Eur J Cancer Clin Oncol, Vol. 20, No. 1, pp. 115–121, 1984.
Printed in Great Britain.

0277-5379/84$3.00+0.00

Cellular Pharmacology of Detorubicin and Doxorubicin in L1210 Cells*

A. ZENEBERGH, R. BAURAIN and A. TROUET

Université de Louvain and International Institute of Cellular and Molecular Pathology, Avenue Hippocrate, 75, B-1200 Brussels, Belgium

Abstract—*Detorubicin (DET), a semi-synthetic analog of daunorubicin, releases at neutral pH doxorubicin (DOX) upon hydrolysis. DET enters faster than DOX into the cultured L1210 cells and reaches higher intracellular levels. When the cells are incubated for* 120 min *at* pH 6.5, *in spite of its rapid hydrolysis, one third of the intracellular fluorescence was due to undegraded DET. DET, like the other anthracyclines studied, is found associated intracellularly only to the lysosomes and to the nuclei. Unchanged DET is found mainly inside the lysosomes where the drug is stabilized, while DOX is found essentially associated to the nuclear DNA of L1210 cells. DET can therefore be viewed as an hydrophobic prodrug of DOX characterized, however, by a distinct subcellular localization in L1210 cells.*

INTRODUCTION

DETORUBICIN (14-diethoxyacetoxydaunorubicin; DET) is a semi-synthetic analog of daunorubicin [1] undergoing clinical trials for hematological neoplasia [2]. At neutral pH DET releases doxorubicin (DOX) upon hydrolysis and the drug stability is increased at acidic pH or when DET is complexed to DNA [3]. Despite its rapid hydrolysis into DOX, DET is characterized by a decreased overall toxicity and a greater potency against the s.c. inoculated L1210 leukemia [3]. Recent results have also shown that DET is less toxic than DOX for the hemopoietic pluripotent and committed stem cells [4] and slightly less cardiotoxic than DOX in the chronic rabbit test developed by Jaenke [5]. The reduced cardiotoxicity of DET has been attributed to a lower uptake by the heart muscle cells [6].

To analyse the role played by DET hydrolysis in its distinct pharmacologic and therapeutic properties we have compared the cellular pharmacology of DET and DOX in cultured L1210 cells.

MATERIALS AND METHODS

DET and DOX were obtained, respectively, from Rhône-Poulenc S.A. (Paris, France) and from Farmitalia-Benelux (Brussels, Belgium). Stock solutions at 6 mg/ml for DOX and 7.2 mg/ml for DET in 0.1 M acetate buffer at pH 4.5 were further diluted with the culture medium buffered at pH 6.5. DET or DOX were added to the complete culture medium at an equimolar concentration ranging between 10 and 85 μM.

L1210 ascitic cells obtained from leukemic DBA_2 mice were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum [7].

For the accumulation experiments 4 ml of L1210 cells (0.8–1 mg cell protein) were incubated in 5 ml Falcon tubes at 37°C. After incubation the cells were washed twice with ice-cold phosphate-buffered saline (PBS) at pH 6.5, centrifuged for 10 min at 1200 rpm (IEC Centrifuge, rotor 253) and resuspended in 1 ml distilled water.

The drugs and their fluorescent metabolites were analysed on 0.1 ml aliquots by high-pressure liquid chromatography (HPLC) and fluorometry as described previously [8]. The proteins were determined by the method of Lowry *et al.* [9].

For cell fractionation experiments, the cells were washed twice with PBS pH 6.5 and resuspended in 0.25 M sucrose, 3 mM imidazole pH 7.0. The homogenization, post-nuclear supernatant (E) preparation, and the assays for marker enzymes, protein and nucleic acids were performed as described previously [10, 11].

RESULTS

The ester bond in position C-14 of DET is hydrolyzed releasing DOX as anthracycline.

