Biochimica et Biophysica Acta, 1149 (1993) 79–85

BBAMEM 76000

In-vivo and in-vitro mitochondrial membrane damages induced in mice by adriamycin and derivatives

Michel Praet and Jean-Marie Ruysschaert

Laboratoire de Chimie-Physique des Macromolécules aux Interfaces, Université Libre de Bruxelles, Brussels (Belgium)

(Received 4 November 1992)

Key words: Adriamycin; Adriamycin derivative; Daunorubicin; Anthracycline; Cardiotoxicity; Free radical; (Mouse)

A major limitation to a prolonged use of adriamycin (ADM) during a clinical treatment is its dose-dependent cardiotoxicity. This toxicity has been related to a general disturbance of the inner mitochondrial membrane structure and its essential biological functions, associated to the production of free radicals by the anthracyclines. 4′-Epiadriamycin (4′-epiADM), 4′-deoxyadriamycin (4′-deoxyADM), 4′-deoxy-4′-iodoadriamycin (4′-deoxy-4′-iodoADM) and 4′-demethoxydaunorubicin (4-demethoxyDNR) are ADM and daunorubicin (DNR) derivatives differing from their parent compounds by minor structural modifications. They are nevertheless documented as less cardiotoxic. Our purpose was to establish whether mitochondrial membrane damages induced in vivo in mice heart by those compounds are correlated with the free radical formation. Heart mitochondria of treated mice were isolated 48 h after a single drug injection in order to measure the acute mitochondrial toxicity. Enzymatic activities of complex I–III and complex IV of the mitochondrial respiratory chain, mitochondrial membrane fluidity and lipid peroxidation were measured. None of the ADM and DNR derivatives displayed a significant acute mitochondrial toxicity. A mitochondrial toxicity was however detected for 4-deoxyADM and 4-demethoxyDNR when drugs were given chronically, but it was strongly reduced as compared with ADM and DNR. Electron transfer between NADH and cytochrome *c*, formation of superoxide radicals and lipid peroxidation were measured in vitro for the various drugs. Comparison of the in-vivo and in-vitro results provides evidence that free radical production explains only partly the in-vivo toxicities.

Introduction

Adriamycin (ADM) is an antitumour anthracycline with a broad spectrum of activity against a large variety of human cancers [1]. A major limitation to its prolonged use during a clinical treatment is its dose-dependent cardiotoxicity, the recommended safe cumulative dose being 550 mg/m^2 [2,3]. It has been proposed that ADM binds with a high affinity to cardiolipin (CL), a phospholipid specific of the inner mitochondrial membrane [4–6], and is subsequently converted by NADH dehydrogenase in a semiquinone radical [7–10]. In the presence of O_2, this radical generates superoxide anions and hydroxyl radicals which peroxidize unsaturated membrane lipids [11–16]. This results in a general disturbance of the inner mitochondrial membrane structure and of its essential biological functions. ADM inhibits in vitro and in vivo the activity of complex I–III and complex IV of the mitochondrial respiratory chain, peroxidizes mitochondrial lipids and modifies the fluidity of the mitochondrial membrane [10,17–21]. Free radical scavengers have been shown to abolish or to delay these toxic effects [1,22–24] that were not observed with ADM analogs having no affinity for CL (*N*-acetylADM, steffimycine) or unable to generate free radicals (5-iminodaunorubicin) [7,8,17,18,20,25,26]. It is worthwhile to mention that those derivatives are less cardiotoxic than ADM, as demonstrated by electrocardiograms on rats treated chronically and by toxicity experiments carried out on cultured cardiocytes [27–31].

Other anthracyclines (4′-epiADM, 4′-deoxyADM, 4′-deoxy-4′-iodoADM and 4-demethoxydaunorubicine) differ from their parent compound (ADM and daunorubicine (DNR)) by minor modifications that are not supposed to modify their affinity for CL or their ability to generate toxic radical species. Those molecules are nevertheless documented as being less cardiotoxic than ADM [32–38]. Our purpose here is to correlate the mitochondrial membrane damages (complex I–III and IV, membrane fluidity and lipid peroxidation) induced by these drugs in mice heart in vivo

Correspondence to: J.-M. Ruysschaert, Laboratoire de Chimie-Physique des Macromolécules aux Interfaces, Université Libre de Bruxelles, C.P. 206/2, Boulevard du Triomphe, 1050 Brussels, Belgium.

with the anthracycline induced in-vitro free radical production.

