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A NEW CLASS OF FREE RADICAL SCAVENGERS REDUCING ADRIAMYCIN MITOCHONDRIAL TOXICITY

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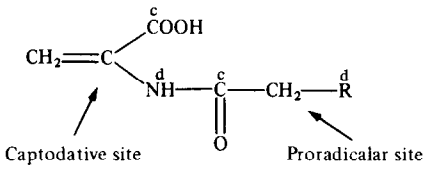
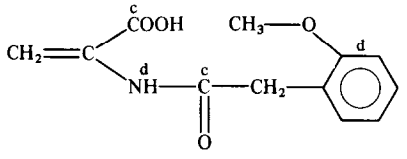
Abstract—Beef heart mitochondria were incubated with ADM and NADH. An adriamycin semiquinone radical was detected using ESR spectroscopy. The semiquinone radical production rate is decreased upon addition of a scavenger (AD 20) in the reaction medium. NMRI mice were treated with AD 20 (70 mg/kg, i.p.) 15 min prior ADM injection (20 mg/kg, i.p.) or with ADM alone. Heart mitochondria were isolated 48 hr later. The enzymatic activities of complex I–III and complex IV of the mitochondrial respiratory chain were strongly depressed in animals receiving ADM alone, whereas these activities were almost completely restored in animals receiving AD 20 and ADM. Fluorescence depolarization measurements indicated that only mice treated with ADM alone presented a decreased fluidity of their cardiac mitochondrial membrane.

Adriamycin (ADM) displays antineoplastic activity against a broad spectrum of human cancers, including various hematologic malignancies and carcinomas of the breast, lung, ovary, brain and gastrointestinal tract [1, 2]. Clinical use of ADM is limited by its unique cardiotoxicity, the total cumulative dose tolerated being 550 mg/m^2 [3–5]. Impairment by ADM of the cardiac mitochondrial respiratory chain functioning is mainly responsible for cardiotoxicity [6–8]. From a molecular point of view, ADM shows a high affinity for cardiolipin (CL), a major phospholipid specific of the mitochondrial inner membrane [9, 10]. The ability of ADM to bind and segregate CL in a separate phase inaccessible for mitochondrial enzymes has been suggested as responsible for the inactivation of complex **I–III** (NADH-dehydrogenase/cytochrome *c* reductase) and **IV** (cytochrome *c* oxidase) of the respiratory chain [11, 12]. Indeed, these complexes require CL in their immediate environment for full enzymatic activity [13, 14]. ADM bound to the mitochondrial membrane can also act as an electron carrier between NADH and cytochrome *c* [15]. It was recently shown [16] that ADM is reduced to a semiquinone form at complex **I** of the mitochondrial electron transfer chain. The redox cycling of the semiquinone radical leads to the formation of superoxide anion, hydrogen peroxide and hydroxyl radicals [16–21], the latter being responsible for major injuries to cardiac mitochondria (membrane lipids peroxidation [22, 23], subsequent membrane rigidification [15] and inactivation of the enzymatic complexes of the respiratory chain [24]). It is of interest to notice that several authors [17, 25] underlined the peculiar sensitivity of the heart to free radical damages, because of a less developed antioxidant defence system. Attempts made in order to minimize ADM cardiotoxicity assumed that the antitumor efficiency of the drug can be dissociated from its toxic side effects [26]. Different approaches exist aiming to increase ADM therapeutic index: association of ADM with DNA [27], simultaneous injection of ADM with a specific antibody [28], injection of ADM encapsulated in various types of liposomes [29–31], design of ADM derivatives based on the molecular understanding of the cardiotoxicity process, use of free radicals scavengers. Among these, the most frequently tested are α-tocopherol [32–34], sulfhydryl compounds such as *N*-acetylcysteine, reduced glutathione and cysteamine [35–38], cofactors of free radicals detoxification enzymes such as selenium [39, 40]. So far, *in vivo* results obtained on the efficiency of these various compounds towards ADM detoxification are often conflicting and not fully convincing. We study here a new class of molecules, exhibiting free radical trapping properties, hence capable of reducing ADM cardiotoxicity due to free radical production: the N-acyl-dehydroalanines, indexed as AD compounds [41], most particularly AD 20 (Fig. 1), the AD derivative which proved to be the most active against ADM acute and chronic toxicity in rodents (submitted). These molecules are capable of stabilizing free radicals as a consequence of their capto-dative properties. This stabilization of free radicals through capto-dative effect was demonstrated theoretically [42] and experimentally [43, 44]. Figure 1 indicates the two main sites of the AD 20 molecule able to react with a free radical, either by direct addition of the radical (on the capto-dative site), or by subtraction of a hydrogen atom (to the proradicalar site). The newly formed species is stabilized by the substituents on each side of the carbon atom bearing the radical (c, captor group; d, donor group). Moreover, the aromatic core acts as a trapper of OH radicals, giving hydroxylated aromatic derivatives. Considering the problem, in biological fluids, of trapping all kinds of free radicals with different polarities and structures, the AD compounds should present some interesting potentialities.