Accepted 11 July 1983.
*This work was supported by Rhône-Poulenc, Paris, France and by a grant of the Fonds Cancérologique de la Caisse Générale d'Epargne et de Retraite, Brussels, Belgium.

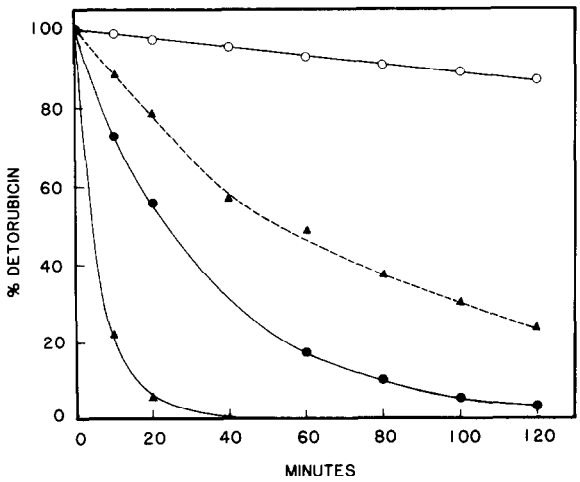

Fig. 1. Stability of DET in solution. DET solutions were incubated at 37°C in 0.5 M acetate buffer at pH 4.5 (○), culture medium-buffered at pH 6.5 (●) and phosphate-buffered saline pH 7.4 in absence (▲——▲) and in presence of DNA ▲----▲). After various times, the amounts of DET remaining in the solution were determined by HPLC and expressed as percentage of remaining DET.

Figure 1 shows the amount of DET remaining unhydrolyzed after various periods of time at pH 7.4, 6.5 and 4.5. We have estimated a half-life value of 24 min at pH 6.5 which is to be compared with 4.6 and 600 min at pH 7.4 and 4.5, respectively. At pH 6.5, after 20 and 120 min incubation at 37°C, respectively, 56 and 3% of DET remained intact. Therefore, all the incubation experiments were performed with a medium at pH 6.5 to slow down the hydrolysis of DET.

When cells are incubated for 20 min with increasing concentrations of DET, the total level of anthracycline fluorescence increases linearly up to 85 μM (Fig. 2a). As determined by HPLC and fluorometry, DET and its hydrolysis product, DOX, account, respectively, for 40 and 44% of the total intracellular fluorescence. The remaining fluorescence is due essentially to the aglycone of DOX (Fig. 2a). Cells incubated in the same conditions in presence of DOX accumulate the drug linearly up to 50 μM, and above this external concentration the uptake seems to become saturated (Fig. 2b). Only a small amount of aglycone is measured by HPLC and fluorometry.

Figure 3 illustrates the uptake of DET and DOX as a function of time when L1210 cells are incubated in presence of the drugs at 85 μM. For cells incubated in the presence of DET, the total fluorescence reaches a plateau after 2 hr incubation. However, the parent drug, DET, levels off after 30 min and thereafter the accumulation is due mainly to its hydrolysis product, DOX.

Cells incubated with DOX accumulate the drug more slowly, the levels reached are lower, and a plateau level is not reached after 120 min.

