Biochemical Pharmacology, Vol. 35, No. 17, pp. 2923–2928, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00
Pergamon Journals Ltd.

IN VIVO AND *IN VITRO* MODIFICATIONS OF THE MITOCHONDRIAL MEMBRANE INDUCED BY 4′ EPI-ADRIAMYCIN

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(*Received* 10 *December* 1985; *accepted* 3 *March* 1986)

Abstract—Mice received 4′ Epi-adriamycin (4′ Epi-ADM) i.p. in increasing doses. After 48 hours, hearts were removed and mitochondria were isolated. 4′ Epi-ADM had no effect on the enzymatic activities of complexes I–III and complex IV of the mitochondrial respiratory chain; no modification of the mitochondrial membrane viscosity was observed and only a slight membrane lipid peroxidation was measured. On the contrary, *in vitro* studies showed a 4′ Epi-ADM dependent enzymatic inactivation of complex I–III and IV, correlated with a mitochondrial membrane rigidification and an enhanced lipid peroxidation rate.

The clinical use of the antitumor anthracycline adriamycin (ADM) is limited by its cardiotoxicity [1, 2] and intensive effort has been made to synthesize ADM analogs in order to improve the therapeutic ratio (minimal cumulative cardiotoxic dose/optimal non toxic antimitotic dose) of this antimitotic. 4′ Epi-adriamycin (4′ Epi-ADM) is an ADM derivative differing from the parent drug only by the epimerization of the hydroxyl group at the C4′ position of the aminosugar moiety (Fig. 1). Nevertheless, 4′ Epi-ADM intercalates between the two strands of the DNA with an association constant very similar to that of ADM ($2.2 \cdot 10^6$ L/M) [3–5]. It was shown [3, 4] that 4′ Epi-ADM like ADM inhibits *in vitro* the growth of mouse embryo fibroblasts and DNA synthesis. Meanwhile, Casazza *et al.* showed [6, 7] on mice receiving i.v. injections of the drugs, that the LD_{50} of 4′ Epi-ADM was 18.76 mg/kg whereas the LD_{50} was 11.76 mg/kg for ADM. In mice bearing L1210 leukemia, the therapeutic ratio was 5.9 for 4′ Epi-ADM and 3 for ADM. Clinical tests on human (ECG studies), comparing ADM and 4′ Epi-ADM, indicated a reduced cardiotoxicity of the latter compound, at doses going up to 550 mg/m^2 [8], which was shown to be the total limit dose of ADM tolerated in human [2]. The results obtained so far suggest that 4′ Epi-ADM is as effective as ADM against a broad spectrum of human neoplasms and better tolerated [9, 10]. A comparative study on disposition of ^{14}C labelled 4′ Epi-ADM in the rat [11], indicated a lower 4′ Epi-ADM concentration, after 24 hr and later times, in nearly all tissues, particularly in heart auricles. Weenen observed in patients participating in a clinical phase II trial, that 4′ Epi-ADM eliminates faster than ADM [12], while several authors noticed that contrary to ADM, the major metabolite is not the 13-dihydro derivative (adriamycinol) but the β-glucuronide of 4′ Epi-ADM and 4′ Epi-adriamycinol [13–15]. There is now a general agreement to correlate the ADM cardiotoxicity with disturbances of the inner mitochondrial membrane biological functions [16–18]. We previously reported on the inactivation *in vivo* of two enzymes of the heart inner mitochondrial membrane respiratory chain (cytochrome *c* oxidase and NADH dehydrogenase–cytochrome *c* reductase) in ADM treated mice [19].

