Role of the quinone structure in the mitochondrial damage induced by antitumor anthracyclines

Comparison of adriamycin and 5-iminodaunorubicin

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Adriamycin cardiotoxicity has been correlated with a disturbance of heart mitochondrial functions. Here, 5-iminodaunorubicin was compared with adriamycin for its capability to interfere in the mitochondrial electron transport with subsequent membrane damaging. The results suggest that minor chemical modifications of the anthraquinone moiety of anthracycline glycoside drugs should be a promising way to decrease mitochondrial membrane damage induced by this class of antitumor drugs.

Adriamycin *5-Iminodaunorubicin* *Cardiotoxicity* *Mitochondrial membrane* *Liposome* *Respiratory chain*

1. INTRODUCTION

Chemotherapeutic usefulness of adriamycin (ADM) (fig.1a) and derivatives, members of the antitumor anthracyclines class, is limited by a unique cardiotoxicity [1]. Many reports correlate this cardiotoxicity of ADM with a disturbance of heart mitochondrial functions and ATP synthesis [2–5]. In [6] we showed that the anthraquinone moiety of the ADM molecule catalyses electron transport from NADH to cytochrome *c*. This resulted in an enhanced chemical reactivity of ADM with formation of covalent-like linkage with membrane components and subsequent membrane damage most likely due to polymerization of chemically activated phospholipids (free radicals, peroxides). Binding of ADM on cardiolipin (CL), a phospholipid specific of the inner mitochondrial membrane, was a prerequisite since the strong electrostatic complex ADM–CL [6] has a ubiquinone-like structure capable to replace native ubiquinone as membraneous electron transporter.

This paper tests the new ADM derivatives modified in their anthraquinone ring to decrease their capability to transport electrons. 5-Iminodaunorubicin (5-IDRB) (fig.1b) was compared with ADM for its capability to interfere in the mitochondrial electron transport with subsequent membrane damaging.

2. MATERIALS AND METHODS

NADH (grade III), beef heart cardiolipin, complex I–III and cytochrome *c* were purchased from Sigma. 1-6 Diphenylhexatriene (DPH) was an Aldrich product and NaN_3 a Merck product. Adriamycin and 5-iminodaunorubicin were provided by the National Cancer Institute. All chemicals were of analytical grade and water was triple-distilled. Fluorescence polarization measurements were performed on an Elscint Microviscosimeter MV_{1a}. Absorbance measurements were realized on a Shimadzu UV-190 double

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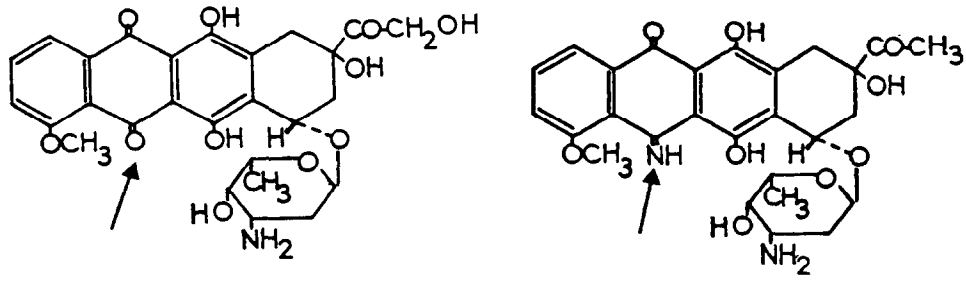

a b

Fig.1. Structure of (a) adriamycin (ADM); (b) 5-iminodaunorubicin (5-IDRB).

beam spectrophotometer, thermostatized at 25°C. The vibration electrode technique was employed to measure the surface potential [7]. For all experiments, Tris–HCl 10^{-2} M buffer (pH 7.4) containing 50 mM NaN_3 was used.