L1210 cells incubated for 20 or 120 min in presence of DET or DOX (85 μM) were

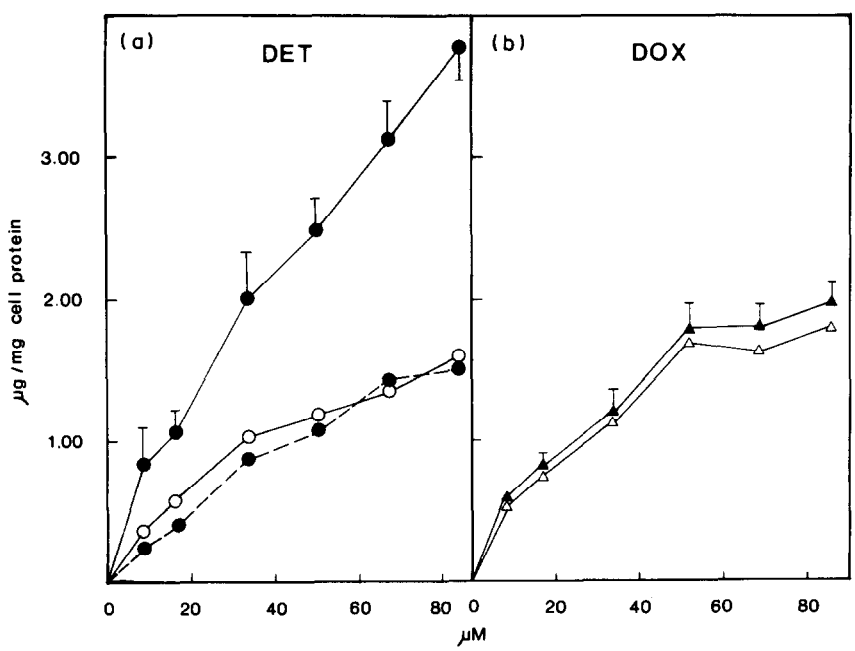

Fig. 2. Intracellular uptake of detorubicin and doxorubicin by L1210 leukemia cells in vitro in function of the drug concentrations. Cells were incubated at pH 6.5 for 20 min, washed twice with PBS at pH 6.5 and resuspended in 1 ml water. Aliquots of 0.1 ml cell homogenate were added to 0.1 ml daunorubicin (internal standard) at 2 μg/ml borate buffer pH 9.8 and 1.8 ml chloroform-methanol (4:1). After vortexing an aliquot of the organic phase was injected into the HPLC. (a) Cells incubated with DET. ●----●, DET; ○——○, DOX; ●——●, sum of the parent drug and its fluorescent metabolites. (b) Cells incubated with DOX. △——△, DOX; ▲——▲, sum of the parent drug and its fluorescent metabolites.

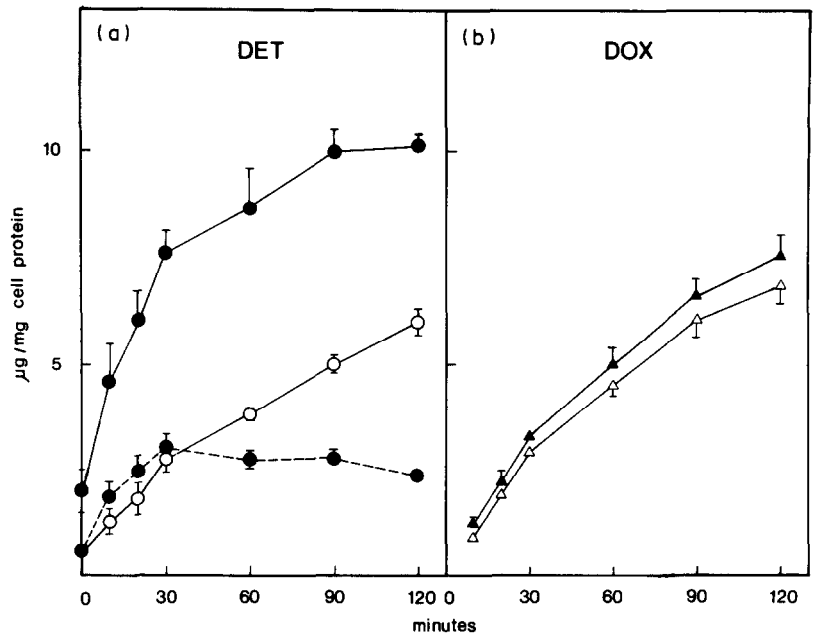

Fig. 3. Uptake of detorubicin and doxorubicin by L1210 cells in culture. Cells were incubated in 4 ml *medium supplemented with* 10% *foetal calf serum and buffered at* pH 6.5. *After incubation the cells were washed and the drugs determined by flow fluorometry after HPLC. (a) L1210 cells incubated with DET at a concentration of* 85 µM. ●----●, *DET;* ○——○, *DOX;* ●——●, *sum of the parent drug and its fluorescent metabolites. (b) L1210 cells incubated with DOX at a concentration of* 85 µM. △——△, *DOX;* ▲——▲, *sum of the parent drug and its fluorescent metabolites.*