Materials and Methods

Materials

ADM, 4′-epiADM, 4′-deoxyADM, 4′-deoxy-4′-iodo-ADM and 4-demethoxyDNR were provided by Farmitalia (Milan). DNR, yeast NADH (grade III), horse heart cytochrome *c*, bovine liver superoxide dismutase (SOD), horse heart catalase, diphenylhexatriene (DPH), sodium deoxycholate and bovine serum albumine (BSA) were purchased from Sigma. Trichloroacetic acid (TCA) and EDTA were Aldrich products; thiobarbituric acid (TBA), NaN_3, Tris, sucrose, dithionite ($Na_2S_2O_4$), NaOH and HCl were from Merck. All chemicals were of analytical grade. Drugs were dissolved in Tris-HCl (pH 7.4) just before use and protected against light exposure. OF1 Swiss male and female mice, average weight 25 g, were provided by IFFA CREDO Belgium. Absorbance measurements were performed on a Shimadzu UV-190 double beam spectrophotometer; fluorescence polarization measurements on an Elscint Microviscosimeter MV 1a.

Methods

In-vivo studies. Acute treatment: Groups of six mice were treated as follows; in each group, three mice received drug by the i.p. route and three mice received 10 mM Tris-HCl buffer (pH 7.4). For each drug and for each dose, two groups of mice (six males and six females) were treated. Mice were killed by cervical dislocation 48 h after the injection.

Mice heart mitochondria were extracted as follows: hearts were removed, rinsed in 10 mM Tris-HCl buffer (pH 7.4) at 4°C and gently homogenized (25%, w/v) in 0.25 M sucrose/1 mM EDTA/1% BSA/10 mM Tris-HCl (pH 7.4) with a glass homogenizer fitted with a Teflon pestle. The homogenate was centrifuged a first time for 5 min at $480 \times g$ (2000 rpm) in a Sorvall RC2-B centrifuge fitted with a SS-34 rotor. The supernatant was then centrifuged for 10 min at $7700 \times g$ (9000 rpm), yielding a light brown pellet of mitochondria, a fraction of which was resuspended and repelleted twice in Tris-HCl at $11500 \times g$ during 6 min, in order to remove the BSA which would interfere in the Folin test. Mitochondria used for lipid peroxidation measurements were treated in the same way in order to remove the EDTA and sucrose which interfere in lipid peroxidation assays. To perform the enzymatic assays, 100 μl of mitochondria were diluted ten-fold in Tris-HCl. 25 μl of deoxycholate 10% were added to the solution. The protein concentration of each mitochondrial suspension was determined with a Folin test [39].

Complex I–III (NADH dehydrogenase-cytochrome-*c* reductase) activity was assayed at 25°C by following the NADH-dependent cytochrome *c* reduction at 550 nm in a 1-ml reaction mixture containing 725 μl of Tris buffer, 100 μl of NADH 1 mg/ml, 50 μl of oxidized cytochrome *c* (15 mg/ml) and 25 μl of NaN_3 10^{-2} M. The reaction was initiated by the addition of 100 μl of the mitochondrial suspension.

Cytochrome-*c* oxidase activity was assayed spectrophotometrically at 550 nm by following the oxidation of reduced cytochrome *c*. Cytochrome *c* was reduced by dithionite. Excess of dithionite was eliminated by gel filtration on a Sephadex G-25 column. The reaction mixture contained 850 μl of Tris-HCl buffer, 50 μl of reduced cytochrome *c* and the reaction was initiated by the addition of 100 μl of the mitochondrial suspension (25°C).

In fluorescence depolarization experiments, 100 μl of mitochondria were suspended in Tris-HCl buffer and 40 μl of DPH as a tetrahydrofuran solution (20 μg/ml) was then added (final volume 2 ml). The mixture was incubated 30 min before measuring fluorescence depolarization at a constant temperature of 25°C on a Elscint Microviscosimeter MV 1a.

For lipid peroxidation measurements, groups of 18 mice were treated as before. Each peroxidation rate was determined on a mitochondrial extract coming from hearts of three mice from the same group. 1 ml of TCA 10% was added to 1 ml of mitochondrial solution and the suspension was centrifuged. 1 ml of TBA 1% in NaOH 0.01 M was then added to the supernatant. This solution was heated at 95°C for 20 min, cooled at room temperature and the amount of malonaldehyde-TBA adduct produced was measured at 532 nm, subtracting the absorbance at 580 nm and considering an ϵ equal to 156 $mM^{-1}\,cm^{-1}$ [40].

Chronic treatment: For each drug, two groups of 12 mice were treated as follows; one i.p. injection (4 mg/kg) two times a week up to a total dose of 20 mg/kg. This total dose was limited to 12 mg/kg in the case of 4-deoxyADM and 4-demethoxyDNR; this dose was also considered for ADM and DNR which are the reference compounds. Two control groups of mice received only buffer. Mice were killed two weeks after the last injection. Six hearts were used for enzyme activity and membrane fluorescence measurements; eighteen hearts were used for lipid peroxidation measurements. Heart mitochondria were extracted as described above.

