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Metabolism of Daunorubicin in Sensitive and Resistant Ehrlich Ascites Tumor Cells

Determination by High Pressure Liquid Chromatography

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Summary. *The intracellular metabolism of daunorubicin (DNR) has been studied in sensitive and resistant Ehrlich ascites tumor (EAT) cells. The subcellular localization of metabolites has been followed by normal-phase and reverse-phase high-pressure liquid chromatography (HPLC). The metabolism of DNR by either sensitive or resistant EAT cells is not significant; unmetabolized DNR is always the main intracellular compound. Daunorubicinol (DOL) accounts for less than 5% after 24 h and an unidentified product is also observed. This highly apolar compound, having an intrinsic fluorescence one order of magnitude greater than that of DNR is formed in acellular conditions and could be a chemical artifact. DNR and DOL are mainly associated with DNA-containing fractions. No significant differences can be observed in the metabolism of DNR in sensitive and resistant EAT cells.*

Introduction

The anthracycline antibiotic daunorubicin (DNR) is widely used in human cancer chemotherapy [7]. The drug intercalates between the base pairs of native DNA, thus leading to an inhibition of DNA-dependent enzymes, such as DNA and RNA polymerases and DNAases [3, 4, 16, 22, 23]. The development of resistance against the action of DNR is an important cause of treatment failure [8]. At the molecular level it has not been possible to observe differences in the affinity of DNR for DNA or chromatin extracted and purified from sensitive and resistant Ehrlich ascites tumor (EAT) cells [21] or in the inhibition of RNA polymerases A and B prepared from the two types of cells [4]. The cellular uptake of the drug is 2–4 times lower in resistant cells, but the subcellular localization is similar in both types of cells [14]. These results suggest an increased outward transport of drug in resistant cells.

In vivo, two mammalian enzyme systems cytoplasmic aldoketoreductases and microsomal glycosidases, are involved in the metabolism of anthracyclines [1, 2]. Several fluorescent metabolites have been identified in vivo in mammalian organs and in plasma, urine, and leukemic cells of patients with acute myeloblastic leukemia [15, 19].

To try to increase our knowledge of the acquired resistance of EAT cells, we carried out a comparison of the intracellular metabolism of DNR and of the subcellular localization of metabolites in the sensitive and resistant EAT cells. In the course of this work, we observed the appearance of an unknown product. Therefore we made a thorough investigation of the metabolism of DNR not only by normal-phase but also by reverse-phase high-pressure liquid chromatography (HPLC).

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Materials and Methods

Solutions of *daunorubicin* (a generous gift from Rhône-Poulenc, Paris, France) in saline were prepared as needed (2 mg/ml). *Polyploid cells from Ehrlich ascites tumor* (sensitive and resistant) (provided by Dr R. Maral, Rhône-Poulenc, Centre Nicolas Grillet, Vitry, France) were cultured in spinner flasks containing RPMI 1640 medium supplemented with essential amino acids, antibiotics, 20% of decomplemented fetal calf serum, and 0.02 *M* HEPES pH 7.4, as described earlier [13]. EAT cells (500×10^6) were incubated in 500 ml culture medium containing 2.5 mg DNR. Incubation was stopped at various times (1–24 h). The culture media in which the cells were grown were kept frozen and anthracycline contents were assayed. After 24 h' incubation in the presence of DNR, a 10%–15% increase of dead cells was observed for sensitive cells and a 0–5% increase for resistant cells; the pH never fell by more than 0.5.

Cell Fractionation. Homogenization of the cells and fractionation by differential centrifugation have been described elsewhere [9, 13]. Cells were fractionated to yield the following fractions: A nuclear fraction, N; a heavy mitochondrial fraction, M; a light mitochondrial fractions, L; a microsomal fraction, P; and a final supernatant, S.

Biochemical Assays. Assay conditions for marker enzymes, DNA, RNA, and proteins have been described elsewhere [13].