homogenized and a post-nuclear supernatant (E) was separated from a nuclear fraction (N). Table 1 gives the amount of anthracycline found in E and N and the amount of fluorescence due to the parent drug and their hydrolysis products.

After an incubation in presence of DET, the nuclear fraction contained mainly DOX while the parent drug DET reaches a comparable level in E and N. After an incubation in presence of DOX most of the drug is associated with the nuclear fraction and the total amount of drug accumulated is lower.

Table 1. Subcellular distribution between the nuclear fraction (N) and the postnuclear supernatant (E) of the fluorescence in L1210 cells incubated 20 *and* 120 min *with DET or DOX at an external concentration of* 85 µM *at* pH 6.5. *Results are expressed in µg per* mg *cell protein*

	20 min		120 min	
Cells incubated	E	N	E	N
In presence of DET				
DET	0.75	0.81	1.40	1.23
DOX	0.69	2.12	1.21	5.44
Aglycone	0.58	0.12	0.49	0.44
Total uptake	2.02	3.05	3.10	7.11
In presence of DOX				
DOX	0.18	0.73	0.46	3.72
Aglycone	0.17	0.18	0.12	0.20
Total uptake	0.35	0.91	0.58	3.92

The post-nuclear supernatant of cells incubated for 20 min with DET was submitted to isopycnic centrifugation. The distribution patterns of DET and its metabolite DOX, DNA and some marker enzymes are represented in Fig. 4. The distribution of DET is bimodal, the main part having a distribution profile similar to that of the lysosomal marker enzyme, *N*-acetyl-β-glucosaminidase, and the remaining being localized in the heavy fractions of the gradient where the DNA present in E is found. The DOX resulting from the hydrolysis of DET is localized mainly in the fractions of the gradient rich in DNA. After an incubation of 120 min the distribution of DET is still superposable to that of the lysosomal enzyme and the distribution pattern of DOX corresponds closely to the distribution of the nuclear contaminating DNA (results not shown).

The distribution pattern of DOX, DNA and marker enzymes for cells incubated for 20 min in presence of DOX is shown in Fig. 5. DOX was found in increasing concentrations in the heavier fractions of the gradient, where the DNA present in E is also found.

The percentage of drug present in E which could be attributed to the lysosomes and the DNA has been calculated, assuming biochemical homogeneity for the two components [10].