(a)



(b)

Fig. 1. (a) AD compounds, general structure: c, captor groups; d, donor groups. (b) AD 20 structure.

We report here on the reduced mitochondrial toxicity of ADM in mice previously treated with AD 20. Mitochondrial enzymes activities (complex **I–III** and complex **IV**), lipid peroxidation and mitochondrial membrane structure are compared to those obtained with ADM alone [24, 45].

MATERIALS AND METHODS

Materials

ADM was supplied by Farmitalia Milan (Italy) and AD 20 by Professor Roberfroid from the Université Catholique de Louvain (Ecole de Pharmacie). NADH (grade III), cytochrome c, dimethylsulfoxide (DMSO) and bovine serum albumine were purchased from Sigma Chemical Co. (St. Louis, MO). Diphenylhexatriene (DPH), trichloroacetic acid (TCA) and EDTA were Aldrich products; thiobarbituric acid (TBA), NaN_3, Tris, sucrose, KH_2PO_4, Na_2HPO_4 (phosphate buffer) and HCl, Merck products. All chemicals were of analytical grade. ADM was dissolved in saline and AD 20 was suspended in gum arabic 2%. NMRI male mice, 3 months old, average weight 25 g, were provided by Iffa Credo, France. They were housed by group of 10 per cage in a constant temperature environment (22°) with alternating 12 hr wake-sleep cycles and they received standard food and water *ad libitum*.

Methods

Beef heart mitochondria isolation. A freshly isolated beef heart was kept on ice and brought to the laboratory. All subsequent operations were made at 4°. Fat and neighbouring tissues were separated from the heart muscle. The muscle was finely minced in cubes, 1 cm side-length. Fifty grammes of cubes were placed in a 100 ml Tris–HCl 10 mM, pH 7.4/sucrose 0.25 M and EDTA 0.2 mM buffer. It was ground in a mixer (2 × 5 sec). Between the grindings, the pH was adjusted at 7.8 if necessary. The homogenate was centrifuged at 1200 *g* for 20 min in a Sorvall RC2-B centrifuge fitted with a GSA rotor. The supernatant was filtered through a piece of cheesecloth in order to discard the lipid granules and recentrifuged for 15 min at 15,000 *g*. Only the fraction of the pellet strongly associated to the bottom of the tube (heavy mitochondria) was recuperated and rehomogenized. The volume of the homogenate was brought to 50 ml with the Tris buffer. The centrifugation–rehomogenization process was repeated twice, in order to wash mitochondria. The protein concentration of the final mitochondrial suspension is then determined (Folin test) [46].

In vitro *studies.* ESR measurements were performed on a Varian Century Series E-109 spectrometer. The semiquinone radical production was measured in solutions containing mitochondria (13 mg protein/ml), ADM (5×10^{-4} M) and NADH (5×10^{-4} M). AD 20 was added (3 mM) in one series of experiments. Wyard's double integration method [47] was used in order to measure the intensity of the ESR signals, proportional to the semiquinone radical concentration.

In order to measure complex **I** activity, 50 μl of mice heart mitochondria (0.13 mg protein) in 10 mM phosphate buffer pH 7.4 and 20 μl of DMSO alone or containing various concentrations of AD 20 were added to 880 μl of phosphate buffer. The reaction was initiated by the addition of 50 μl of NADH (1 mg/ml) and the NADH oxidation followed spectrophotometrically by the decrease of the absorbance at 340 nm. The reference well contained no NADH. Complex **I–III** activity was measured on 1 ml solutions containing 810 μl of phosphate buffer, 50 μl of mitochondria, 20 μl of NaN_3 10^{-2} M, 50 μl of oxidized cytochrome c 15 mg/ml, 20 μl of DMSO alone or containing various concentrations of AD 20 and 50 μl of NADPH 1 mg/ml to initiate the reaction. The reduction of cytochrome c was measured spectrophotometrically by following the increase of the absorbance at 550 nm. The reference cell contained no NADH.

