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SUBCELLULAR LOCALIZATION OF SOME ANTHRACYCLINE DERIVATIVES IN EHRLICH ASCITES TUMOR CELLS

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SUMMARY

With the whole set of the tested anthracyclines, a bimodal localization, nuclear and lysosomal, is observed. But the percentage of the drug which is stored in the nuclei is different according to the drug. With some derivatives which have a high therapeutic efficiency, 82-87 % of the drug is recovered in the nuclei. In a second group whatever the biological activity of the drug only 49-52 % of the drug accumulates in the nuclei.

INTRODUCTION

Anthracycline antibiotics such as daunorubicin (DNR) and doxorubicin (DOX) have an antitumoral activity and are widely used in human cancer chemotherapy (1,2). The use of these compounds is limited by their peculiar cardiotoxicity and the development of resistance against these drugs. One way to improve the oncostatic arsenal is the development of analogs in order to find new derivatives with less toxicity and/or more activity. Elucidation of the mechanism of the action of drugs may permit new approaches in the development of cancer chemotherapy.

It has been postulated that the high affinity of the anthracyclines for DNA is the main cause of the nuclear trapping of the drugs (3,4,5). The presence of NH_2 at the C-3' position is essential for the formation of an intercalative complex (4,6). A modification of the amino function such as in N-acetyl-DNR or in N-acetyl-DOX markedly decreases the affinity for DNA and can

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be the cause of the low concentration of these drugs in fibroblast nuclei (7). This explanation is not convenient for the DNR analogs which are the subject of this present work since the whole set of analogs have an NH_2 in the appropriate position. In spite of striking differences in biological activity, DNR analogs intercalate *in vitro* between DNA base pairs (8,9,10) and do not show significant differences in their affinity for DNA (3). Therefore it would be hazardous from *in vitro* studies to extrapolate an explanation of an *in vivo* mechanism and activity.

In order to improve the understanding of the pharmacology of DNR analogs it seems relevant to compare these analogs on the basis of their effect on a tumor target (11). We have chosen Ehrlich ascites tumor (EAT) cells to follow *in vivo* the intracellular uptake and the subcellular localization of some DNR derivatives using the cell fractionation technique (12).

MATERIAL AND METHODS

Daunorubicin and daunorubicin analogs

Anthracycline derivatives were obtained from Rhône Poulenc (Paris, France). Solutions in saline (2 mg/ml) were prepared extemporaneously.

Daunorubicin derivative formulae and antitumor activities are given in table 1.

All derivatives, except RP 38 422, used in the present work, have already been studied (3,13). By thermal denaturation and analytical centrifugation, we have shown that RP 38 422 gives an intercalation complex with DNA. The value of its affinity constant is of the same order of magnitude as the values found with other DNR analogs, but RP 38 422 has no therapeutic efficiency.

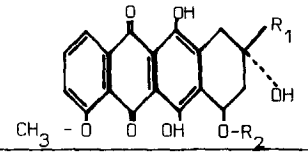
Ehrlich ascites tumor cells

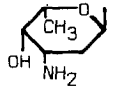
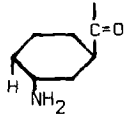
CD 1 female mice (Charles River, France), 6-8 weeks old were inoculated i.p. with 10^6 EAT cells per mouse. After eight days, 25 to 50 mg/kg of drug were given i.p.. After one hour, mice were sacrificed and ascitic fluid was collected. In all cases, due to the short time interval following the treatment, the death rate of tumor cells, as determined by the trypan blue exclusion test, was never higher than 10 %.

Cell fractionation and sucrose gradient centrifugation

Homogenization of the cells, fractionation by differential centrifugation and analysis by sucrose gradient centrifugation were described earlier (13,14). Cells were fractionated to yield the following four fractions : a nuclear fraction N, a heavy mitochondrial fraction M, a light mitochondrial fraction L, a microsomal fraction P and a final supernatant S.

Table I - Daunorubicin analogs.