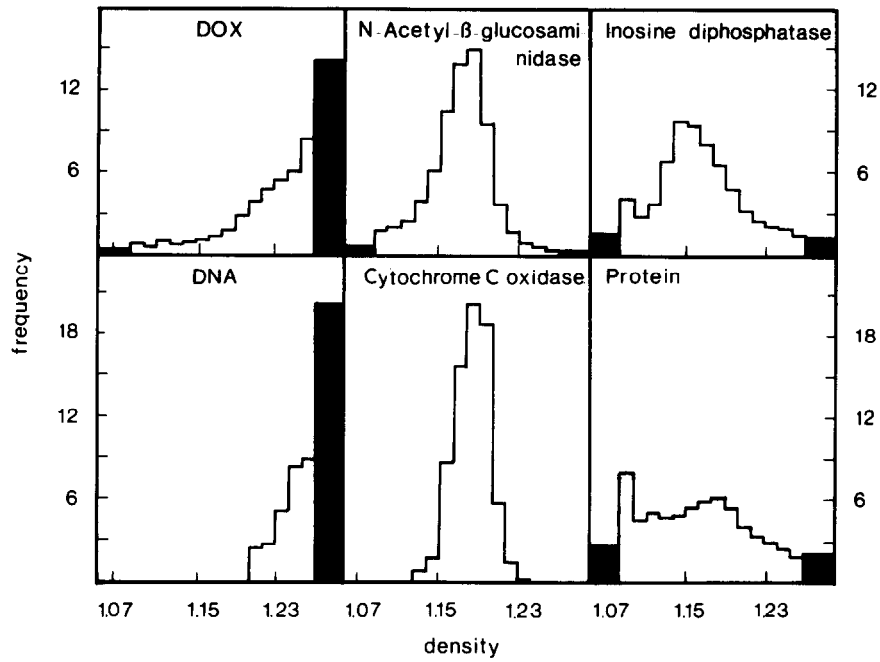

Fig. 4. Distribution patterns of DET, its metabolite DOX, marker enzymes and DNA in the post-nuclear supernatant of L1210 cells incubated for 20 min *with DET at an extracellular concentration of* 85 μM *at* pH 6.5. *The frequency (ordinate) is* $\Delta Q\,(Q \times \Delta\delta)$, *where* ΔQ *is the amount of constituent in the fraction,* Q *is the total amount of constituent in all fractions and* $\Delta\delta$ *is the density increment for each fraction* (0.0133). *Filled areas represent the amount of constituent equilibrating at densities below* 1.07 *and above* 1.27, *respectively.*

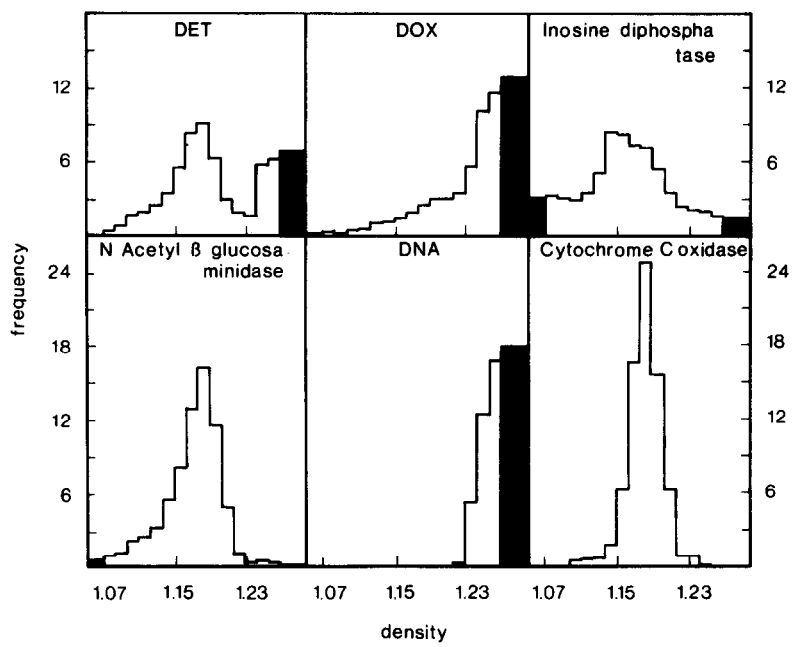

Fig. 5. Distribution patterns of DOX, marker enzymes, proteins and DNA in the post-nuclear supernatant of L1210 cells incubated for 20 min *with DOX at an extracellular concentration of* 85 μM *at* pH 6.5.

The percentage of total anthracycline (A) which can be attributed either to *N*-acetyl-β-glucosaminidase (L) and to DNA (D) in each fraction is given by the relation: $A = x \cdot L + y \cdot D$, where x and y are the fraction content in lysosomal enzymes and DNA, respectively. Solving the equation for each fraction of E gives the repartition of DET and DOX between the lysosomes and DNA (Table 2).

