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A time study on the uptake of estramustine into prostatic tumour 1013L cells *in vitro*

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The nuclear protein framework, the nuclear protein matrix, NPM, has attracted much attention during the last years due to its probable role in several important biological processes [1], e.g. DNA synthesis. RNA synthesis, processing and transport and hormone action. We have earlier investigated the role of the NPM in estramustine (EM) induced cell death. In HeLa cells, highly sensitive to EM, we found a high amount of intact EM and several metabolites hydrofobically bound to the NPM [2]. This preferential binding was also found in the human prostatic tumour 1013L cell line, with an increase in NPM uptake at higher cell densities [3]. Other results have shown that EM acts as an antimitotic agent arresting cells in metaphase [4] and causing an inhibition of the assembly and a disassembly of microtubules via interaction with the microtubule-associated proteins [5, 6]. However, other data indicate that cytotoxicity is also mediated via binding to the NPM [7].

Although no indication of DNA damage has been found for EM [2], the important role of the NPM gives cause for further studies at the nuclear level. In fact, we have earlier studied the effect of EM on specific RNA labelling in 1013L cells. An inhibition of all the different RNA-species was found, indicating a mode of action involving nuclear targets [8]. From our previous experiments in 1013L cells, we also know that EM and its oxidative metabolite estromustine are retained by the NPM for a long time, 45–50% being still bound after a 7 hr drug free recovery period [7]. This is higher than that found for other anti-mitotic agents, such as vinblastine and vincristine, where after 3 hr recovery 10 and 30% respectively were found to be retained intracellularily [9]. Thus, although estramustine exhibits typical characteristics of an anti-mitotic agent, this indicates that its uptake kinetics might differ from that of the vinca-alkaloids. We therefore deemed it of interest to study the uptake kinetics of EM over an extended time period at the cellular, nuclear and NPM level.

Materials and methods

^{3}H-estramustine, estradiol 3-*N*-bis[2-chloroethyl] carbamate (2, 4, 6, 7-^{3}H; 102 Ci/mmol) was synthesized at AB Leo. The purity of the compound was at least 98% as determined by high performance liquid chromatography, HPLC (Waters Bondapak C18 column, acetonitrile: water: acetic acid (63:36:1); 0.4 ml/min). The substance, stored in 9:1 toluene: ethanol, was evaporated with N^2 (g) and dissolved in 95% ethanol. The final concentration of solvent in the incubation mixture did not exceed 0.2%.

The human prostatic 1013L tumour cell line [10] was kindly donated by Dr. D. Mickey, Duke University, U.S.A. The cells were grown as described earlier [3]. HeLa S_3 cells were grown in minimum essential medium (S-MEM) supplemented with 10% FCS. The cells were constantly stirred (40 rpm) and diluted at 10^6 cells/ml. Cells in late log phase ($0.5–0.7 \times 10^6$ cells/ml) were incubated with 10 nM ^{3}H-EM at 37° from 5 min to 24 hr. Incubations were terminated by centrifugation of the cells in a chilled centrifuge (1000 rpm) and subsequent washes with ice-cold medium without serum. Nucleus and nuclear protein matrix were isolated as described earlier [3].

For determinations of drug uptake 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 for 10 min. Protein measurements were carried out according to Lowry [11].

Results and discussion

Figure 1 shows the cellular, nuclear and NPM uptake of ^{3}H-EM in 1013L cells. After 1 hr, approximately 30% of the final binding was found associated with the nuclear and NPM fractions, but the maximum was not reached until 15 to 20 hr from the start of the incubation. ^{3}H-EM binding to whole cells was, however, a faster process, after 5 min

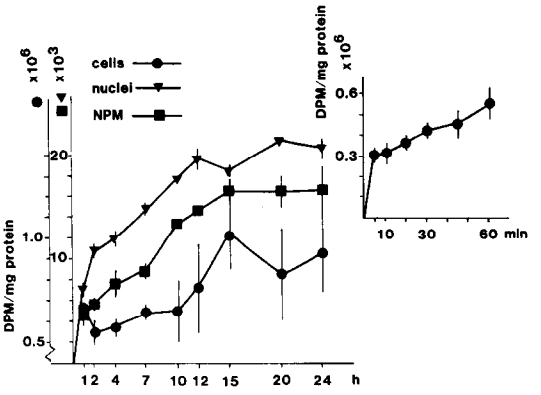

Fig. 1. The uptake of radioactivity, after incubation with ^{3}H-EM (10 nM), by prostatic 1013L tumour cells, nuclei and nuclear protein matrix *in vitro*. The initial cellular uptake up to 1 hr is amplified in the insert. Values are given in DPM/mg protein. Each point is the mean of two experiments.

incubation, 50–60% was bound and by 1 hr the maximum was reached. Comparative uptake studies in the human tumour HeLa S_3 cell line indicated a similar slow and time-dependent uptake pattern for EM, to the nucleus and NPM (see Table 1). Maximum cellular uptake was reached after a 1 hr incubation whereas after 9 hr incubation only 33–34% of the maximum nuclear and NPM uptake was found.