Mitochondrial membranes were extracted from beef heart as in [8]. They were stored at −20°C for several weeks without loss of activity. Complex I–III was assayed for its NADH-cyt. *c* oxidoreductase activity by following cyt. *c* reduction at 550 nm. Multilamellar proteoliposomes were obtained by dispersing the dry lipid film with a purified enzyme suspension [9]. Liposome suspension was not sonicated in order to avoid enzyme damage. In this procedure, an ethanolic solution of cardiolipin was evaporated under nitrogen, and then dispersed in Tris–HCl buffer with proteins. The drug, both electrostatically and covalently bound to cardiolipin, can be extracted by a $CHCl_3$ phase. To distinguish between the two types of binding, the electrostatically bound drug was first dissociated by 6 M $CaCl_2$ [10].

3. RESULTS

Since the ability of an ADM derivative to interfere with mitochondrial electron transport is related to its capability to form an electrostatic complex with CL [11], we first measured the association constant between 5-IDRB and CL. This was carried out by measuring the surface potential of a monolayer of CL mixed with DPPC, a neutral phospholipid inert towards 5-IDRB. If we consider the reaction between an anionic site of CL (P^-) and the positively ionized drug (D^+), one can write:

$$D^+ + P^- \rightleftharpoons DP \quad (1)$$

with an association constant:

$$K = \frac{[DP]}{[D^+]_s[P^-]} = \frac{\sigma_0 - \sigma}{\sigma} \frac{1}{[D^+]_s} \quad (2)$$

where:

σ and σ_0 = the surface charge density after and before complexation, respectively;

$[D]_s$ = the molar concentration of D^+ at the interface.

$[D^+]_s$ is related to the bulk concentration through a Boltzmann distribution:

$$[D^+]_s = [D^+]_\infty \exp(-e\psi/kT) \quad (3)$$

where:

e = the electronic charge;

k = the Boltzmann constant;

ψ = the negative surface potential after complexation.

From a general point of view, the electrostatic potential ψ (mV) associated to the lipid monolayer is described by the Gouy–Chapman theory. At 25°C:

$$\psi = 50.4\, sh^{-1}(134\, \sigma/\sqrt{C}) \quad (4)$$

where:

σ = the surface charge density in charge/Å^2;

C = the molar salt concentration in the solution.

To determine the surface charge density of the monolayer after the drug has been added (σ) in the subphase, we increased 10-times the $[Na^+]$ of the

bulk by injecting an appropriate amount of a saturated $NaNO_3$ solution into the subphase. The increase of the surface potential $\Delta(\Delta\psi)$ after the Na^+ injection is related to σ by relation 5 [12]. At 25°C:

$$\Delta(\Delta\psi) = 50.4\ (sh^{-1}\frac{134\ \sigma}{\sqrt{C_2}} - sh^{-1}\frac{134\ \sigma}{\sqrt{C_1}}) \qquad (5)$$

where:

C_1 and C_2 are the concentrations in monovalent ions before and after $NaNO_3$ injection, respectively;

C_1 was chosen equal to 10^{-2} M/l and C_2 equal to 10^{-1} M/l. For a detailed discussion on this surface potential technique see [12]. Results are reported in table 1.

K-Value for ADM is in good agreement with the value determined in [11]. The K-values found for ADM and 5-IDRB are of the same order of magnitude. The effect of ADM and 5-IDRB on the electron transport between NADH and cyt. c was therefore tested on extracted beef heart mitochondria. Fig.2 shows that ADM increases the rate of electron flow between NADH and cyt. c by ~80%, whereas in presence of 5-IDRB no increase is observed. Even a slight decrease was recorded. To test the chemical reactivity of the drug towards the membrane, proteoliposomes of CL and purified NADH-cyt. c oxidoreductase were incubated with the drug in presence or in absence of NADH. The fact that even in presence of 6 M/l Ca^{2+} (in this condition the electrostatic complex is dissociated [10]) the water-soluble drug is extracted from the aqueous phase into a chloroform phase in partition experiments, suggests the formation of a covalent linkage between the drug and CL. Results are reported in table 2. The amount of drug was evaluated spectrophotometrically.