Anthracycline Determinations. Anthracyclines present in the biological samples (culture media, cells, and fractions) were determined by the following methods:

Method A: Total fluorescence (excitation and emission wavelengths of 485 and 580 nm, respectively) was measured after precipitation of the proteins and nucleic acids of the sonicated homogenates with 40% trichloracetic acid (TCA),

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following the method of Noël et al. [17]. Mean recovery was 102 ± 5%.

Method B: Anthracyclines were extracted from the biological samples, analyzed by normal-phase HPLC. To 0.1 ml of biological sample, 0.1 ml doxorubicin (DOX) used as internal standard at 2 μg/ml in borate buffer, pH 9.8, was added and the drug extracted by 1.8 ml chloroform-methanol mixture (4 : 1 by volume). After mixing and centrifugation, an aliquot of the organic layer was injected into the chromatograph [5]. The stationary phase consisted of 7 μm silica gel (Hibar Lichrosorb, Merck, Darmstadt, FRG) and the mobile phase at 1.2 ml/min was a mixture of chloroform, methanol, glacial acetic acid and 0.3 *M* $MgCl_2$ (720 : 210 : 40 : 30 by volume). Drugs were evaluated with a Gilson Spectro/glo fluorometer (Gilson, Middleton, Wisc., USA) with narrow bandwidth interference filters at 520 and 600 nm for the excitation and emission wavelengths, respectively. Mean recovery was 100.8 ± 3.5%. With this method the most hydrophobic compounds were not retained on the column: daunomycinone (DNR-aglycone) and doxorubicinone (DOX-aglycone) were recovered in the void volume (Fig. 1a).

Method C: Anthracyclines were extracted from the biological samples by rapid purification on Sep-pack C-18 mini-column (Waters associates, Milford, Ma., USA) as described by Robert [20], and eluted with chloroform-methanol (2 : 1 by volume). After evaporation of the solvent under nitrogen at 40° C the residue was dissolved in the mobile phase of the reverse-phase HPLC system. HPLC conditions were nearly the same as those described by Israel et al. [12]; the stationary phase consisted of micro Bondapak-phenyl (Waters Associates) and the mobile phase was an isocratic mixture of acetonitrile and 0.1% ammonium formate, pH 4 (32 : 68 by volume). Drugs were evaluated by fluorometry using an excitation wavelength of either 254 or 482 nm, according to the desired sensitivity [20], and an emission cut-off filter at 560 nm (Schoeffel fluorometer, San Francisco 970, Cal., USA). Recovery was greater than 98%. In this method, the order of retention of the anthracyclines is reversed, DNR-aglycone being the last to be eluted (Fig. 1b).

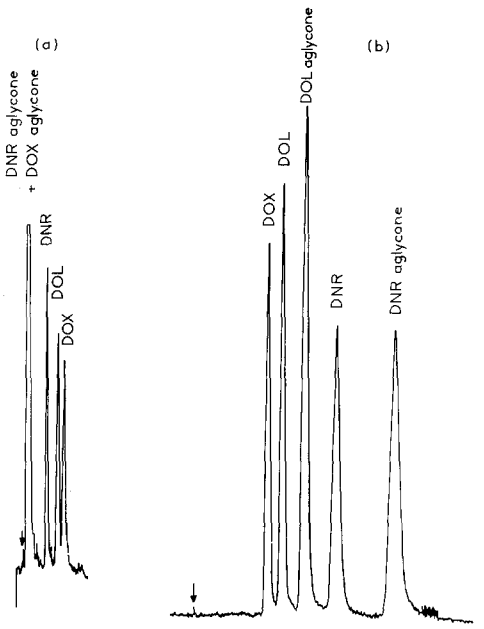

Fig. 1. Separation of doxorubicin (DOX), daunorubicinol, daunorubicinol aglycone, daunorubicin and daunorubicin aglycone by HPLC. The different anthracyclines were separated from a mixture by normal-phase HPLC (a) and by reverse-phase HPLC (b) and estimated by fluorometry