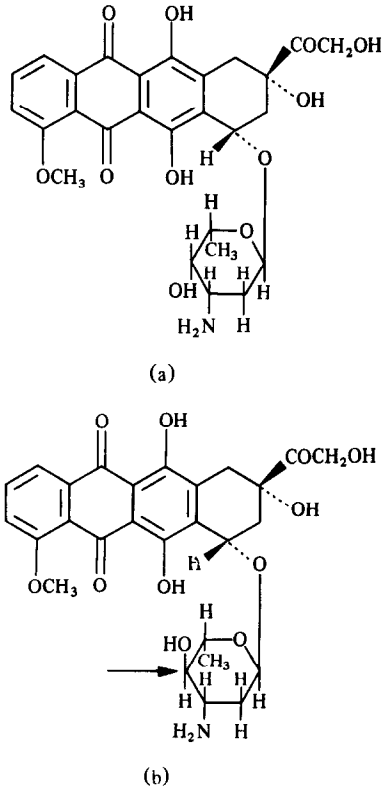

Fig. 1. Structures of ADM (a) and 4′ Epi-ADM (b).

The inactivation was accompanied by an increase of the mitochondrial membrane viscosity and was correlated to the ability of ADM to enhance mitochondrial lipid peroxidation. These mitochondrial membrane modifications were attributed to the ability of the anthraquinone moiety of ADM to be converted by NADH dehydrogenase in a semiqui-

none radical which reacts with the unsaturated lipids of the mitochondrial membrane [20, 25]. We investigate here the ability of 4′ Epi-ADM to induce such damages in the heart mitochondrial membrane of 4′ Epi-ADM treated mice as compared to ADM.

MATERIALS AND METHODS

Materials

ADM and 4′ Epi-ADM were supplied by Farmitalia Milan (Italy). NADH (grade III), cytochrome *c* and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO). Diphenylhexatriene (DPH), trichloracetic acid (TCA) and EDTA were Aldrich products; thiobarbituric acid (TBA), NaN_3, Tris, sucrose and HCl, Merck products. All chemicals were of analytical grade and water was triple distilled. The 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 and fluorescence polarization measurements on an Elscint Microviscosimeter MV 1a.

Methods

Our studies *in vivo* were performed with groups of 4 or 5 mice treated as follows: in a group, each mouse received an i.p. dose of 40, 50, 60 and 70 mg/kg of 4′ Epi-ADM with a control mouse receiving no drug. The animals were killed after two days since previous work indicated that adriamycin induced enzyme inactivation was observed after this period. For peroxidation measurements *in vivo* three mice were used in each experiment. Each result is the average of three experiments. Mice were sacrificed 72 hr after the i.p. injection of 4′ Epi-ADM and ADM.

For *in vivo* and *in vitro* experiments, mitochondria were extracted from mice heart as follows: animals were killed, their hearts removed, rinsed in Tris–HCl 10 mM pH 7.4 buffer at 4° and gently homogenized (25%, w/v) in 0.25 M sucrose: 1 mM EDTA: 1% bovine serum albumin (BSA): Tris–HCl 10 mM pH 7.4 with a glass homogenizer fitted with a Teflon pestle. The homogenate was centrifuged a first time for 5 min at 480 *g* (2000 rpm). The supernatant was then centrifuged for 10 min at 7700 *g* (9000 rpm) yielding a light brown pellet of mitochondria, which was resuspended and repelleted twice in Tris–HCl at 11,500 *g* during 6 min, in order to remove the BSA which would interfere in the Folin test and the EDTA and sucrose which interfere in the lipid peroxidation assays. In some *in vitro* experiments beef heart mitochondria were used; they were extracted as described by Smith [26]. To perform the enzymatic assays, 100 μl of mitochondria were diluted tenfold in Tris–HCl and deoxycholate 10%. The protein concentration of each mitochondrial suspension was determined with a Folin test [27].