Clearly, 5-IDRB remains much more chemically inert than ADM. Do the above described phenomena modify the membrane properties? A possible way to evaluate a general modification of the membrane properties is to measure the fluorescence depolarization of a dye embedded in the membrane. The fluorescence depolarization P can be correlated to the membrane viscosity [13]. We first measured P using the fluorescence of the drug itself on proteoliposomes containing CL,

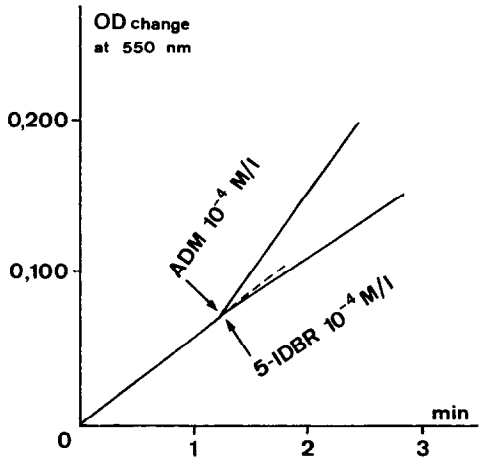

Fig.2. Effect of ADM and 5-IDRB on the rate of electron transport between NADH and cyt. c in a mitochondrial suspension 6×10^{-3} mg protein/ml. Cytochrome c was: 0.7 mg/ml; Tris–HCl 10^{-2} M buffer (pH 7.4) containing 50 mM NaN_3 was used.

Table 1

Association constant and surface potential data of the ADM–CL and 5-IDRB–CL complex

	ADM	5-IDRB
$\Delta(\Delta\psi)$[a]	17 mV	25 mV
σ_0[b]	5.53×10^{-3} (charge/Å^2)	5.53×10^{-3} (charge/Å^2)
σ[c]	3.93×10^{-4} (charge/Å^2)	6.18×10^{-4} (charge/Å^2)
ψ[d]	8 mV	13 mV
$[D^+]_\infty$[e]	10^{-5} M	10^{-5} M
$[D^+]_s$[f]	1.38×10^{-5} M	1.60×10^{-5} M
K[g]	1×10^{-6} l/M	2×10^{-5} l/M

[a] Experimental values of the increase of the surface potential following Na^+ injection

[b] σ_0 is determined by the monolayer composition. A CL/DPPC molar ratio of 27/100 was chosen. Considering an area/molecule of 60 Å^2 for DPPC (uncharged) and 120 Å^2 for CL (bearing two negative charges) for a close-packed monolayer, σ_0 was calculated

[c] Determined from $\Delta(\Delta\psi)$, eq. (5) with the use of a numerical technique developed in [12]

[d] From σ using eq. (4)

[e] Chosen equal to 1×10^{-5} M/l for ADM and 1×10^{-5} M/l for 5-IDRB

[f] From $[D^+]_\infty$ using eq. (3)

[g] Equation (2)

Table 2

Formation of a new linkage between drug and CL as a consequence of the electron flow between NADH and cyt. *c*

	− NADH	+ NADH
ADM extracted with chloroform	0%	81%
5-IDRB extracted with chloroform	0%	3%

The values in percent represent the excess of drug extracted by chloroform in the conditions described below as compared to a simple system containing only buffer, chloroform and the drug. In this system, the amount of drug extracted by chloroform is only 1% for ADM and 16% for 5-IDRB. Multilamellar proteoliposomes were obtained by dispersing the lipid film (CL) with Tris–HCl buffer (pH 7.4) containing the purified enzyme (NADH-cyt. *c* oxidoreductase). Proteoliposomes 1 mg CL/ml, 6.25 units of NADH-cyt. *c* oxidoreductase were incubated for 3 h with the drug 2×10^{-4} M in the presence or absence of NADH 1.2×10^{-3} M. After incubation, excess of drug and NADH was eliminated by centrifugation of the proteoliposomes (10 min, 800 × *g*) followed by 5 washings. The bound drug was estimated as in section 2