Complex I–III and complex IV activities, fluorescence depolarization and lipid peroxidation measurements on mice heart mitochondria were performed as described above.

In-vitro studies. Mice heart mitochondria were extracted as described in the in-vivo studies and stored at −20°C for several weeks without loss of activity.

Complex I–III activation by anthracyclines was measured at 25°C in a reaction medium containing 50 μl of oxidized cytochrome *c* (15 mg/ml), NADH (10^{-3} M), 25 μl NaN_3 (10^{-2} M) in Tris-HCl buffer alone or Tris-HCl buffer containing antimitotic (final concentration 10^{-4} M). At $t = 0$, 100 μl of mitochondrial suspension (0.5 mg protein) are added to this reaction medium (final volume 1 ml). The enzymatic activity of complex I–III is then measured as described before.

In-vitro lipid peroxidations were measured on mice heart mitochondria (1 mg/ml) incubated 3 h at 25°C in the presence of NADH (2.5 mM) and the different anthracyclines (10^{-4} M). After incubation the solution was centrifuged (10 min, $800 \times g$) and the pellet was resuspended in 600 μl of Tris buffer. 1 ml of TCA 10% was added and the solution was centrifuged. 2 ml of TBA 1% in NaOH 0.01 M was added and the lipid peroxidation rate is determined as decribed above. A zero-time blank (lipid peroxidation after an incubation time $t = 0$) was always subtracted from the result obtained at $t = 3$ h, in order to include any interference by anthracycline or other additions to the reaction mixture in the measurement of peroxidation.

O_2 consumption was determined at 20°C with a Clark type electrode in a 2-ml reaction mixture containing 1 mg of mitochondrial proteins, $2 \cdot 10^{-4}$ M NADH and $3 \cdot 10^{-3}$ M NaN_3. The oxygen consumption was measured after addition of the antimitotic (10^{-4} M). Thereafter, SOD (100 units) and catalase (40 units) were added sequentially.

Superoxide anion production is evaluated at 25°C from the rate of SOD-inhibitable acetylated cytochrome *c* reduction as described in Ref. 4. The reaction medium (1 ml) contained 50 μg of mitochondrial proteins, NADH (10^{-3} M), 50 nmol of acetylated cytochrome *c*, antimitotic (10^{-4} M) and 0 or 10 μg SOD, in Tris-HCl buffer. Acetylated cytochrome *c* reduction was followed spectrophotometrically at 550 nm. The difference $\Delta A_{550-SOD} - \Delta A_{550+SOD}$ allows the calculation of superoxide anion production, considering an ϵ of 19.3 mM^{-1} cm^{-1}. Cytochrome *c* acetylation was performed as described by Azzi and Wada [41,42]. Briefly, 100 mg cytochrome *c* are dissolved in 10 ml of a sodium acetate saturated solution at 0°C. Acetate anhydride is added in excess with respect to the lysine content of the protein (10:1, mol/mol). The reaction is interrupted after 30 min and the mixture is dialysed during 12 h at 0°C against 10 mM Tris-HCl buffer ((pH 7.4) 2 l, four buffer changes).

Results

In-vivo acute mitochondrial toxicity of anthracyclines

Table I reports the enzymatic activities of complex I–III and complex IV of heart mitochondria extracted from mice treated with the different antimitotics at a dose of 40 mg/kg. ADM has the strongest inhibiting capacity. Analogs modified in C-4′ position do not modify the enzymatic activities. DNR is slightly less inhibitory than ADM. Weak inhibitions are observed with 4-demethoxyDNR.

Table I indicates also that only ADM, DNR and 4-demethoxyDNR enhance the lipid peroxidation measured on heart mitochondria of treated mice. Enzymatic inhibition and membrane rigidification (fluorescence polarization data, Table I) resulting from these lipid peroxidations are only observed with ADM and DNR. Peroxidation mediated by 4-demethoxyDNR does not significantly modify the membrane fluidity and the enzymatic activities. This suggests that the lipid peroxidations measured after 4-demethoxyDNR treatment do not reach a treshold beyond which membrane damages become drastic. Such a treshold

TABLE I

In-vivo acute mitochondrial toxicity of anthracyclines

Mice received an i.p. dose of anthracycline (40 mg/kg) and heart mitochondria were extracted 48 h later. Enzymatic activities of complex I–III and complex IV of the mitochondrial respiratory chain are expressed as the percentage of the activity measured in mice which received no drug. Lipid peroxidation is expressed in pmol of malonaldehyde equivalents/mg protein per hour. *P* is the fluorescence polarization value, proportional to the loss of fluidity of the mitochondrial membrane. Each value is the average of six experiments. All measurements were done as described in Materials and Methods.