The biphasic cellular uptake of EM into prostatic tumour cells shown in this report, is consistent with the uptake pattern of vincristine (VCR) by murine leukemia cells [12]. The authors demonstrated on serial uptake measurements between 30 sec and 40 min, a biphasic pattern, temperature dependence and competitive inhibition by the structural analogue, vinblastine, VBL. The results indicated the presence of an energy-dependent transport mechanism for translocation of VCR into the cells. Ferguson *et al.* [9] reported studies on the uptake of VCR and VBL in mouse leukemia L1210 cells showing that cellular uptake reached an equilibrium by 4 hr. Also, after a 3 hr recovery period, 30% VCR and 10% VBL, in comparison to the maximum uptake, was retained in the cells. On the contrary, uptake of chlorambucil by L5178Y lymphoblasts *in vitro* [13] and of busulphan, melphalan and chlorambucil by Yoshida ascites sarcoma cells *in vitro* [14] showed a completely different pattern. Cellular uptake for these substances was restricted to the first 5 min, i.e. 45 sec, of incubation. Efflux of intact chlorambucil was also found to be very rapid, more than 60% of the drug being lost from the cells in 1 min [13]. These results indicated a simple diffusion mechanism for transport of alkylating agents in cultured tumour cells. Hence, the present report demonstrates differences between the uptake of EM and that of alkylating agents indicating that the nor-nitrogen mustard moiety probably is not involved in the uptake of EM.

We have earlier reported on EM metabolism in both HeLa and 1013L cells. The accumulated radioactivity in HeLa cells after a 24 hr ^{3}H-EM treatment and a 7 hr recovery period originates mainly from EM while a relatively large amount of lipophilic metabolites together with the parent substance are located in the NPM of the same cells. The metabolite scheme in 1013L cells exhibits a different pattern. Estradiol and estrone are found in the cellfraction as well as EM and a large amount of estromustine, EoM. In the NPM, however, only EM and EoM are found [7].

Table 1. Time course of radioactivity uptake in HeLa cell fractions

Incubation time (hr)	Cells	Nuclei	NPM
1	103.6	16.3	13.7
3	102.4	26.6	20.8
6	101.2	35.4	27.5
9	107.3	33.4	34.1
24	100	100	100

Figures represent percentage of total DPM uptake, the value obtained after 24 hr incubation is represented by 100%. Variation did not exceed ± 5.0% in the two experiments.

This implies that the 1013L cellular radioactivity is due to uptake of EM and its metabolites. Uptake into the 1013L nucleus and NPM, on the contrary, can only be due to the existence of EM and EoM.

The very slow nuclear and NPM-uptake of EM may be related to the slow release of the drug from the NPM. We have demonstrated that 50% of NPM associated drug was still bound after a 7 hr recovery period in drug free medium [7]. These two factors indicate either that a low number of drug binding sites are available or that a tight binding occurs. Interest has previously been focused on cellular uptake while no information is available on nuclear uptake of anti-mitotic agents. Thus it is impossible to say if the slow nuclear uptake and release of EM metabolites is unique or a facit of all anti-mitotic agents. Further studies on other cell lines and other substances are needed before these results can be said to be specifically related to EM and its metabolites.

In summary, the uptake of ^{3}H-estramustine, into prostatic tumour 1013L cells and HeLa cells was investigated. Drug uptake of 10 nM estramustine was followed in whole cells, pure nuclei and nuclear protein matrix, over a 24 hr period. This investigation shows a surprisingly slow nuclear and nuclear protein matrix uptake of estramustine, in 1013L and HeLa cells *in vitro*. Maximum whole cell uptake was reached after 1 hr incubation, whereas only 30% (1013L) and 13–16% (HeLa), of the maximum binding associated with the nuclei and nuclear protein matrix fractions, was reached by this time. Maximum binding to the two later fractions was not achieved until after 15 to 20 hr exposure.

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