IN VIVO EFFECTS OF SEVERAL ANTHRACYCLINES ON DNA INTEGRITY

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1. Introduction

Anthracycline antibiotics are widely used in human cancer chemotherapy. Their activity has been attributed mainly to their intercalation between the base pairs of native DNA. In the last few years, however, a number of authors have attributed their cytotoxic action to DNA damage, such as single strand breaks or alkaline labile regions. Using neutral and alkaline sucrose gradient centrifugation [1] or hydroxyapatite column chromatography at 60°C [2], it was shown that doxorubicin needs the cellular environment to induce DNA single-strand breaks. By use of alkaline sucrose-gradient centrifugation [3], doxorubicin was shown to induce both single- and double-strand breaks. Doxorubicin and daunorubicin induce the formation of regions in nuclear DNA which can be susceptible to hydrolysis by *Neurospora crassa* endonuclease and which can be demonstrated by change in the sedimentation properties of nuclear DNA in neutral sucrose gradients [4]. Doxorubicin [5], daunorubicin [5,6] and aclacinomycin A [7] induce single strand scissions in the presence of reducing agents. Both free radical of anthracycline quinones and hydroxyl radical may react with DNA strands.

The in vivo effects of anthracycline upon the integrity of DNA has been studied using cell lysates or nuclear structures of various kinds of cells: human lymphoblastic cells [1], leukemia cells [2], HeLa, L_{1210} leukemia and Me-180 cells and a rat excision repair-deficient line [3] and mouse fibroblasts [4]. In [5–7] DNA cleavage was followed with a superhelical DNA. In [8] no degradation of Ehrlich ascites tumor cell DNA was shown by treatment of the animals with daunorubicin or doxorubicin, contrary to the damage observed with L_{1210} tumor cells. Results obtained with one population of tumor cells, in our case Ehrlich ascites tumor cells, cannot be applied to another kind of cells. For example, using leukemia cells from several patients, DNA damages were different depending on the population of cells [9] when uptake and retention of the drug are characteristic of each drug and uniform for the different cell population.

Here, we report the results obtained with daunorubicin and some of its derivatives after administration of the drugs to mice bearing an Ehrlich ascites tumor. The use of a technique based on the isopycnic centrifugation of nuclei extracted from Ehrlich ascites tumor cells followed by a CsCl gradient allows the separation of undegraded DNA [10]. The sedimentation constant of this DNA has been determined in an analytical ultracentrifuge, before and after alkaline degradation and subsequently the number of single strand breaks has been calculated.

2. Materials and methods

2.1. *Daunorubicin and daunorubicin analogs*

Daunorubicin (DNR), [14-^{14}C]DNR (spec. act. 50 mCi/mM) and DNR analogs were a generous gift from Dr R. Maral (Rhône Poulenc SA, Paris). The analogs are: doxorubicin or adriamycin (DOX), detorubicin (DETO), daunorubicinol or duborimycin (DOL), RP 38422, RP 21080 and its isomers RP 32885, RP 32886, RP 33366 and *N*-L-leucyl-daunorubucin (LEU-DNR) (table 1). Solutions in saline were prepared extemporaneously.

2.2. *Ehrlich ascites tumor (EAT) cells*

CDl female mice (20–25 g body wt, Charles River) were inoculated i.p. with 10^6 cells/mouse. Resistance was developed by treatment with subtherapeutic doses of DNR. Eight days after inoculation for sensitive Ehrlich ascites tumor (EAT) cells and 15 days for the

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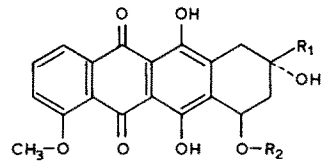
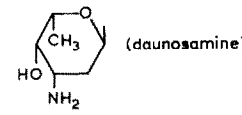
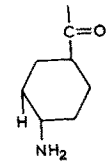
Table 1



Daunorubicin analogs

Compound	R_1	R_2
Daunorubicin (RP 13057) DNR	$-COCH_3$	 (daunosamine)
Doxorubicin (RP 25253) DOX	$-COCH_2OH$	Daunosamine
Daunorubicinol or duborimycin (RP 20798) DOL	$-CHOH-CH_3$	Daunosamine
Detorubicin (RP 33921) DETO	$-CO-CH_2-O-CO-CH(OC_2H_5)_2$	Daunosamine
RP 38422	$-CO-CH_2-S-CH_2-COOCH_3$	Daunosamine
RP 21080[a]	$-COCH_3$	
RP 32885	$-COCH_3$	As above
RP 33366	$-COCH_3$	As above
N-L-Leucyl daunorubicin (Leu-DNR)		L-Leucyl daunosamine

[a] RP 21080 is composed of 4 stereoisomers which have been isolated: the 2 *trans*-isomers RP 33365 and 33366; and the 2 *cis*-isomers RP 32885 and 32886

resistant cells, the ascitic fluid was collected. 'EAT' cells were cultured as in [11].