Table 1. Incubation of daunorubicin (16.5 μg/ml) in culture medium[a]

Incubation time (h)	Unidentified product	Daunorubicin (μg/ml)
0 (4)	0	16.52
4 (2)	0.71	16.54
24 (3)	7.30	15.97

[a] Numbers in brackets give the number of experiments. Normal-phase HPLC, with DOX as an internal standard. The concentration of the unidentified product has been calculated as if this product has the same quantum fluorescence yield as Dox

Table 2. Comparison of the evaluation of antracycline concentration in subcellular fractions with three different techniques[a]

Cell fraction	Extraction method			
	TCA 40%	Methanol-chloroform		Sep-Pak
	Estimation of anthracycline concentration (μm/ml)			
	Fluorescence, total anthracyclines	High-pressure liquid chromatography		
		Normal-phase		Reverse-phase
		DNR + DOL	Unidentified compound	DNR + DOL
N	37.95	39.92	6.20	38.20
M	5.68	5.55	1.70	5.23
L	1.68	1.73	1.02	1.41
P	1.72	1.74	0.74	1.58
S	0.76	0.92	0.23	0.74

[a] DOX was the internal standard for HPLC evaluation. The concentration of the unidentified product has been calculated as in Table 1. Sensitive EAT cells were incubated with DNR (5 μg DNR/ml per 10^6 cells) for 24 h

Table 3. Intracellular fluorescent compounds found in sensitive and resistant EAT cells incubated in presence of DNR (5 μg/ml per 10^6 cells)[a]

Incubation time (h)	Sensitive cells			Resistant cells		
	Unidentified product	DNR	DOL	Unidentified product	DNR	DOL
	μg product/mg protein					
1	0.12	8.14	0.12	0.12	2.61	0.05
3	0.25	8.25	0.23	0.26	3.58	0.13
18	0.34	7.60	0.21	0.32	6.87	0.07
24	0.87	9.29	0.53	0.92	3.42	0.14

[a] Drugs were extracted by methanol-chloroform and separated by normal-phase HPLC (see *Materials and Methods*). The concentration of the unidentified product has been calculated as in Table 1. DOX was the internal standard for the HPLC evaluation