Drug	Complex I–III	Complex IV	Lipid peroxidation	*P*
No drug	100	100	411 ± 52	0.242 ± 0.004
ADM	43 ± 9 [a]	48 ± 8 [a]	1138 ± 64 [b]	0.270 ± 0.001 [a]
4′-EpiADM	100 ± 9 [b]	100 ± 6 [b]	412 ± 61 [b]	0.240 ± 0.007 [b]
4′-DeoxyADM	100 ± 7	100 ± 8	439 ± 60	0.241 ± 0.002
4′-Deoxy-4′-iodoADM	100 ± 7	100 ± 9	450 ± 21	0.242 ± 0.003
DNR	50 ± 5	61 ± 8	882 ± 20	0.261 ± 0.003
4-DemethoxyDNR	82 ± 3	82 ± 8	607 ± 17	0.242 ± 0.003

[a] See Ref. 18.
[b] See Ref. 19.

was already mentioned during acute treatment of mice with high doses of 4'-epiADM (60–70 mg/kg) [21].

In-vivo chronic mitochondrial toxicity of anthracyclines

Originally, we intended to give each mouse a total cumulative dose of 20 mg/kg of antimitotic, the treatment consisting in an injection of 4 mg/kg twice a week during two weeks and an half. This schedule is classically used in ADM chronic toxicity trials and allows to reach maximal cumulative doses even higher than the one here considered [43].

However, the maximum total cumulative dose of 4-deoxyADM and 4-demethoxyDNR here tolerated was 12 mg/kg, beyond which the number of surviving mice in each treatment group became too small (up to 80% of the animals died before a total cumulative dose of 16 mg/kg could be reached).

4'-DeoxyADM and 4-demethoxyDNR, given chronically (12 mg/kg), inhibit complex I–III and complex IV activities. The inhibition was however less pronounced than with ADM or DNR, the reference compounds, at the same total cumulative dose of 12 mg/kg (Table II). 4'-epiADM and 4'-deoxy-4'-iodoADM do not affect significantly the activity of complex I–III and complex IV. Lipid peroxidation and depolarization fluorescence values are also shown in Table II. Clearly, ADM, DNR, 4-demethoxyDNR and 4'-deoxyADM enhance significatively the lipid peroxydation rate and the membrane rigidity, while the other compounds have no effect. This chronic toxicity trial revealed toxic effects undetectable during an acute treatment. The data gathered in Table II are in agreement with the following sequence of mitochondrial toxicity: ADM > DNR > 4-demethoxyDNR > 4'-deoxyADM > 4'-deoxy-4'-iodoADM = 4'-epiADM ≃ no drug.

In-vitro enhancement of electron transport, production of oxygen free radicals

ADM was shown to increase the rate of electron flow between NADH and cytochrome *c* in extracted beef heart mitochondria [8,44]. The same observation

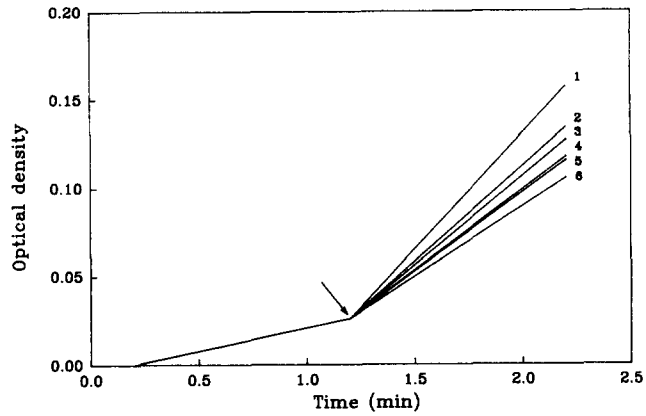

Fig. 1. Effect of anthracyclines on the rate of electron transfer between NADH and cytochrome *c* through complex I–III of mitochondria extracted from mice heart (measure of the reduction of cytochrome *c* at 550 nm). The experimental procedure is described in Materials and Methods. 1, ADM; 2, DNR; 3, 4-deoxyADM; 4, 4-demethoxyDNR; 5, 4-epiADM; 6, 4-deoxy-4-iodoADM. The arrow corresponds to the addition of the antimitotic.