NADH-cyt. *c* oxidoreductase and the drug in the presence or in absence of NADH. When 5-IDRB was used, $P^{5\text{-IDRB}}$-values were almost independent on the incubation in presence of NADH. However, when ADM was used, a dramatic increase of P^{ADM} was observed (table 3). As both a decrease of the membrane fluidity or a decrease of the life-time of the excited state of the chemically modified ADM molecule can account for the P^{ADM} increase, we can only conclude that the ADM membrane system was dramatically modified after incubation in the presence of NADH, whereas the 5-IDRB membrane system was not. More quantitative data about the membrane viscosity were obtained by measuring the *P*-value of diphenylhexatriene (DPH), a classical probe of the membrane fluidity. Incubation of proteoliposomes with NADH did not modify the P^{DPH} when 5-IDRB was used. However, a large increase of P^{DPH} was recorded when ADM was used. As the DPH molecule has not been modified since it was added at the end of the reaction, the increase of P^{DPH} must correspond to a loss of mobility of DPH which can be correlated to a rigidification of its environment. It must be pointed out from table 3 that in the absence of NADH, P^{DPH} is significantly different for proteoliposomes containing 5-IDRB or ADM. D^{DPH} in the presence of ADM corresponds to P^{DPH} in the absence of drug demonstrating that ADM does not perturb the microviscosity of the membrane. This is in good agreement with preceding data which showed that ADM is excluded from the hydrophobic part of the bilayer [11].

Table 3

Effect of electron transport between NADH and cyt. *c* on fluorescence depolarization of drugs and DPH

	Proteoliposomes + ADM	Proteoliposomes + ADM + NADH	Proteoliposomes + 5-IDRB	Proteoliposomes + 5-IDRB + NADH
P^{drug}	0.070	0.163	0.096	0.090
P^{DPH}	0.190	0.226	0.217	0.219

P is defined as:

$$P = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp})$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities polarized parallel and perpendicular to the direction of polarization of the excitation

Precision on *P*-values, 0.004; P_{DPH} without drug, 0.190. In fluorescence depolarization experiments, proteoliposomes (2.13 mg CL/ml; 2.33 mg mitochondrial protein/ml were incubated for 24 h with the drug (ADM or 5-IDRB) 3×10^{-4} M in the presence or absence of NADH (20 mg/ml). After the incubation, proteoliposomes were washed 5-times by centrifugation, then diluted to obtain a constant fluorescence intensity. This solution was incubated with DPH (0.6 μg DPH/ml mitochondrial suspension) and diluted 20-times. At this concentration, only DPH gave a measurable signal

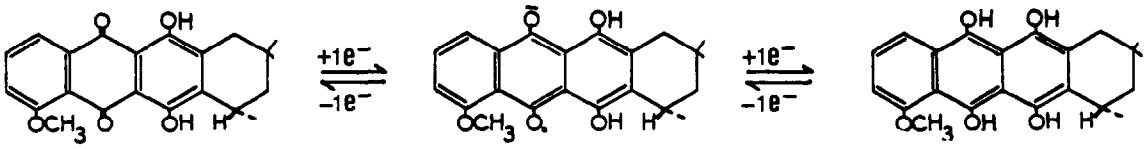

Fig.3. Hypothetical reactions of the anthraquinone moiety of ADM during electron transport.

On the other hand, the slight rigidification observed in the presence of 5-IDRB means that this drug modifies the movement of the phospholipid hydrocarbon chain.

4. DISCUSSION

We have shown here that ADM is capable of facilitating electron transport between NADH and cyt. *c* in the inner mitochondrial membrane whereas 5-IDRB is not. The difference of behaviour between ADM and 5-IDRB can in fact not arise from a different ability to form the electrostatic complex with CL [11] since the measured association constants of the drug–CL complex were of the same order of magnitude for both drugs. We therefore tentatively attributed the electron transport through ADM to the reaction described in fig.3.

The consequences of the electron transport through ADM are:

(i) An enhancement of ADM chemical reactivity followed by a covalent-like binding to CL (table 2);

(ii) A damaging of the membrane properties (microviscosity) (table 3).

Adriamycin radicals are spontaneously formed by adriamycin solution at physiological pH whereas 5-IDRB does not form semiquinone radicals [14]. It is likely that ADM-free radicals enhance mitochondrial lipids peroxidation as has been already observed on other membrane systems [15–19]. As we know that peroxidized phospholipids can undergo polymerization reactions [20,21] (and therefore increase of membrane microviscosity) and that the inner mitochondrial membrane is very sensitive to the peroxidation of its phospholipids [22], it is not surprising that ADM-mediated electron transport affects so drastically the mitochondrial membrane [2–5]. Recent studies seem to confirm that in fact 5-IDRB is much less cardiotoxic than ADM [23,24]. The cumulative dose of 5-IDRB required to produce significant widening of the QRS complex in the rat electrocardiogram, was 6-times the cumulative dose of ADM [25]. It was confirmed that 5-IDRB did not induce irreversible changes in the vacuole ultrastructure of myocardiac cells of rats. These changes were caused by an equal dose of ADM [23].