In vivo *studies*. Complex I–III (NADH dehydrogenase–cyt. *c* reductase) activity was assayed by following the NADH dependent cytochrome *c* reduction at 550 nm in a 1 ml reaction mixture containing 625 μl of Tris, 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 ($Na_2S_2O_4$). Excess of dithionite was eliminated by gel filtration on a Sephadex G-25 column. The reaction mixture contained 850 μl of Tris–HCl, 50 μl of reduced cytochrome *c* and was initiated by the addition of 100 μl of the mitochondrial suspension. In fluorescence depolarization experiments, 100 μl of mitochondria were suspended in 2 ml of Tris–HCl buffer and 40 μl of DPH as a tetrahydrofuran solution (20 μg/ml) were then added. The mixture was incubated half an hour before measuring fluorescence depolarization at a constant temperature of 25°.

For lipid peroxidation measurements, 1 ml of mitochondrial solution was treated with 1 ml of TCA 10% and centrifuged.

One millilitre of TBA 1% was then added to the supernatant. This solution was heated at 95° for 20 min, cooled at room temperature and the quantity of malonaldehyde-TBA adduct produced was measured at 532 nm substracting the absorbance at 580 nm and considering an ε of 156 $mM^{-1}\,cm^{-1}$ [28, 29].

In vitro *studies*. One hundred microlitres of mitochondria were incubated for an hour with 25 μl of NaN_3 10^{-2} M, 100 μl of the drug in various concentrations, 25 μl of deoxycholate 10%, 650 μl of the Tris buffer. 50 μl of NADH 1 mg/ml and 50 μl of oxidized cytochrome *c* were added to this mixture in order to measure the activity of complex I–III at 550 nm with the Shimadzu spectrophotometer. For complex IV (cytochrome *c* oxidase), 100 μl of mitochondria were incubated for an hour with 100 μl of the drug in various concentrations, 25 μl of deoxycholate 10% and 750 μl of Tris buffer. Fifty microlitres of reduced cytochrome *c* are added to the mixture in order to measure the activity of cytochrome *c* oxidase at 550 nm.

Beef heart mitochondria were used for the fluorescence depolarization measurements *in vitro* since high concentrations of mitochondria were required, which demands the sacrific of a large number of mice. Two hundred and fifty microlitres of beef heart mitochondria (13 mg protein/ml) were incubated for 4 hr at 25° with 15 mg of NADH and ADM or 4′ Epi-ADM at desired concentration. One hundred microlitres of the incubate were centrifuged and washed twice with 200 μl of Tris–HCl; Tris was then added up to 3 ml and 15 μl of DPH in tetrahydrofuran (1 mg/ml) added half an hour before fluorescence depolarization is measured.

In order to measure mitochondrial lipid peroxidation *in vitro*, 1 ml of mice heart mitochondria (1.3 mg protein/ml) were incubated for an hour at 37° under an O_2 atmosphere with ADM or 4′ Epi-ADM (final concentration 10^{-4} M), NADH (2.5 mM) in a final volume of 1750 μl. After incubation, lipid peroxidation was measured on 600 μl of this mixture by addition of TCA 10%, centrifugation and addition of 2 ml of TBA 1%. The mixture was heated