Compound	R_1	R_2	MTD (mg/kg i.p.)	Activity (%)
Daunorubicin (RP 13 057) DNR	$-COCH_3$		0.75	180
Doxorubicin (RP 25 253) DOX	$-COCH_2OH$	"	0.75	190
Daunorubicinol or Duborimycine (RP 20 798) DOL	$-CHOH-CH_3$	"	3.00	160
Detorubicin (RP 33 921) DETO	$-CO-CH_2-O-CO-CH(OC_2H_5)_2$	"	1.2	190
RP 38 422	$-CO-CH_2-S-CH_2-COOCH_3$	"	50	100
RP 21 080*	$-COCH_3$		10	180
RP 32 885	"	"	∿ 10	190
RP 32 886	"	"	∿ 15	176
RP 33 365	"	"	∿ 10	132
RP 33 366	"	"	∿ 10	140

*RP 21 080 is composed of 4 stereoisomers which have been isolated : the two trans-isomers RP 33 365 and 33 366 and the two cis-isomers RP 32 885 and 32 886. MTD : 5 consecutive treatments, maximal tolerated dose, intraperitoneal dose (i.p.) which induces neither weight loss nor mortality, mg/kg i.p. (mice). Activity : Leukemia L 1210, grafted i.p. in $B_6D_2F_1$ mice (10^3 cells) on day 0, activity is expressed by the following ratio :

$$\frac{\text{mean survival time (treated mice)}}{\text{mean survival time (control)}} \times 100$$

, mice are treated at the MTD, on days 0,1,2,3 and 4, the activity is significative when the percentage is > 150.

Table II - Accumulation of daunorubicin analogs in Ehrlich ascites tumor cells.

Compound	Accumulation (µg of drug/mg of protein)*	
	A	B
Daunorubicin	2.75 (1)	12.74 (4)
Doxorubicin	2.33 (1)	9.24 (2)
Daunorubicinol	3.17 (1)	8.77 (2)
Detorubicin	4.87 (3)	16.26 (1)
RP 38 422	0.93 (1)	3.71 (3)
RP 21 080	2.68 (1)	5.52 (1)
RP 32 885	1.44 (3)	-
RP 32 886	0.50 (1)	-
RP 33 365	1.28 (1)	-
RP 33 366	1.14 (2)	-

EAT bearing mice were inoculated i.p. with 25 mg/kg (A) or 50 mg/kg (B) of the drug and were sacrificed one hour after injection. Accumulation of drug is expressed by the ratio µg of drug/mg of protein. Number experiments are given in parenthesis.
* This value represents the sum of intact drug and fluorescent metabolites when metabolisation occurs.

Biochemical assays

Assay conditions for marker enzymes, nucleic acids and proteins were previously described (13). Anthracycline drug concentration was evaluated according to Noel *et al.* (15).

RESULTS

In vivo cellular uptake

The cellular uptake of DNR and DNR analogs after i.p. injection of 25 mg/kg or 50 mg/kg of drug per mouse is given in table II. One can notice that there are differences in the cellular uptake according to the drug : for example DNR, DOX, DOL and DETO accumulate at an higher rate than the other drugs.

Subcellular localization of the drugs

One hour after the treatment of mice with the anthracyclines, EAT cells were collected and cellular localization was performed. Taking into account the relative amount of β-N-acetylglucosaminidase and α-L-fucosidase in the N and M fractions, the true distribution of the drug between nuclei and cyto-

Table III - Percentage of daunorubicin analogs recovered in DNA containing fractions (N and M fractions).

Compound	Percentage in N + M fractions*	Number of experiments
Daunorubicin	87.40 ± 2.72	4
Doxorubicin	83.99	2
Daunorubicinol	82.67	2
Detorubicin	84.23	2
RP 38 422	50.18	3
RP 21 080	51.82	3
RP 32 885	49.64	3
RP 32 886	52.30	1
RP 33 365	50.32	1
RP 33 366	52.05	3

*Corrected values taking into account the lysosomal contamination (see text).

plasm has been calculated. As shown in table III, according to the percentage of drug recovered in the DNA containing fractions, the drug fall into two groups : in the first group (DNR, DOX, DOL and DETO) 83 to 87 % of the drug is recovered in the DNA containing fractions, whereas in the second group (RP 38 422, RP 21 080 and its four isomers) only 49 to 52 % is recovered.

For RP 38 422, the distribution of marker enzymes, protein and nucleic acids is given in figure 1. A similar pattern is observed for the anthracyclines belonging to the second group (Fig. 2). The distribution pattern of these drugs is clearly bimodal and follows the distribution of DNA and that of lysosomal hydrolases (β-N-acetylglucosaminidase and α-L-fucosidase) and this distribution is distinct from that of mitochondrial enzyme (cytochrome *c* oxidase), of microsomal fraction enzymes (NADPH cytochrome *c* reductase and inosine diphosphatase) and of soluble enzyme (phosphoglucomutase).