2.3. *DNA*

Chicken erythrocyte DNA, highly purified, was extracted as in [12]. Total EAT DNA was extracted from EAT cells as in [13]. Nuclear EAT DNA: The nuclear fraction was prepared as in [14]. The DNA was prepared by a method derived from [10]. The nuclear pellet was lysed with 6 M guanidine, viscosity was reduced by homogenization with a Dounce homogenizer (Kontes Glass Co., Vineland NJ). Aliquots of nuclear lysates were centrifuged (72 h, 30 000 rev./min) in a CsCl gradient with a Spinco L 75, using an SW 50.1 rotor at 20°C. Ten drop fractions were collected.

2.4. *Physicochemical methods*

The determination of the sedimentation coefficients was performed in a Beckman Model E ultracentrifuge, equipped with a UV light source and a monochromator (260 nm) using cells with Epon centerpieces.

Melting curves were recorded with an UNICAM SP 800, in 0.1 M SSC (15 mM NaCl, 1.5 mM trisodium citrate). Temperature was increased at 1°C/min.

2.5. *Biochemical assays*

DNA and DNR concentrations were determined according to [11].

2.6. *Estimate of the number of single-strand breaks*

The M_r-values in neutral and alkaline medium were estimated from the variation of the sedimentation coefficients using the equations in [15].

Neutral medium (1.0 M NaCl): $s^{o}_{20,w} = 0.0882\, M^{0.346}$

Alkaline medium (0.9 M NaCl–0.1 M NaOH): $s^{o}_{20,w} = 0.0528\, M^{0.400}$

These equations agree well with results relating $s^{o}_{20,w}$ to *Mw* (weight-average molecular weight) [16].

The number of single strand breaks (Sn) can be deduced from the relation:

$$2\left[\frac{Mn\ (\text{neutral})}{2\,Mn\ (\text{alkaline})} - 1\right]$$

where *Mn* is the number-average molecular weight either in neutral or alkaline medium. For native double-helical DNA molecule *Mw*/*Mn* has been experimentally determined and is ~2. Therefore, if we use the weight-average molecular weights calculated from the sedimentation coefficients in neutral and alkaline medium, we obtain:

$$Sw = 4\left[\frac{Mw\ (\text{neutral})}{2\,Mw\ (\text{alkaline})} - 1\right]$$

To obtain comparative values, the number of single strand breaks has always been calculated for a DNA of $M_r = 10^6$.

No single strand breaks can be detected in chicken erythrocyte DNA or in EAT DNA.

3. Results and discussion

3.1. *Integrity of native DNA molecules in the presence of anthracyclines in vitro*

Before starting the study of the postulated formation of single-strand breaks in DNA molecules after the action of anthracyclines in vivo, it was necessary to test the integrity of the DNA molecules after complexation by anthracyclines in vitro.

The intercalation of all the tested derivatives between the DNA basepairs stabilizes the double-helical structure of DNA [17–19]. No single-strand breaks can be detected by comparing the sedimentation constant of the whole set of compounds measured by neutral and alkaline ultracentrifugation (table 2).

Table 2
Molecular parameters of the DNA after complexation with daunorubicin or daunorubicin analogs

Compounds	*P/D* 10			*P/D* 20		
	ΔT_m (°C)	S_N	S_A	ΔT_m (°C)	S_N	S_A
Daunorubicin	8.3	29.2	31.1	5.0	26.4	29.9
Doxorubicin	11.0	27.4	34.5	7.6	32.2	31.3
Detorubicin	8.7	27.9	29.2	5.0	28.9	30.6
Daunorubicinol	–	–	–	7.1	29.2	29.0
RP 21080	9.0	28.9	32.2	6.1	27.6	30.1
RP 32885	7.6	29.6	28.3	5.8	28.6	28.4
RP 33366	6.4	27.7	28.0	6.6	30.1	30.0
N-L-Leucyl-dauno-rubicin	4.2	28.4	30.9	3.2	28.1	31.2
RP 38422[a]	11.0	17.2	16.2	7.5	18.6	17.5