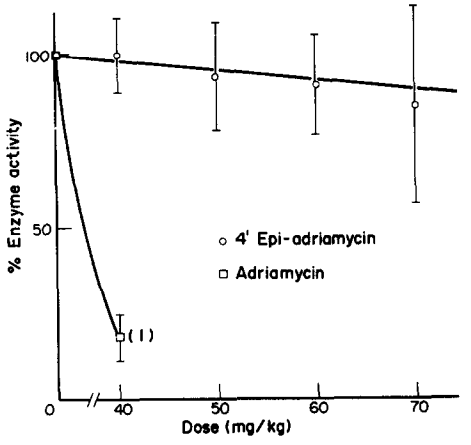

Fig. 2. Enzymatic activity of the complex I–III. OF1 Swiss male and female mice received ip doses of ADM (□) or 4' Epi-ADM (○). 100% activity (337 ± 25 μmole cytochrome *c* reduced/mg protein × min) accounts for results obtained with untreated mice. Mitochondria were extracted from mice hearts 48 hr after drug administration. 100 μl of these mitochondria were suspended with 25 μl of 10% deoxycholate and 875 μl of Tris–HCl buffer (pH 7.4; 10^{-2} M). The enzymatic activity was measured on 100 μl of this solution in presence of 100 μl of NADH (1 mg/ml), 25 μl of NaN_3 (10^{-2} M) and 625 μl of Tris–HCl. At time $t = 0$, 50 μl of oxidized cytochrome *c* (15 mg/ml) were added. The enzymatic activities are given for the same protein concentration in the mitochondrial suspension (Folin). Bars represent standard deviations from 4 or 5 mice. (1) See ref. 19.

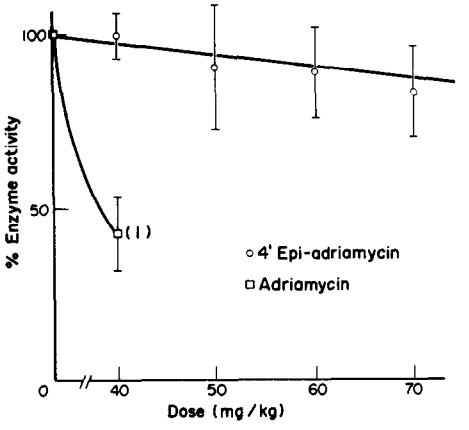

Fig. 3. Enzymatic activity of cytochrome *c* oxidase. OF1 Swiss male and female mice received i.p. doses of ADM (□) or 4' Epi-ADM (○). The activity was measured on 100 μl of the mitochondrial suspension in presence of 850 μl of Tris–HCl buffer. At time $t = 0$, 50 μl of cytochrome *c* reduced with dithionite were added. Other experimental conditions are the same as in Fig. 2. 100% activity corresponds to 3700 ± 275 μmole cytochrome *c* oxidized/mg protein × min. (1) See ref. 19.

at 95° for 20 min and lipid peroxidation was measured as indicated previously.

RESULTS

Effect of 4' Epi-ADM on the complex I–III and complex IV activities in vivo

Figure 2 shows the evolution of the enzymatic activity of complex I–III for increasing doses of 4' Epi-ADM *in vivo* (100% of activity accounts for untreated animals). Clearly, 4' Epi-ADM doesn't affect significantly the enzymatic activity for doses going from 40 mg/kg to 70 mg/kg, whereas ADM inhibits strongly the enzyme even at 40 mg/kg [19].

The same observation was made for cytochrome *c* oxidase (Fig. 3) in identical experimental conditions. Previous work in our laboratory [19] showed that the inactivation of these two complexes of the mitochondrial respiratory chain was accompanied by a noticeable rigidification of the mitochondrial membrane. It was therefore tempting to verify such a correlation in the case of 4' Epi-ADM using the fluorescence depolarization technique.

Effect of 4' Epi-ADM on the mitochondrial membrane structure in vivo

Fluorescence polarization was used to evaluate the mitochondrial membrane fluidity. The fluorescence depolarization P depends on the mobility of a fluorescent marker (DPH) embedded in the lipid bilayer, thus on the fluidity of the membrane considered. P increases with the membrane rigidification. It appears in Fig. 4 that for 4' Epi-ADM no significant rigidification of the mitochondrial membrane was observed up to dose of 70 mg/kg, whereas for ADM this rigidification was noticeable even at a dose of 40 mg/kg [19]. For both drugs, there is a correlation between their *in vivo* effect on the enzymatic complexes and their ability to cause mitochondrial membrane damage resulting in membrane rigidification. It could be objected that ADM still present in mitochondria extracted from mice heart, interferes with fluorescence depolarization measurements.