As it has been previously done in the case of DNR (14) in order to determine more precisely the extranuclear distribution of DOX, DOL and DETO (Fig. 2) a cytoplasmic fraction corresponding to MLP fraction was analyzed by sucrose gradient centrifugation. The distribution pattern of DOL is clearly

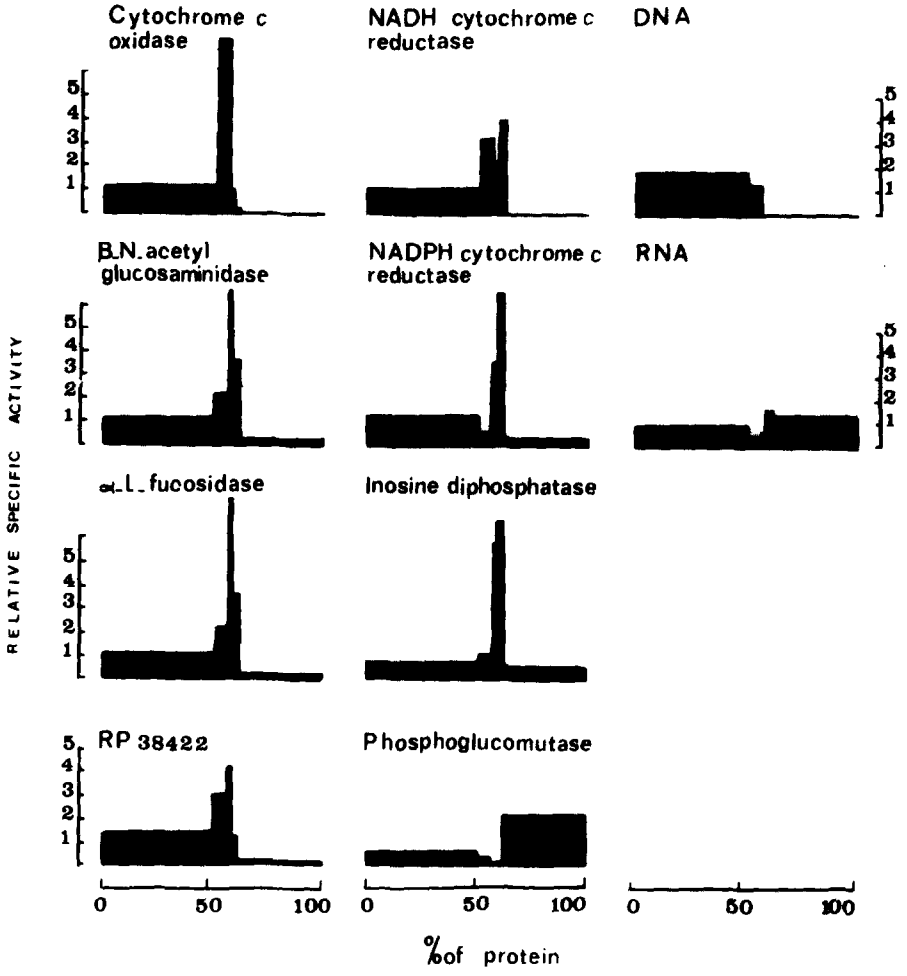


Fig. 1 - Distribution of RP 38 422 in EAT cells as determined by subcellular fractionation. Mice were inoculated i.p. with 0.5 mg of RP 38 422/mouse and sacrificed after one hour. N, M, L, P and S fractions are represented in blocks ordered in the same sequence along the abcissa when the length is proportional to the protein content. The ordinates give the relative specific activity (or the amount of RP 38 422, DNA and RNA) which is the percentage of activity (or amount) recovered in each fraction over the percentage of protein in the same fraction. Graph represents the mean results of three experiments.

bimodal (Fig. 3) : the major part of DOL follows the distribution of the two lysosomal marker enzymes and this distribution is distinct from that of mitochondrial and microsomal fraction enzymes. Part of DOL is recovered with heavy fractions corresponding to nuclear material contamining the MLP fraction. The same pattern was obtained with DOX and DETO.