[a] For RP 38422, another chicken erythrocyte DNA with a lower sedimentation constant has been used: $S_N = 19.2$, $S_A = 17.7$; ΔT_m = increase of the melting temperature of DNA, after complexation by anthracyclines; S_N = sedimentation constant in neutral medium (1 M NaCl); S_A = sedimentation constant in alkaline medium (0.9 M NaCl–0.1 M NaOH); *P* = DNA concentration; mol nucleotides/l (mean nucleotide M_r 326); *D* = anthracycline concentration

Chicken erythrocyte native DNA; T_m, 71°C in 0.1 SSC; $S_N = 29.5$, $S_A = 30.9$

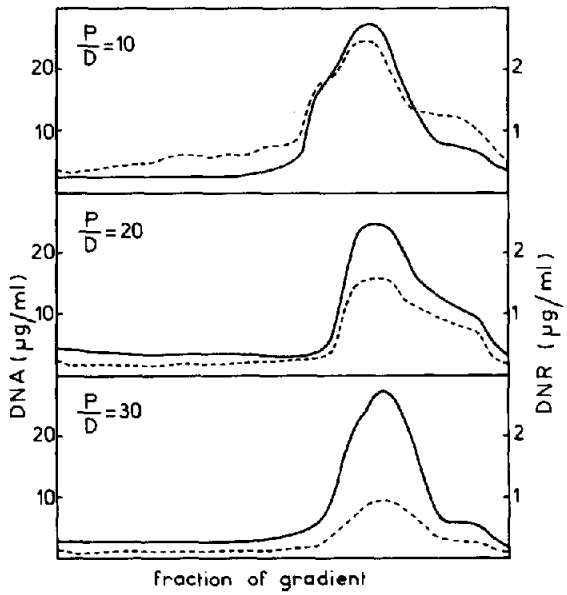


Fig.1. Isopycnic separation of the DNA–DNR complex. One ml of the nuclear pellet dissolved in 6 M guanidine was added to a solution containing 30.72 g CsCl, 0.22 ml 1 M Tris–HCl (pH 7.9), 0.02 ml 0.5 M EDTA (pH 7.9) and 22.20 ml H_2O. Aliquots of 5 ml of this final solution were centrifuged at 30 000 rev./min for 72 h at 20°C in an SW 50.1 rotor. Fractions of 10 drops were collected: (P) [DNA] in nucleotides/l (mean nucleotide M_r = 326) (——); (D) [DNR] (– – –).

The use of CsCl gradients to extract nuclear DNA does not break the anthracycline–DNA complex as shown in fig.1 where the absorbance at 260 nm of DNA and the fluorescence at 580 nm of DNR are presented. It is easy to see that the two curves can be superimposed.

The CsCl gradient does not induce the formation of single-strand breaks in the DNA since the double-helical structure is maintained after gradient centrifugation and subsequent dialysis of the DNA–DNR complex whatever the DNR concentration. No significant changes in the molecular parameters of the DNA can be observed (table 3).

3.2. *In vivo, influence of some anthracycline drugs on the integrity of the DNA from EAT sensitive cells*

The number of single-strand breaks induced in EAT nuclear DNA by the various anthracyclines tested as well as the therapeutic efficiency and the intranuclear localization of the same drugs are given in table 4. The number of single-strand breaks is always higher than in native DNA or DNA complexed by anthracyclines in vitro.

3.3. *Comparison of the in vivo influence of DNR on the integrity of DNA molecules from EAT-sensitive and -resistant cells*

We compared the number of single-strand breaks induced in DNA prepared from nuclei isolated from sensitive and resistant EAT cells. No significant differences were observed between the results obtained with the 2 types of cells prepared from mice after i.p. inoculation of DNR at the maximum tolerated dose or from EAT cultured cells incubated with DNR.

In conclusion, if in vitro the binding of the analogs of daunorubicin studied here does not induce single-strand breaks in the double-helical DNA, it is not the same in vivo after treatment of mice bearing an Ehrlich ascites tumor by the same anthracycline derivatives:

Table 3
Molecular parameters of EAT DNA complexed by DNR, before and after CsCl gradient centrifugation

	Control		*P/D* = 10		*P/D* = 20	
	A	B	A	B	A	B
T_m (°C)	72	71.8	81.7	80.8	78.3	78.0
ΔT (°C)	5.9	6.1	10.4	8.7	11.1	7.9
S_N	23.2	23.4	24.6	25.3	27.5	25.2
S_A	19.2	19.6	26.9	27.2	29.5	25.8
sw	0.1	0.1	0.05	0.02	0	0