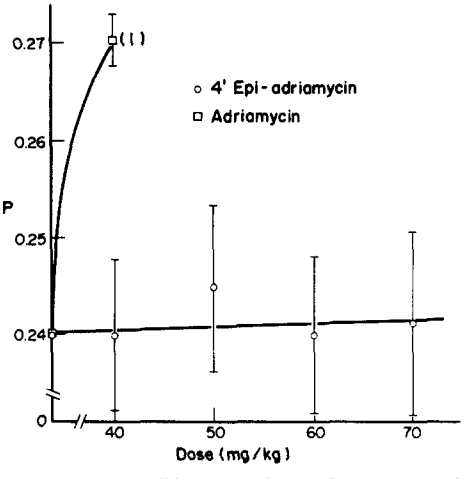

Fig. 4. *In vivo* modification of the fluorescence depolarization. OF1 Swiss male and female mice received i.p. dose of ADM (□) or 4' Epi-ADM (○). 100 μl of extracted mitochondria were suspended in 2 ml of Tris–HCl buffer and DPH (0.4 μg DPH/ml of mitochondrial suspension) was added as a tetrahydrofuran solution (20 μg/ml). After 1 hr of incubation, depolarization measurements were made at a constant temperature of 25°. (1) See ref. 19.

Table 1. Effect of ADM and 4′ Epi-ADM on lipid peroxidation in mice heart mitochondria *in vivo*

	Lipid peroxidation (malonaldehyde equivalents in pmoles/mg protein)
No drug	411 ± 52
4′-Epi-ADM 40 mg/kg	412 ± 61
4′Epi-ADM 50 mg/kg	552 ± 46
4′ Epi-ADM 60 mg/kg	653 ± 39
4′ Epi-ADM 75 mg/kg	707 ± 42
ADM 40 mg/kg	1138 ± 64

In each experiment, three mice were used in order to obtain sufficient lipid concentration. Mice were killed and heart mitochondria extracted 48 hr after drug administration. Mitochondria were treated with TCA and TBA as described in Materials and Methods. The amount of malonaldehyde-TBA adduct produced, representative of lipid peroxidation, is measured spectrophotometrically at 532 nm, subtracting the absorbance at 580 nm. Each result is the average of three or four experiments.

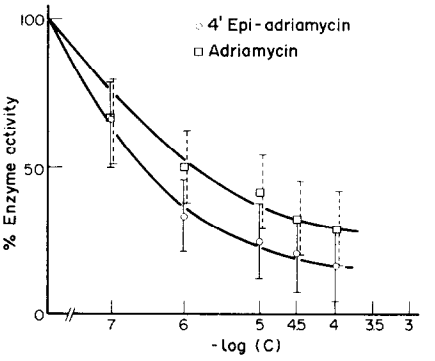

Fig. 5. Inhibition of complex I–III activity *in vitro*. 100 μl of freshly extracted mice heart mitochondria were incubated for an hour with 100 μl of ADM or 4′ Epi-ADM at various concentrations, 25 μl of NaN_3 10^{-2} M, 25 μl of deoxycholate 10% and 650 μl of Tris–HCl buffer. The activity was measured spectrophotometrically at 550 nm after addition of 50 μl of NADH 1 mg/ml and 50 μl of oxidized cytochrome *c* (15 mg/ml). 100% of activity (300 μmoles cytochrome *c* reduced/mg protein × min) accounts for mitochondria which were incubated without drug. Each point is the average of three experiments.

However, using ^{14}C labelled ADM (data not shown) we were unable to detect ADM in our mitochondrial fraction. Table 1 gives the amount of malonaldehyde-TBA adduct produced in heart mitochondria of mice treated with various doses of 4′ Epi-ADM and 40 mg/kg of ADM. There is an enhancement in the rate of lipidic peroxidation with increasing doses of 4′ Epi-ADM; however, at 40 mg/kg this rate is identical for 4′. Epi-ADM as for untreated mice whereas for ADM a threefold increase can be noted. All these results show that, regarding mitochondrial toxicity *in vivo*, 4′ Epi-ADM is much less toxic than ADM. Considering the minor structural difference between the two molecules, it can be argued that, rather than an altered interaction with the mitochondrial membrane, the metabolic fate of 4′ Epi-ADM in the organism could be modified.