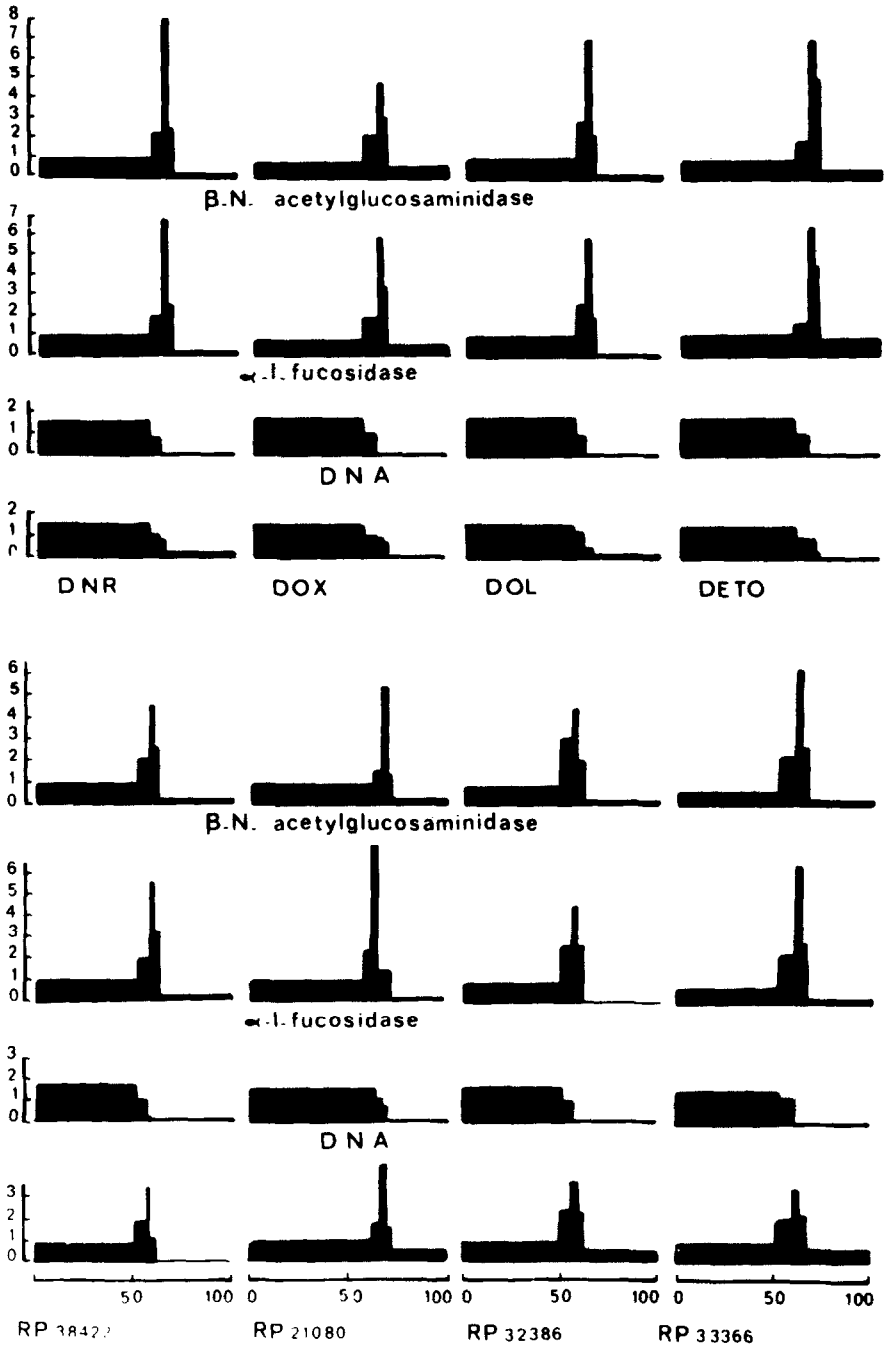


Fig. 2 - Fractionation patterns of acid hydrolases (β-N-acetylglucosaminidase and α-L-fucosidase), DNA and anthracyclines (daunorubicin, doxorubicin, detorubicin, daunorubicinol, RP 38 422, RP 21 080, RP 32 886, RP 33 366). Mice were inoculated i.p. with 0.5 mg of drug/mouse and sacrificed one hour after inoculation. Results are expressed as in figure 1.