P = DNA concentration, mol nucleotides/l (mean nucleotide M_r 326); D = DNR concentration; A = before CsCl gradient centrifugation; B = after CsCl gradient centrifugation; T_m = in 0.1 SSC; ΔT (°C) = width of the transition between 17–83% of hyperchromicity; S_N = sedimentation constant in neutral medium (1 M NaCl); S_A = sedimentation constant in alkaline medium (0.9 M NaCl–0.1 M NaOH); sw = number of single strand breaks/10^6 DNA M_r

Table 4
Number of single strand breaks induced in DNA of EAT cells after treatment of mice by DNR and DNR analogs in vivo

Drug	T_m (°C)	sw	Intranuclear drug[a] (μg drug/mg protein)	Activity (%)
–	71.5	0.11 (2)	–	–
Daunorubicin	74.9	2.41 (2)	2.40 (4)	180
Doxorubicin	76.6	0.68 (3)	1.96 (2)	190
Daunorubicinol	74.6	1.41 (1)	3.83 (2)	160
Detorubicin	73.1	0.90 (1)	4.10 (2)	190
RP 38422	71.3	3.76 (2)	0.47 (3)	100
RP 21080	71.5	0.47 (1)	1.39 (3)	180
RP 33366	72.0	0.77 (1)	0.59 (3)	140
RP 32885	–	0.16 (1)	0.72 (3)	190
RP 32886	–	0.48 (1)	0.27 (1)	176
Leu-DNR	73.5	1.80 (1)	–	163

[a] Corrected values taking into account the lysosomal contamination [20]

Mice were inoculated i.p. with 1 mg drug/mouse and sacrificed after 60 min. Number in parentheses: no. expt. T_m in 0.1 SSC; sw = number of single strand breaks. 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 maximal tolerated dose on days 0, 1, 2, 3 and 4, the activity is significant at >150%

the number of single-strand breaks in DNA molecules extracted from EAT cells is always higher than in native DNA or in DNA complexed in vitro. In all cases, there is no correlation between the number of single-strand breaks and the therapeutic activity. The antitumor activity of the drugs are very different: one of them, RP 38422, in spite of an important mutagenic activity can be considered as completely devoid of any therapeutic effect. RP 21080 is a mixture of 4 stereoisomers, RP 32885, RP 32886, RP 33365 and RP 33366. The latter two stereoisomers have a very low therapeutic action, but RP 32885 and RP 32886 have an activity of the same order of magnitude as DNR. All the other derivatives are active against Ehrlich ascites tumor. Two of these drugs, one active (DNR) and one inactive (RP 38422) induce an important number of single-strand breaks. But DOX, DOL and DETO which have a high therapeutic activ-

Table 5
Number of single-strand breaks induced in DNA from EAT-sensitive and -resistant cells after treatment of mice or cultured cells by DNR

DNR	Sensitive cells					Resistant cells				
	T_m (°C)	ΔT (°C)	S_N	S_A	sw	T_m (°C)	ΔT (°C)	S_N	S_A	sw
–	71.5	6.2	16.8	15.1	0.41	71.6	6.9	25.4	22.7	0.21
11.5 μg/mouse	71.2	6.1	21.6	13.4	1.46	72.8	6.9	20.6	12.8	1.63
5 μg/10^6 cells	74.0	10.6	18.6	12.4	1.60	72.4	8.6	19.9	11.5	2.00

Mice were inoculated i.p. with 11.5 μg [14-^{14}C]DNR/mouse and sacrificed after 60 min. Cells were incubated with 5 μg DNR/ml incubation medium and incubation was stopped after 24 h. T_m in 0.1 SSC; ΔT = width of the transition between 17–83% of hyperchromicity; S_N = sedimentation constant in neutral medium (1 M NaCl); S_A = sedimentation constant in alkaline medium (0.9 M NaCl–0.1 M NaOH); sw = number of single-strand breaks/10^6 DNA M_r

ity induce a lower number of single-strand breaks.

We have shown that 2–4-times less DNR is found associated with nuclear DNA in resistant cells [11]. However, the number of single-strand breaks induced in nuclear DNA from resistant cells is of the same order of magnitude as the number of single-strand breaks in nuclear DNA from sensitive cells.

Our results confirm that the anthracyclines need the cellular environment to induce single-strand breaks in the DNA. The enzymatically formed 'site-specific free radicals' postulated in [21] could interact with DNA molecules, producing damage to the DNA structure.

One important fact is that no correlation can be established between the number of single-strand breaks induced by an anthracycline and its therapeutic activity and (or) the quantity of drug accumulated in the nuclei. One cannot establish a direct relation between the activity of the drug and the integrity of the double-helical structure of the DNA molecule. Therefore, it is tempting to postulate that, beside the intercalation in the DNA molecule, there exists another mechanism of action.

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