TABLE III

Oxygen consumption by complex I – III of mice heart mitochondria

O_2 consumption was determined in vitro at 20°C with a Clark-type electrode in a 2-ml reaction mixture containing 1 mg of mitochondrial proteins, $2 \cdot 10^{-4}$ M NADH and $3 \cdot 10^{-3}$ M NaN_3. The oxygen consumption was measured after addition of the antimitotic (10^{-4} M). Thereafter, SOD (100 units) and catalase (40 units) are added sequentially. Results are the average of six experiments.

Drug	O_2 consumption (nmol/min per mg protein)		
		+SOD (100 units)	+catalase (40 units)
None	11 ±2	11 ±2	11 ±2
ADM	63 ±7	49.5±6	36 ±4
4'-EpiADM	28 ±2	23.5±3	20 ±1
4'-DeoxyADM	45.5±5	35 ±3	24 ±1.5
4'-Deoxy-4'-iodoADM	22 ±2	20 ±2	16.5±2
DNR	47 ±6	38 ±5	34.5±4
4-DemethoxyDNR	40 ±4	31 ±3	22 ±2

TABLE II

In-vivo chronic mitochondrial toxicity of anthracyclines

Mice were treated chronically with the different anthracyclines as indicated in Materials and Methods and heart mitochondria were extracted two weeks after the last injection. The total cumulative dose is indicated between brackets. Each result is the average of six experiments. Parameter description is given in Table I. Measurements were done as described in Materials and Methods.

Drug				
	100	100	428±11	0.241±0.002
ADM (20 mg/kg)	54±7	52 ±6	947±19	0.265±0.003
ADM (12 mg/kg)	56±9	51 ±4	802±17	0.258±0.003
4'-EpiADM (20 mg/kg)	100±6	97 ±5	453±14	0.241±0.002
4'-DeoxyADM (12 mg/kg)	77±4	75.5±9	617±14	0.250±0.002
4'-Deoxy-4'-iodoADM (20 mg/kg)	97±8	100 ±9	462±20	0.241±0.002
DNR (12 mg/kg)	60±6	59.5±6	744±16	0.255±0.002
4-DemethoxyDNR (12 mg/kg)	69±3	69 ±6	664±20	0.250±0.003

TABLE IV

Superoxide anion production

Superoxide anion production is determined in vitro from the rate of SOD-inhibitable acetylated cytochrome *c* reduction as described in Refs. 11 and 41. The reaction medium (1 ml) contained 50 μg of mitochondrial proteins, NADH (10^{-3} M), 50 nmol of acetylated cytochrome *c*, antimitotic (10^{-4} M) and 0 or 10 μg SOD, in Tris-HCl buffer. Acetylated cytochrome *c* reduction was followed spectrophotometrically at 550 nm. The results are the average of six experiments.

Drug	Superoxyde radical production (nmol reduced acetylated cytochrome *c* / min per mg protein).
None	0.5 ± 0.05
ADM	23.5 ± 1.9
4′-EpiADM	11 ± 0.8
4′-DeoxyADM	16.5 ± 1.1
4′-Deoxy-4′-iodoADM	2.5 ± 0.48
DNR	16.8 ± 1.7
4-DemethoxyDNR	14.5 ± 1.2

was made with mice heart mitochondria (Fig. 1). Other anthracyclines enhance to a lesser extent, both the electron transport between NADH and cytochrome *c* (Fig. 1), and the oxygen consumption by complex I–III of the mitochondria (Table III). Addition of SOD and catalase in the reaction chamber results in a partial decrease of the oxygen consumption, for each drug, indicating that H_2O_2 and superoxide anion are produced in the reaction medium.

Superoxide anion formation is followed by the rate of SOD-inhibitable acetylated cytochrome *c* reduction, as described in Refs. 11 and 41. The amount of superoxide produced in our experimental conditions in the presence of ADM is close to the one measured by Doroshow [11] (Table IV) and is always higher than with any other anthracycline. The formation of superoxide anion is paralleled by the enhancement of in-vitro mitochondrial lipid peroxidation (Table V).

TABLE V

In-vitro lipid peroxidation measured on mitochondria

Mitochondria (1 mg protein/ml) were incubated during 3 h with NADH (2.5 mM) and the different anthracyclines (10^{-4} M). After incubation, the solution was centrifuged (10 min, 800× *g*) and the pellet was resuspended in 600 μl of Tris buffer, before lipid peroxidation was measured. A zero-time blank (lipid peroxidation after an incubation time $t = 0$) was always subtracted from the result obtained at $t = 3$ h, in order to include any interference by anthracycline or other additions to the reaction mixture in the measurement of peroxidation. The results are the average of six experiments.