The amount of anthracycline associated to DNA present in E has been added to the amount present in the fraction N to afford the true distribution of the drugs between the lysosomes and the nuclei (Fig. 6). After incubation in

Table 2. Percentage of DET or DOX, in the post-nuclear supernatant, attributed to lysosomes (N-acetyl-β-glucosaminidase) and DNA

	Incubation time			
	20 min		120 min	
	Lysosomes	DNA	Lysosomes	DNA
In presence of DET				
DET	61	39	98	2
DOX	22	78	39	61
In presence of DOX				
DOX	19	81	39	61

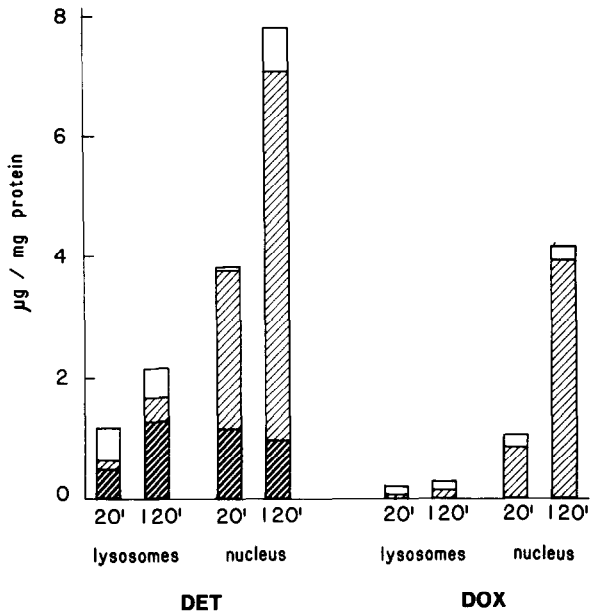

Fig. 6. Lysosomal and nuclear distributions of DET and DOX in L1210 cells incubated for 20 and 120 min at an extracellular drug concentration of 80 µM. The results are corrected for the DNA content in the post-nuclear fraction as explained in the text and expressed in µg drug/mg protein. ▨, DET; ▨, DOX; □, aglycone.

presence of DET, the nuclear concentration is greater than the concentration reached in the lysosomes. In the nucleus the majority of the anthracycline is in the form of the hydrolysis product, DOX, while in the lysosomes intact DET is found predominantly. When the cells are incubated in presence of DOX, the drug levels in both the nucleus and the lysosomes are lower than after incubation in presence of DET, the nucleus being, however, the main site of accumulation of DOX.

DISCUSSION

In cultured L1210 cells DET accumulates more rapidly and reaches higher intracellular levels than DOX. At pH 6.5 and assuming that 1 mg cell protein corresponds to 5 µl [12] we can calculate that after 1 hr indubation the intracellular concentrations of DET and DOX are, respectively, 30 and 20 times higher than those of the extracellular ones.

In the conditions chosen, it has not been possible to avoid the hydrolysis of DET into DOX. However, it has been slowed down and after 20 min more than 50% of the drug remained intact, while at pH 7.4 only 5% of DET remained as such. After incubation in presence of DET, the intracellular level of unmetabolized drug increases up to 30 min (Fig. 3). This could be due only to an intracellular protection of DET from hydrolysis. Previous results [3] have shown that such a protection could result from a complex formation with DNA or an acid pH (Fig. 1) and results from Figs 4 and 6 show that the drug is in fact distributed only between the lysosomes and the DNA. No DET was found associated to the mitochondria or to the plasma membrane, which does not preclude but reduces drastically the probability that the drug does play a role at those sites.