Our results strongly suggest that the ADM quinone function is essential to induce mitochondrial membrane damage and that minor modifications at this level could modify strongly the cytotoxicity.

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REFERENCES

[1] Praga, C. (1979) Cancer Treat. Ref. 63, 5, 827–833.
[2] Bachmann, E., Weber, E. and Zbinden, G. (1975) Agents Actions 5, 383–390.
[3] Arena, E., Arico, M., Biondo, F., D'Alessandro, N., Dusonchet, L., Gebbia, N., Gerbasi, F., Sanguedolce, R. and Rausa, L. (1974) in: Adriamycin Reviews, pp.160–172, European Press, Ghent.
[4] Necco, A. and Dasdia, T. (1974) IRCS Med. Sci. Biochem. 2, 1293–1294.
[5] Mailer, K. and Petering, D.H. (1976) Biochem. Pharmacol. 25, 2085–2089.
[6] Goormaghtigh, E., Pollakis, G. and Ruysschaert, J.-M. (1983) Biochem. Pharmacol., in press.
[7] Caspers, J., Landuyt-Caufriez, M., Deleers, M. and Ruysschaert, J.-M. (1979) Biochim. Biophys. Acta 554, 23–38.
[8] Smith, A.L. (1967) Methods Enzymol. 10, 81–86.
[9] Racker, E. (1973) Biochem. Biophys. Res. Commun. 55, 224–230.

[10] Goormaghtigh, E., Vandenbranden, M., Ruysschaert, J.-M. and De Kruijff, B. (1982) Biochim. Biophys. Acta 685, 137–143.
[11] Goormaghtigh, E., Chatelain, P., Caspers, J. and Ruysschaert, J.-M. (1980) Biochem. Pharmacol. 29, 3003–3010.
[12] Goormaghtigh, E., Caspers, J. and Ruysschaert, J.-M. (1980) J. Colloid Interface Sci. 1, 163–170.
[13] Shinitzky, M. and Barenholz, Y. (1978) Biochim. Biophys. Acta 515, 367–394.
[14] Pietronigro, D.D., McGinness, J.E., Koren, M.J., Crippa, R., Seligman, M.L. and Demopoulos, H.B. (1979) Physiol. Chem. and Physics II, 405–414.
[15] Naoki, Y., Taketoshi, K., Keiko, N., Tomoko, F., Masanori, F. and Kazuo, O. (1979) Cancer Chemother. Pharmacol. 3, 223–227.
[16] Ryohei, O., Hinoyuki, T. and Tukasa, K. (1979) Vitamin 53, 569–570.
[17] Myers, C.E., McGuire, W.P., Liss, R.H., Ibrim, I., Grotzinger, K. and Young, R.C. (1977) Science 197, 165–168.
[18] Thayer, W.S. (1977) Chem. Biol. Interact. 19, 265–278.
[19] Goodman, J. and Hochstein, P. (1977) Biochem. Biophys. Res. Commun. 77, 797–803.
[20] Nielsen, H. (1979) Lipids 14, 900–906.
[21] Nielsen, H. (1981) Lipids 16, 215–222.
[22] Vladimirov, Y.A., Olenev, V.I., Suslova, T.B. and Cheremisina, Z.P. (1980) Adv. Lipid Res. 17, 173–249.
[23] Lowe, M.C. and Smalmood, J.I. (1980) Cancer Chemother. Pharmacol. 5, 61–65.
[24] Tony, G.L., Henry, D.W. and Acton, E.M. (1979) J. Med. Chem. 22, 2, 36–39.
[25] Peters, J.H., Evans, M.J., Jensen, R.A. and Acton, E.M. (1980) Cancer Chemother. Pharmacol. 4, 263–266.