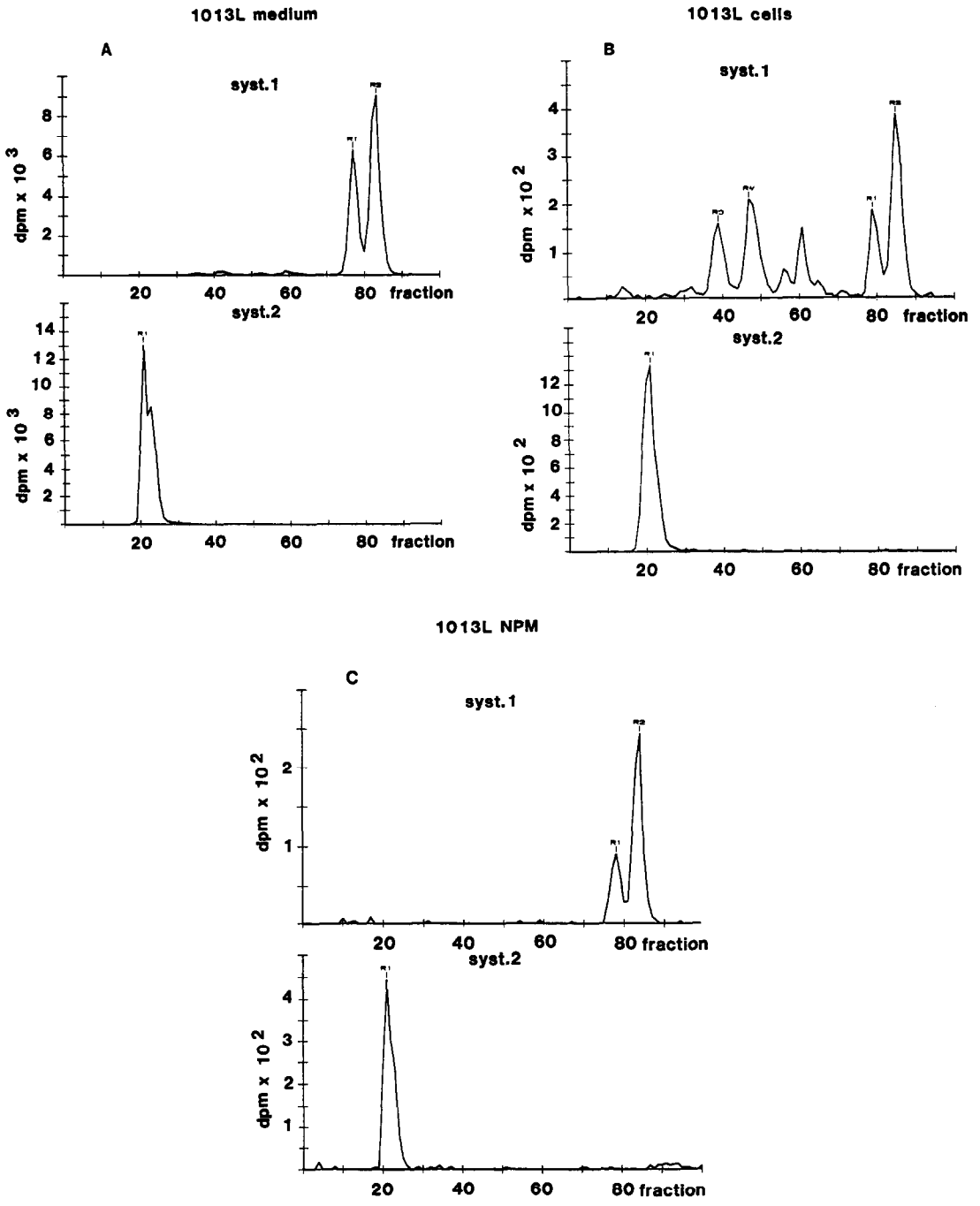

Fig. 3. Metabolism of estramustine by human prostatic 1013L tumour cells. HPLC diagrams of extracts obtained from 1013L cells treated for 24 hr with 1 nM ^{3}H-estramustine plus a 7 hr recovery period: (A) RPMI 1640 medium; (B) whole cells; (C) nuclear protein matrix. References are: R1-estramustine, R2-estromustine, R3-estradiol, R4-estrone.

found still associated with the NPM, i.e. 45% (Table 2).

Other cell lines

To determine if lipophilic metabolites were specific to HeLa cells, HPLC studies were carried out on cell and nuclear fractions from PC-3, MCF-7 and the Dunning G line. These studies demonstrated that oxidation of EM to EoM occurred in all these cell lines but no further metabolism was found (Table 3).