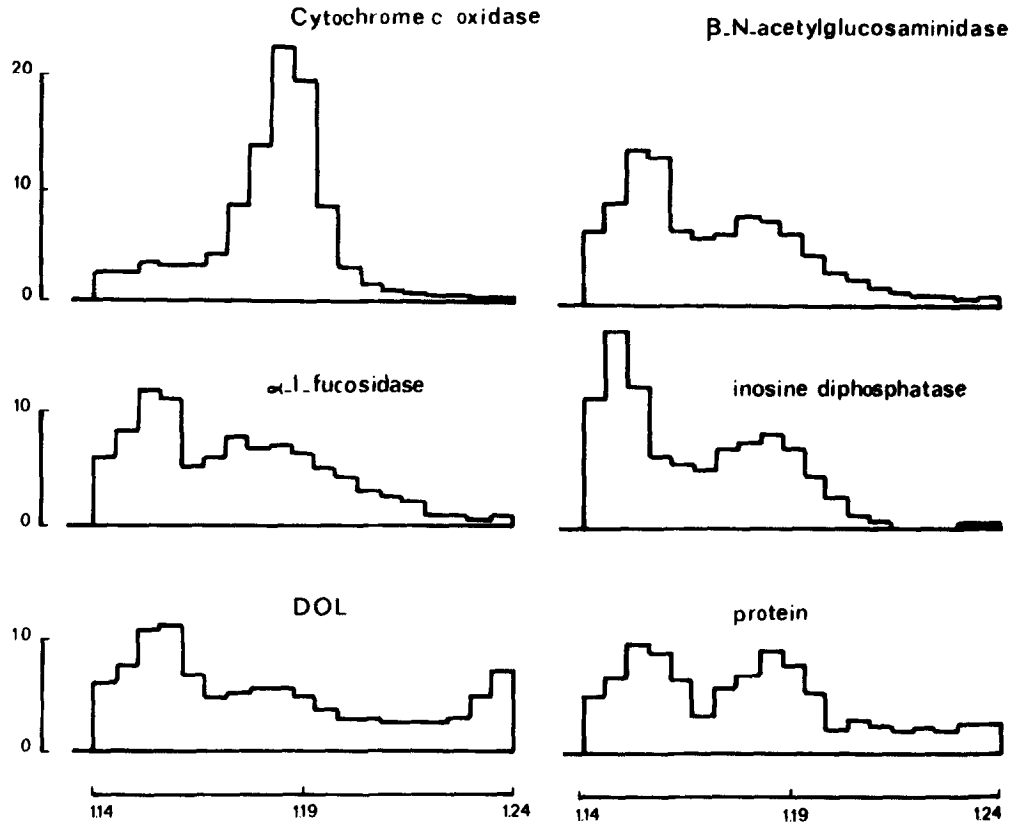


Fig. 3 - Distribution of daunorubicinol, protein and marker enzymes of the MLP fraction after sucrose gradient centrifugation. Mice were inoculated i.p. with 1 mg DOL/mouse and sacrificed after one hour. The ordinates indicate the percentage of total enzymatic activity or the amount of DOL and protein. The abcissa indicate sucrose density.

CONCLUSION

As previously described with DNR (14), anthracycline analogs accumulate, *in vivo*, in EAT cells. The cellular uptake differs according to the drug. Differences in cellular uptake could be explained by differences in the diffusion across the cellular membrane. It has been shown for EAT cells (16,17) that anthracyclines penetrate in the cell by diffusion and are actively transported outwards by a "leak and pump" mechanism.

A bimodal localization, nuclear and lysosomal, is observed with all derivatives. Whatever the tested anthracycline, given at the same dose, lysosomes accumulate the same quantity of drug, while in nuclei the accumulation is different. In the first group (DNR, DOX, DOL and DETO) 82 to 87 % of the drug

accumulates in the nuclei, while the remaining is recovered in lysosomes. These drugs have an high therapeutic efficiency, three of them (DNR, DOX and DETO) are used in human cancer chemotherapy. In the second group (RP 38 422, RP 21 080 and its four isomers) 49 to 52 % of the drug accumulates in the nuclei, while the major part of the extranuclear drug is recovered in lysosomes. In this group one can find drugs which have an experimental antitumoral activity (RP 21 080, RP 32 885 and RP 32 886), drugs which are poorly active (RP 33 365 and RP 33 366) or inactive (RP 38 422).

The four isomers of RP 21 080 set a problem : in spite of a same affinity for DNA, a same cellular uptake and a same intracellular localization, they exhibit different biological activity. A possible explanation could be difference in the *in vivo* metabolisation process of these isomers. Work is in progress on the study of the metabolites of these anthracycline analogs.

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