Our results confirm the general trend observed for the subcellular localization of various anthracyclines such as DNR [13, 14], DOX [7], ACM [7] and *N*-L-leucyl-*DNR* [15], which are found associated intracellularly only to the lysosomes and to the nuclear DNA in cultured fibroblasts [13, 14], L1210 cells [7], Ehrlich ascites cells [16] and rat myocardial cells [17]. The subcellular localization of the anthracyclines is affected quantitatively by the balance between their pK_a, their DNA-binding constant K_a and their hydrophobicity. The hydrophobicity modulates the speed with which the anthracyclines enter the cells by permeation and therefore the cytosolic concentrations [7, 14, 18]. Therefore, the faster uptake of DET by cells could be related to its greater lipophilicity. The lysosomal localization could be explained by the weak base character of the anthracyclines and the acidity of the lysosomes [19, 20]. The nuclear localization results from the high affinity of the drugs for the DNA, which is due to the intercalation of the planar aglycone moiety between bases pairs of

DNA and the stabilization of the intercalation by an ionic bond between the positively charged amine function of the daunosamine and a negatively charged phosphate group of the DNA [21].

Previous results on the accumulation of DNR and DOX in cultured fibroblasts have shown that the nuclei becomes more rapidly saturated than the lysosomes [14]. Results from Fig. 6 indicate that DET, like DOX, saturates first the nuclei. There the parent drug is slowly converted into DOX and after 2 hr of incubation more than 78% of the fluorescence in the nuclei is under the form of DOX. The protection from the hydrolysis is better in the lysosomal compartment since there the amount of intact DET increases up to 2 hr and represents at that time more than 61% of the intralysosomal drug fluorescence.

The higher potency of DET, as compared to DOX, on the s.c. inoculated L1210 murine leukemia [3] could be related to the greater lipophilicity, the higher rate of uptake and the higher nuclear concentration reached by DET in the target cells. The greater lipophilicity allows the drug to cross more easily the biological membranes separating the blood from the target cells and therefore to reach higher levels in the vicinity of the tumor cells. The decreased chemotherapeutic activity of DET, as compared to DOX, against the L1210 leukemia inoculated i.v. could be due to the more rapid plasma clearance of DET [3].

In conclusion, our results show that despite a rapid hydrolysis DET has a different subcellular distribution than its hydrolysis product DOX. Unchanged DET is found mainly inside the lysosomes where the drug is stabilized, while DOX is found essentially associated to the nuclear DNA of L1210 cells. The subcellular localization of various anthracyclines is therefore the same qualitatively, they distribute only between the lysosomes and the nuclei and only quantitative differences are observed resulting from differences in the pK_a, DNA-binding affinity K_a and lipophilicity of the various anthracyclines.

The potentialities of DET as lipophilic anthracycline which generates both *in vitro* and *in vivo* DOX remain to be investigated in the case of brain tumors. Higher anthracycline levels could accumulate in the brain due to better crossing of the blood brain barrier by this lipophilic anthracycline.

Acknowledgements—We are indebted to Mrs. B. Vande Voorde-Aerts and Miss T. Aerts for their skillful technical assistance.