Drug	Lipid peroxydation (nmol malonaldehyde/ mg protein per h)
None	4.74 ± 0.53
ADM	25.3 ± 2
4′-EpiADM	10.04 ± 0.8
4′-DeoxyADM	16.27 ± 1.9
4′-Deoxy-4′-iodoADM	6.34 ± 0.7
DNR	18.04 ± 1
4-DemethoxyDNR	11.53 ± 1.3

Anthracyclines can be classified according to their ability to transport electrons, to generate free radicals and to enhance lipid peroxidation as follows: ADM > DNR ≧ 4′-deoxyADM > 4-demethoxyDNR ≧ 4′-epiADM > 4′-deoxy-4′-iodoADM > no drug.

Discussion

Our objective, since several years, has been to establish a relationship between the cardiotoxicity, the mitochondrial toxicity and the molecular structure of anthracyclines [8,18,20,21,44,45]. Two sites on the ADM molecule have been identified as crucial: the amine in position 3′ of the daunosamine, essential for the interaction with CL, a phospholipid specific of the inner mitochondrial membrane, and the quinone moiety on the aglycone, involved in the production of free radicals. Modification of these functions generates either compounds which have no affinity for CL (*N*-acetyl-ADM, steffimycine) or are unable to induce the formation of free radicals (5-iminodaunorubicine), although they bind CL. Those derivatives are devoid of mitochondrial toxicity, and are less cardiotoxic than ADM or DNR, their parent compounds [28–31].

Acute treatment of mice with high doses of C-4′ and C-4-modified anthracyclines (4′-epiADM, 4′-deoxy-ADM, 4′-deoxy-4′-iodoADM, 4-demethoxyDNR) is characterized by an absence of cardiac mitochondrial toxicity (4′-epiADM, 4′-deoxyADM and 4′-deoxy-4′-iodoADM) or a weak toxicity (4-demethoxyDNR), as indicated by measurement of complex I–III and IV activities, mitochondrial membrane fluidity and lipid peroxidations.

A chronic mitochondrial toxicity is observed in the case of 4′-deoxyADM and 4-demethoxyDNR; it modifies the properties of the mitochondrial membrane, though in a lesser extent than ADM and DNR. 4′-epiADM and 4′-deoxy-4′-iodoADM do not affect the mitochondrial functions.

The following sequence summarizes the mitochondrial toxicity of the ADM derivatives: ADM > DNR > 4-demethoxyDNR > 4′-deoxyADM > 4′-deoxy-4′-iodoADM = 4′-epiADM ≃ no drug.

It provides evidence that minor structural changes in the ADM molecule can significantly modify the mitochondrial toxicity. The relationship between the decreased mitochondrial toxicity of ADM and DNR derivatives and their lower cardiotoxicity, already described for *N*-acetylADM, steffimycine and 5-imino-DNR is confirmed. Indeed, the weak cardiotoxicity of 4′-epiADM, 4′-deoxyADM, 4′-deoxy-4′-iodoADM and

4-demethoxyDNR has been demonstrated by histological studies on treated animals, cardiac biopsies and electrocardiograms on patients receiving the compounds during an antitumour treatment [1,33–38].

The attenuated cardiotoxicity of 4′-epiADM has partially been explained on the basis of a modified metabolic pathway, consisting in the conjugation to a glucuronic acid, which is not observed with ADM because of an inadequate configuration of the hydroxyl group in position C-4′ [46,47]. However, this original metabolic pathway is specific for humans and does not occur in murine [48]. Other factors must be envisaged to explain the difference of mitochondrial and cardiac toxicities between ADM and 4′-epiADM.

The maximum tolerated cumulative dose was of 12 mg/kg during a chronic treatment with 4′-deoxyADM and 4-demethoxyDNR. LD50 measurements in mice indicated that these antimitotics have a higher general toxicity than ADM [3,38,49]. This general toxicity (hematologic toxicity, myelosuppression, gastrointestinal toxicity) was also described in patients treated with 4-demethoxyDNR [38,50]. We show here that it is not related to an increased mitochondrial toxicity. It was not observed during acute treatment of mice with 4′-deoxyADM and 4-demethoxyDNR in spite of the high doses administred, probably as the result of the limited delay (48 h) between the administration of the drugs and the sacrifice of the animals.