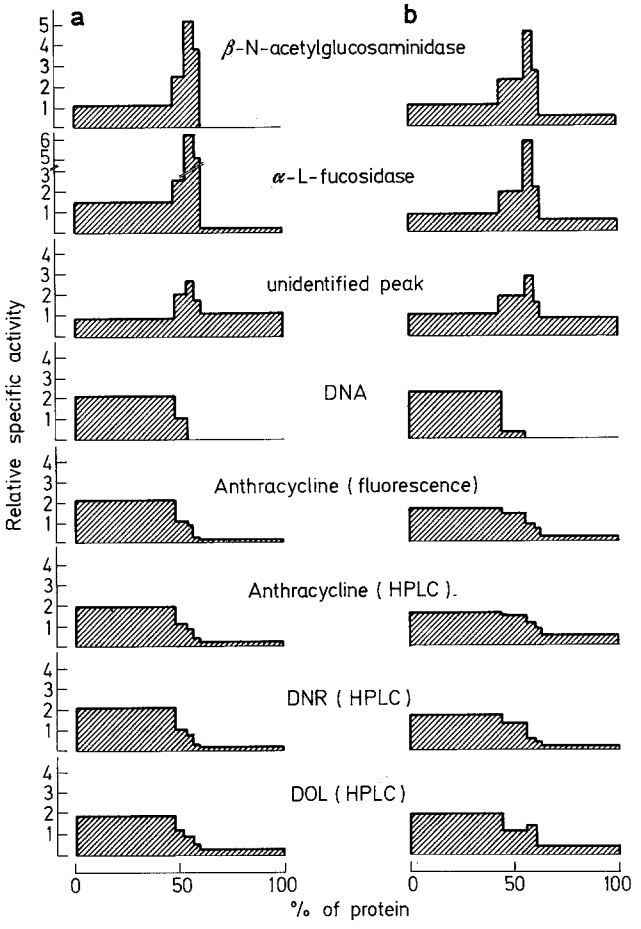

Fig. 2. Distribution of anthracyclines in EAT cells as determined by subcellular fractionation. Sensitive (a) and resistant (b) EAT cells were incubated in vitro with 5 μg DNR/ml incubation medium for 24 h. N, M, L, P, and S fractions are represented in blocks ordered in the same sequence along the *abcissa*, where the length is proportional to the protein content. The *ordinate* give the relative specific activity (or the amount of anthracycline or DNA), which is the % of activity (or amount) recovered in each fraction over the % of protein in the same fraction. Total anthracyclines have been estimated by spectrofluorometry of by normal-phase HPLC, unidentified peak, DNR, and DOL by normal-phase HPLC

Results

During incubation in the presence of DNR, aliquots of acellular culture medium were removed and analyzed by HPLC method B. No DOL formation was observed, but an unidentified peak increased with incubation time (Table 1). The specific fluorescence of this compound must be higher than that of DNR by about one order of magnitude. This unknown product was not DNR- or DOL-aglycone, since it was retained on the reverse-phase column used in HPLC method C.

The anthracycline concentrations in the different EAT cell fractions were evaluated according to the three different methods. The comparative results given in Table 2 show that if the presence of the highly fluorescent peak is not taken into account the two HPLC methods of extraction and determination of anthracyclines give similar results, which are in good agreement with those obtained by measuring the total fluorescence.

We have followed the fluorescent compounds found intracellularly in both sensitive and resistant EAT cells incubated in the presence of DNR. In both cell lines, unmetabolized DNR was the main compound detected by HPLC (Table 3). The metabolite of DNR, DOL, and the unidentified peak increased slightly during incubation period.

The subcellular localizations of anthracyclines, marker enzymes, and DNA in both sensitive and resistant EAT cells incubated for 24 h in the presence of DNR are given in Fig. 2. In sensitive cells, anthracyclines were associated mainly with fractions containing DNA, while in resistant cells higher proportions of DNR and DOL were found in other fractions. The observed differences were weak and may well be of no significance.

Discussion

We have previously observed that intracellular anthracycline concentration reaches a plateau after 7 h of incubation of EAT cells with DNR and is two to four times lower in resistant than in sensitive cells [14].

In this paper, we show that the intracellular metabolism of DNR by the two cell lines is not quantitatively important. In addition to the main compound, unmetabolized DNR, two minor fluorescent products are found intracellularly: DOL, which accounts for at most 5% of the total fluorescence after 24 h of incubation, and an unidentified fluorescent compound, which cannot be quantitated with accuracy due to its unknown specific fluorescence. This highly apolar compound seems to be one order of magnitude more fluorescent than DNR, and it is formed in acellular conditions. The structure of this unidentified compound, which might well be a chemical artifact, remains to be determined. We have observed, on the other hand, that bis-anhydroaklavinone, and apolar aglycone-type compound obtained by chemical conversion of the anthracycline aclacinomycin A [18], has a specific fluorescence 20 times higher than that of aclacinomycin A.

The low conversion of DNR to DOL observed in both sensitive and resistant EAT cells with no significant difference has also been observed in leukemic cells of patients with acute myeloblastic leukemia [19] and in L_{1210} cells [6]. In human leukocytes, reduced DOL formation has been connected with clinical resistance to DNR [10, 11]. It seems that in EAT cells

the resistance is not due to a difference in the activity of the DNR reductase, since no significant difference in the metabolism of DNR has been observed between sensitive and resistant EAT cells.

The small difference observed in the subcellular localization of DNR and DOL might not be sufficient to explain the resistance. We have observed a lesser amount of anthracyclines associated with fractions containing DNA in the resistant line, and since the total anthracycline uptake is lower in those cells, the amount of DNR present in the nuclei is still reduced and could be one of the parameters accounting for DNR resistance in EAT cells.

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