REFERENCES

1. MARAL R, DUCEP JB, FARGE D, PONSINET G, REISDORF D. Préparation et activité antitumorale expérimentale d'un nouvel antibiotique semi-synthétique: la diéthoxyacétoxy-14-daunorubicine (33 921 RP). *CR Acad Sci (Paris)* 1978, **286**, 443–446.
2. JACQUILLAT C, WEIL M, AUCLERC MF *et al.* A survey of the anthracycline derivatives in hematology. *Cancer Chemother Pharmacol* 1979, **2**, 53.
3. DEPREZ-DE CAMPENEERE D, BAURAIN R, TROUET A. Pharmacokinetic, toxicologic and chemotherapeutic properties of detorubicin in mice: a comparative study with daunorubicin and adriamycin. *Cancer Treat Rep* 1979, **63**, 861–867.
4. HUYBRECHTS M, TROUET A. Comparative toxicity of detorubicin and doxorubicin, free and DNA-bound for hematopoietic stem cells. *Cancer Chemother Pharmacol* 1980, **5**, 79.
5. JAENKE R, DEPREZ-DE CAMPENEERE D, TROUET A. Cardiotoxicity and comparative pharmacokinetics of six anthracyclines in the rabbit. *Cancer Res* 1980, **40**, 3530–3536.
6. DEPREZ-DE CAMPENEERE D, BAURAIN R, TROUET A. Accumulation and metabolism of new anthracycline derivatives in the heart after i.v. injection into mice. *Cancer Chemother Pharmacol* 1982, **8**, 193–197.
7. ZENEBERGH A, BAURAIN R, TROUET A. Cellular pharmacokinetics of aclacinomycin A in cultured L1210 cells. Comparison with daunorubicin and doxorubicin. *Cancer Chemother Pharmacol* 1982, **8**, 243–249.
8. BAURAIN R, ZENEBERGH A, TROUET A. Cellular uptake and metabolism of daunorubicin as determined by high-performance liquid chromatography: application to L1210 cells. *J Chromatogr* 1978, **157**, 331–336.
9. LOWRY OH, ROSEBROUGH NJ, FARR AL, RANDALL RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951, **193**, 265–275.
10. DE DUVE C, PRESSMAN BC, GIANETTO R, WATTIAUX R, APPELMANS F. Tissue fractionation studies. VI. Intracellular distribution pattern of enzymes in rat liver tissue. *Biochem J* 1955, **60**, 604–617.

11. ZENEBERGH A, SCHNEIDER YJ, LONI MC, TROUET A. Enzymatic characterization and analytical fractionation of L1210 cells. *Biochimie* 1981, **63**, 411–418.
12. TULKENS P, TROUET A. The uptake and intracellular accumulation of aminoglycoside antibiotics in lysosomes of cultured rat fibroblasts. *Biochem Pharmacol* 1978, **27**, 415–424.
13. NOËL G, PETERSON C, TROUET A, TULKENS P. Uptake and subcellular localization of daunorubicin and adriamycin in cultured fibroblasts. *Eur J Cancer* 1978, **14**, 363–368.
14. PETERSON C, TROUET A. Transport and storage of daunorubicin and doxorubicin in cultured fibroblasts. *Cancer Res* 1978, **38**, 4645–4649.
15. MASQUELIER M, BAURAIN R, TROUET A. Cellular pharmacology of amino acid derivatives of daunorubicin. In: NELSON JD, GRASSI C, eds. *Current Chemotherapy and Infectious Dieases*. Washington DC, American Society of Microbiology, 1980, Vol. 2, 1688–1690.
16. LONDOS-GAGLIARDI D, AUBEL-SADRON G, MARAL R, TROUET A. Subcellular localization of daunorubicin in sensitive and resistant Ehrlich ascites tumor cells. *Eur J Cancer* 1980, **16**, 849–854.
17. BLANCHARD JC, SCHNEIDER YJ, BAURAIN R, TROUET A. Accumulation, metabolism and subcellular localization of daunorubicin, doxorubicin and their DNA-complexes in rat heart ventricules. *Eur J Cancer* 1981, **17**, 297–305.
18. BACHUR NR, STEELE M, MERIWETHER WD, HILDEBRAND RC. Cellular pharmacodynamics of several anthracycline antibiotics. *J Med Chem* 1976, **19**, 651–654.
19. DE DUVE C, DE BARSY TH, POOLE B, TROUET A, TULKENS P, VAN HOOF F. Lysosomotropic agents. *Biochem Pharmacol* 1974, **23**, 2495–2531.
20. OKHUMA S, POOLE B. Fluorescence probe measurement of intralysosomal pH in living cells and perturbation of pH by various agents. *Proc Natl Acad Sci USA* 1978, **75**, 3327–3330.
21. PIGRAM WJ, FULLER M, HAMILTON LD. Stereochemistry of intercalation interaction of daunorubicin with DNA. *Nature New Biol* 1972, **235**, 17–19.