We therefore performed *in vitro* experiments to establish the validity of this assumption.

Effect of ADM and 4′ Epi-ADM on the complex I–III and complex IV activities in vitro

Mice heart mitochondria were incubated with increasing concentrations of ADM and 4′ Epi-ADM. The evolution of the activity of complex I–III versus ADM and 4′ Epi-ADM concentration (Fig. 5) shows, in the limit of the experimental error, an obvious and similar inactivation for both drugs *in vitro*, while *in vivo*, only ADM had an inhibitory effect.

Figure 6 indicates that the inhibition of the activity of cytochrome *c* oxidase, in the same experimental conditions, is very similar for ADM and 4′ Epi-ADM. We studied in the next step the possible correlation between the *in vitro* inactivation of the enzymatic complexes of the mitochondrial respiratory chain by ADM and 4′ Epi-ADM and the ability of these drugs to rigidify the mitochondrial membrane.

Effect of ADM and 4′ Epi-ADM on the mitochondrial membrane structure in vitro

Since fluorescence depolarization measurements *in vitro* require high mitochondrial concentrations, we used beef heart mitochondria. Moreover, the sensitivity of the method and interference between the fluorescence of the probe (DPH) and drugs present in the analysed mixture made significant measurements possible only at 5. 10^{-4} and 10^{-4} M drug concentrations. However, at these concentrations, the enzymatic complexes are already inactivated (Figs 4 and 5) *in vitro*. Mitochondria incubated with both drugs display a membrane rigidification indicated by a significatively enhanced polarization degree (Table 2).

Table 2. *In vitro* membrane fluidity change in beef heart mitochondria incubated with ADM or 4′ Epi-ADM and NADH

Concentration	No drug	10^{-4} M	5.10^{-4} M
P ADM	0.240 ± 0.004	0.260 ± 0.005	0.260 ± 0.007
P 4′ Epi-ADM	0.240 ± 0.005	0.260 ± 0.005	0.260 ± 0.005

Mitochondria were incubated at 25° for 4 hr with the drug and NADH. The drug still in solution after incubation was eliminated by centrifugation and washing. 15 μl of DPH in THF (1 mg/ml) were added half an hour before performing fluorescence polarization measurement.

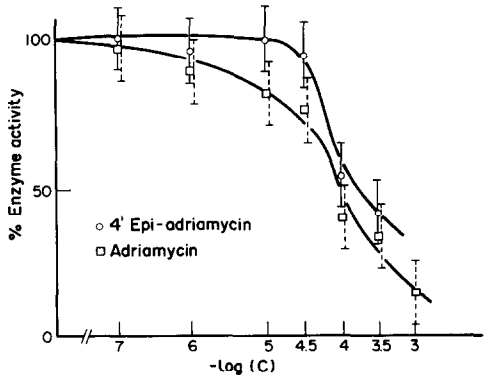

Fig. 6. Inhibition of complex IV activity *in vitro*. 100 μl of freshly extracted mitochondria were incubated for an hour with 100 μl of ADM or 4′ Epi-ADM at various concentrations, 25 μl of deoxycholate 10% and 850 μl of Tris–HCl buffer. The activity was measured at 550 nm after addition of 50 μl of cytochrome *c* reduced by dithionite (15 mg/ml). 100% of activity (500 μmoles cytochrome *c* oxidized/mg protein × min) accounts for mitochondria which were incubated without drug. Each point is the average of three experiments.

To complete this study, lipid peroxidation measurements *in vitro* were performed. We showed previously [19] that an enhancement of mitochondrial lipid peroxidation occurred when mitochondria were incubated in presence of ADM and NADH. The same observation was made when mitochondria were incubated in the same conditions with 4′ Epi-ADM instead of ADM (Table 3).