Two properties are essential in our mind to explain the weaker mitochondrial toxicities of the ADM and DNR derivatives: a weaker affinity for CL and/or a weaker ability to transport electrons and to mediate the free radicals formation. The affinities of ADM, DNR and their derivatives for CL are not significantly different (data not shown). On the contrary, despite their structural analogy, the derivatives do not transport electrons and do not catalyse the formation of superoxide anion as efficiently as their parent compounds. Our data provide experimental evidence that minor structural differences between the anthracyclines are sufficient to modify their ability to transfer electrons. Theoretical calculations showed that the 4′-epiADM epimerisation of the hydroxyl group in C-4′ generates a conformation not observed in the case of ADM, in which the hydrogen of the hydroxyl moiety in C-4′ interacts with the hydroxyl group in C-6 position [51]. This interaction alters indirectly the quinone function as the hydroxyl group in position C-6 interacts with the oxygen in position 5. The decreased ability to transfer electrons would result from the stabilisation of the quinone function, whose reduction would become more difficult. The environment of the quinone function is also modified in 4-demethoxyDNR, since the methoxy group in C-4 position, present in DNR, is replaced by a single hydrogen atom. This replacement could explain its decreased capacity of transferring electrons.

The respective capabilities of anthracyclines to produce free radicals in vitro explain only partly their in-vivo toxicity. Comparison of the sequences of in-vivo mitochondrial toxicity and free radicals production reveals an inversion between 4′-deoxyADM and 4-demethoxyDNR. Moreover, 4-demethoxyDNR produces slightly more free radicals than 4′-epiADM, whereas its in-vivo toxicity is much more pronounced.

Our data indicate that, besides a direct modification of the quinone function, modifications at position 4′ of the daunosamine moiety of ADM or at position 4 of the aglycone can reduce its ability to produce toxic free radicals and its mitochondrial toxicity. Since compounds modified in this way have been shown to display antitumour activity comparable to that of ADM [1,38,53], it is clear that such modifications can be envisaged as a promising way to obtain antimitotics with a better therapeutic index than ADM or DNR.

References

1 Weiss, R.B., Sarosy, G., Clagett-Carr, K., Russo, M. and Leyland-Jones, B. (1986) Cancer Chemother. Pharmacol. 18, 185–197.
2 Praga, C., et al. (1979) Cancer Treat. Rep. 63, 827–834.
3 Kantrowitz, N.E. and Bristow, M.R. (1984) Progr. Cardiovasc. Dis. 27, 195–200.
4 Goormaghtigh, E., Chatelain, P., Caspers, J. and Ruysschaert, J.M. (1980) Biochem. Pharmacol. 29, 3003–3010.
5 Goormaghtigh, E., Chatelain, P., Caspers, J. and Ruysschaert, J.M. (1980) Biochim. Biophys. Acta 597, 1–14.
6 Cheneval, D., Müller, M., Toni, R., Ruetz, S. and Carafoli, E. (1985) J. Biol. Chem. 260, 13003–13007.
7 Pollakis, G., Goormaghtigh, E., Delmelle, M., Lion, Y. and Ruysschaert, J.M. (1984) Res. Commun. Chem. Pathol. Parmacol. 44, 445–459.
8 Pollakis, G., Goormaghtigh, E. and Ruysschaert, J.M. (1983) FEBS Lett. 155, 267–272.
9 Nohl, H. and Jordan, W. (1983) Biochem. Biophys. Res. Commun. 114, 197–205.
10 Marcillat, O., Zhang, Y. and Davies, K.J.A. (1989) Biochem. J. 259, 181–189.
11 Doroshow, J.H. (1983) Cancer Res. 43, 460–472.
12 Myers, C.E., Mac, Guire, W.P., Liss, R.H., Ifrim, I., Grotzinger, K. and Young, R.C. (1977) Science 197, 165–167.
13 Goodman, J. and Hochstein, P. (1977) Biochem. Biophys. Res. Commun. 77, 797–803.
14 Mimnaugh, E.G., Trush, M.A., Bhatnagar, M. and Gram, T.E. (1985) Biochem. Pharmacol. 34, 847–856.
15 Griffin-Green, E.A., Zaleska, M.M. and Erecinska, M. (1988) Biochem. Pharmacol. 37, 3071–3077.
16 Davies, K.J.A. and Doroshow, J.H. (1986) J. Biol. Chem. 261, 3060–3067.
17 Goormaghtigh, E., Brasseur, R. and Ruysschaert, J.M. (1982) Biochem. Biophys. Res. Commun. 104, 314–320.
18 Goormaghtigh, E., Huart, P., Brasseur, R. and Ruysschaert, J.M. (1986) Biochim. Biophys. Acta 861, 83–94.
19 Nicolay, K. and De Kruijff, B. (1987) Biochim. Biophys. Acta 892, 320–330.