DISCUSSION

These studies have shown that the human prostatic tumour cell line 1013L originating from a primary

Table 3. The existence of lipophilic metabolites in different cell lines after 24 hr incubation with 10 nM ^{3}H-estramustine and ^{3}H-estromustine, or 1 nM ^{3}H-estramustine (1013L)

Cell line	Fractions		
	Cell	Nuclei	NPM
HeLa	+	+	+
HeLa*	+	+	+
HeLa†	+	+	ND
1013L*	ND	−	−
PC-3	−	−	ND
MCF-7	−	−	ND
Dunning G	−	−	−

+, present; −, absent; ND, not done.
* Analyzed after a 7 hr recovery period.
† EoM treated cells.

tumour, is capable of oxidizing EM to EoM, and that estradiol is produced resulting from hydrolysis of the carbamate ester bridge. Both of these processes are part of the metabolic pathway for Estracyt in man [14]. In addition we have demonstrated that both EM and EoM are cytotoxic to these cells. Thus, the 1013L cell line is a relevant system for studying the cellular kinetics of the two cytotoxic metabolites of Estracyt *in vitro*.

The two major nuclear fractions obtained in NPM isolation, LSB and HSB (see Introduction), contained in HeLa cells more radioactivity than the corresponding fractions from 1013L cells. This could be due to the existence of the lipophilic metabolites found only in HeLa cells. In contrast, the phospholipid fraction, TX-TM, from 1013L cells, contained higher amounts of both EM and EoM than that from HeLa cells (Table 1), perhaps indicating differences in the drug association to the nuclear envelope between these cell lines. The binding of EM to the nuclear phospholipids might change nuclear membrane structure and thereby inhibit transport of essential macromolecules. In fact, we have recently found evidence which indicates that the transport of RNA from the nucleus of EM treated 1013L cells is inhibited (Kruse and Hartley-Asp, unpublished results). Only 2.5% of bound EM was found in the fraction obtained after final digestion with DNase and RNase in HeLa cells. This is in contrast to that found for colchicine, another anti-mitotic agent, where 70% of the total nuclear bound drug was associated with this particular fraction [20]. This could be associated with the fact that colchicine induces chromosomal aberrations, which are a result of DNA damage [21], whereas EM does not induce aberrations [8].

The present studies also show that once bound, EM and its metabolites are retained, for a long period of time, in both HeLa and 1013L cells; 45–50% was still bound after the 7 hr recovery period. This prolonged drug binding could be involved in the lack of reversibility found for the anti-invasive effect of EM in another human prostate tumour line, the DU 145 [7]. Under any circumstances this provides a drug reserve which can cause delayed cell death.

Although the identity of the metabolites found bound to the NPM of HeLa cells is not yet clarified, the R_f-values clearly show that the metabolites have a very non-polar character. Such metabolites can be formed by conjugation of xenobiotics with lipids or fatty acids [22]. Hence acylation of endogenic lipids and steroids by xenobiotic carboxylic acids may occur, and we have previously shown transacylation of a compound similar to EM, namely prednimustine [23]. Formation of fatty acid esters of estradiol has recently been detected *in vivo* [24]. Thus even if the EM-metabolites, which are retained in the HeLa cell matrix are not yet identified it is tempting to assume that EM has undergone conjugation with different non-polar compounds in HeLa cells. No lipophilic metabolites were found in EM treated 1013L cells, nor in any of the other cell lines investigated. Thus the finding of these lipophilic metabolites indicates the presence of a specific metabolic pathway in HeLa cells.

Several studies have shown that the major plasma metabolite found after administration of Estracyt in man, rat and dog is the 17-keto analogue of estramustine; estromustine (EoM) [14, 15, 25]. This metabolite has also been found in prostate tumour tissue [15] which has been presumed to be a result of uptake from the plasma. However, it could also result from oxidation in the prostate tumour itself, since as seen here, EM is oxidized to EoM by tumour cells in culture.

Hydrolysis of the carbamate bridge also takes place releasing estradiol and estrone in man. Both these metabolites were found in the cytoplasm of 1013L cells indicating the capability of these cells to hydrolyse EM. As no estrogens were found in the nucleus, hydrolysis appears to be a cytoplasmic function.

In conclusion, this work has shown that metabolic processes are present and active in tumour cells *in vitro*, and that these processes may differ from cell type to cell type. It demonstrates that cellular uptake of estramustine is accompanied by oxidative metabolism to EoM, in several cell types, and that further specific metabolism occurs in HeLa cells. These lipophilic metabolites may be responsible for the increased sensitivity of HeLa cells to EM. Also although EoM is less cytotoxic than EM in 1013L cells, these two metabolites probably play contributary roles in EM-induced cell death. An additional facet of these experiments has been that we have demonstrated that the human prostatic cancer cell line 1013L is a relevant model system for studying the effect of drugs on prostatic carcinoma.

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