DISCUSSION

Considerable interest has been devoted in our laboratory to the study of the relationship between the mitochondrial toxicity and the cardiac toxicity of antitumour anthracyclines. Cardiolipine (CL), a lipid specific of the inner mitochondrial membrane, was identified as the membrane target responsible for the mitochondrial toxicity. The CL–ADM interaction has been demonstrated to be involved in: (a) the inhibition of cytochrome *c* oxidase, according to a mechanism described by Goormaghtigh *et al.* [30]; (b) the formation of free radicals responsible for the membrane rigidification altering the functioning of the enzymes embedded in the mitochondrial membrane [19–21, 24, 25].

Considering these two points, several structural modifications in the ADM molecule could be proposed to lead to less toxic derivatives. Since the amine function of the aminosugar moiety is essential for the interaction of ADM with CL, a modification of this function (acetylation) would reduce the affinity of the new compound for CL and its mitochondrial toxicity. *N*-acetyl ADM [19, 30] does not bind to CL but has lost its therapeutic efficacy.

Considering the crucial role of the ADM anthraquinonic moiety in the production of free radicals mediated by NADH dehydrogenase and its cofactor [22, 28], a modification of the quinone function would generate a less cardiotoxic compound. Encouraging results have been obtained with 5-iminodaunorubicine, which produces a low amount of free radicals in several systems, is less toxic and maintains its therapeutic activity [19, 22, 23, 31–33]. We demonstrate here the reduced mitochondrial toxicity of 4′ Epi-ADM *in vivo*. Indeed, no detectable heart mitochondrial membrane rigidification occurred in mice treated with 4′ Epi-ADM, and the inactivations of complex I–III and cytochrome *c* oxidase were negligible as compared with the inactivations observed with ADM at lower doses. About heart mitochondrial lipid peroxidation, a clear enhancement of produced malonaldehyde is observed for high doses of 4′ Epi-ADM (60, 75 mg/kg) indicating that free radical toxicity and subsequent membrane damages still exist for 4′ Epi-ADM. They are, however, not comparable with those caused by ADM and not sufficient to markedly alter the activity of mitochondrial respiratory chain enzymes.

Since apparently the slight structural modification of 4′ Epi-ADM could not lead to a reduced affinity for CL (to be published) or abolish its capacity of transferring electrons between NADH and cytochrome *c*, the origin of the lower cardiotoxicity of 4′ Epi-ADM lies probably in a modified metabolic pathway and/or general uptake by the organism. In fact, modified metabolism and pharmacokinetics of 4′ Epi-ADM have already been observed [11–15]. Synthesis and toxicological studies of some of the 4′ Epi-ADM metabolites could contribute to develop a new class of antimitotics with a reduced cardiotoxicity.

Table 3. Effect of 4′ Epi-ADM on lipid peroxidation in mice heart mitochondria *in vitro*

	Lipid peroxidation (malonaldehyde equivalents in nmoles/hours × mg protein)
Mitochondria + NADH	4.74 ± 0.53
Mitochondria + NADH + 4′ Epi-ADM (10^{-4} M)	10.26 ± 1.04

Mitochondria (1.3 mg protein/ml) were incubated for an hour at 37° under an O_2 atmosphere with 4′ Epi-ADM 10^{-4} M, NADH 2.5 mM, in a final volume of 1750 μl. Lipid peroxidation assays were then performed as described in Materials and Methods. Each figure is the result of five experiments.

Acknowledgements—Financial support was obtained from the "Banque Nationale de Belgique" and the "Caisse Générale d'Epargne et de Retraite". We gratefully acknowledge Dr. J. P. Lobelle and Dr. F. C. Giuliani (Farmitalia Carlo Erba) for the 4'-Epi-adriamycin gift. E.G. is a Senior Research Assistant from the National Fund for Scientific Research, Belgium.

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