20 Praet, M., Pollakis, G., Goormaghtigh, E. and Ruysschaert, J.M. (1984) Cancer Lett. 25, 89–96.
21 Praet, M., Laghmiche, M., Pollakis, G., Goormaghtigh, E. and Ruysschaert, J.M. (1986) Biochem. Pharmacol. 35, 2923–2928.
22 Sonneveld, P. (1978) Cancer Treat. Rep. 621033–1036.
23 Powell, S.R. and Mac Cay, P.B. (1988) Toxicol. Appl. Pharmacol. 96, 175–184.
24 Praet, M., Buc Calderon, P., Pollakis, G., Roberfroid, M. and Ruysschaert, J.M. (1988) Biochem. Pharmacol. 37, 4617–4622.
25 Doroshow, J.H. (1983) Cancer Res. 43, 4543–4551.
26 Lown, J.W., Hsiao-Hsiung, C., Plambeck, J.A. and Acton, E.M. (1979) Biochem. Pharmacol. 28, 2563–2568.
27 Zbinden, G., Bachmann, E. and Holderegger, C. (1978) Antibiot. Chemother. 23, 255–270.
28 Bachur, N.R., Gordon, S.L. and Gee, M.V. (1978) Proc. Natl. Acad. Sci. USA 76, 954–957.
29 Tong, G.L., Henry, D. and Acton, E.M. (1979) J. Med. Chem. 22, 36–39.
30 Peters, J.H., Evans, M.J, Jensen, R.A. and Acton, E.M. (1980) Cancer Chemother. Pharmacol. 4, 263–266.
31 Lowe, M.C. and Smallwood, J.I. (1980) Cancer Chemother. Pharmacol. 5, 61–65.
32 Von Hoff, D.D., Layard, M.W., Basa, P., Von Hoff, A.L., Rosenweiz, M. and Muzio, M. (1979) Ann. Intern. Med. 91, 710–717.
33 Arcamone, F. (1985) Cancer Res. 45, 5995–5999.
34 Barbieri, B., et al. (1987) Cancer Res. 47, 4001–4006.
35 Villani, F., Galimberti, M., Comazzi, R. and Crippa, F. (1989) Eur. J. Cancer Clin. Oncol. 25, 13–18.
36 Villani, F., Galimberti, M., Comazzi, R. and Crippa, F. (1988) Int. J. Clin. Pharmacol. Therapy Toxicol. 26, 185–189.
37 Casazza, A.M. (1979) Cancer Treat. Rep. 63, 835–844.
38 Casazza, A.M. (1986) Cancer Treat. Rep. 70, 43–49.
39 Lowry, H.O., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
40 Wills, E.D. (1969) Biochem. J. 113, 315–324.
41 Azzi, A., Montecucco, C. and Richter, C. (1975) Biochem. Biophys. Res. Commun. 65, 597–603.
42 Wada, K. and Okunoki, K. (1968) J. Biochem. 64, 667–681.
43 Bellini, O. and Solcia, E. (1985) Virchows Arch. (Cell Pathol.) 49, 137–152.
44 Goormaghtigh, E., Pollakis, G. and Ruysschaert, J.M. (1983) Biochem. Pharmacol. 32, 889–893.
45 Goormaghtigh, E. and Ruysschaert, J.M. (1984) Biochim. Biophys. Acta 779, 271–288.
46 Weenen, H., Lankelma, J., Penders, P.G.M., Mac Vie, J.G., Ten Bokkel Huinik, W.W., De Planque, M.M. and Pinedo, H.M. (1983) Invest. New Drugs 1, 59–64.
47 Weenen, H., Van Maanen, J.M.S., De Planque, M.M., Mac Vie, J.G. and Pinedo, H.M. (1984) Eur. J. Cancer Clin. Oncol. 20, 916–919.
48 Robert, J. (1988) Bull. Cancer 75, 167–174.
49 Casazza, A.M., Di Marco, A., Bonadonna, G., Bonfante, V., Bertazzoli, C., Bellini, O., Pratesi, G., Sala, L. and Ballerini, C. (1980) in Anthracyclines: current status and new development (Crooke, S.T. and Reich, S.D. eds.), pp. 400–430, Academic Press, New York.
50 Ganzina, F., Pacciarini, M.A. and Di Pietro, N. (1986) Invest. New Drugs 4, 25–28.
51 Malatesta, V., Tosi, C. and Fusco, R. (1986) Chem. Phys. Lett. 128, 565–568.
52 Doroshow, J.H. and Davies, K.J.A. (1986) J. Biol. Chem. 261, 3068–3074.
53 Gianni, L., Vigano, L., Surbone, A., Ballinari, D., Casali, P., Tarella, C., Collins, J.M. and Bonadonna, G. (1990) J. Natl. Cancer Inst. 82, 469–477.