In vitro *studies.* They were carried out with four groups of six mice treated as follows: group A received no drug; group B received ADM (20 mg/kg); group C received AD 20 (70 mg/kg); group D received AD 20 (70 mg/kg) 15 min before ADM (20 mg/kg). All injections were made by the i.p. route. Mice were killed by cervical dislocation two days after the injection. Heart mitochondria were extracted as described in [24]. For lipid peroxidation measurements, four groups of 18 mice treated as above were used; each result was obtained with the collected hearts of three mice in the same group. Complex **I–III** and complex **IV** activities, fluorescence depolarization and lipid peroxidation measurements on those mice heart mitochondria were performed as described in [24]. Absorbance measurements were performed on a Shimadzu UV-190 double beam spectrophotometer and fluorescence polarization measurements on an Elscint Microviscosimeter MV 1a.

RESULTS

Free radical scavenging properties of AD 20 as detected by ESR

In a preliminary study, we investigated the sca-

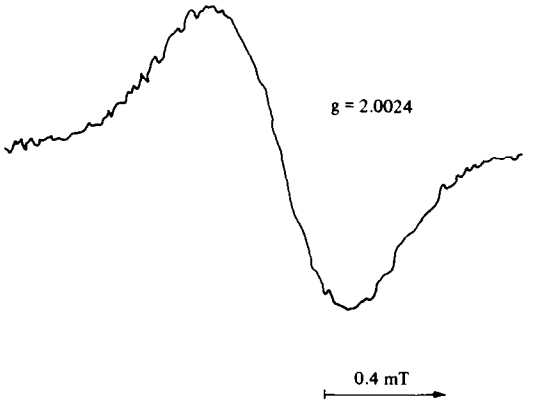


Fig. 2. ESR spectrum of adriamycin semiquinone radical formed when beef heart mitochondria (13 mg proteins/ml) are incubated with NADH (5×10^{-4} M) and ADM (5×10^{-4} M).

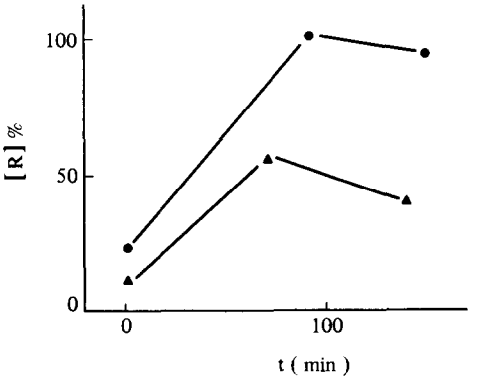


Fig. 3. ADM semiquinone radical concentration as detected by ESR. Beef heart mitochondria (13 mg proteins/ml) are incubated with NADH (5×10^{-4} M) and ADM (5×10^{-4} M), in the presence (▲, 3 mM in gum arabic 2%) or in the absence (●) of AD 20.

venging properties of AD 20 towards the ADM semiquinone radical. Considering the impossibility of performing this kind of experiment *in vivo*, we used heart mitochondria incubated with NADH and ADM as a model. In these conditions, a semiquinone radical is produced, that can be detected by ESR ([48], Fig. 2). It was characterized by a line width of 0.4 mT and a *g* value of 2.0024, in agreement with previous reports [18, 49]. In order to obtain a sufficient amount of mitochondria and to prevent the sacrifice of too many mice, mitochondria were isolated from beef heart. The production rate of the semiquinone radical during a prolonged time period decreased when AD 20 was added to the reaction mixture (Fig. 3). This decrease is not due to a direct effect of AD 20 on the univalent reduction of adriamycin. Indeed, the activities of NADH-dehydrogenase (complex **I**) and NADH-dehydrogenase/cytochrome *c* reductase (complex **I–III**) of mice heart mitochondria incubated with increasing AD 20 concentrations, are not modified up to an AD 20 concentration of 10 mM (Fig. 4), this indicates the inability of AD 20 to accept electrons at this level of the respiratory chain and consequently to prevent the formation of a semiquinone radical upon addition of ADM.

Detoxification of ADM mitochondrial toxicity by AD 20 in vivo

Complex **I–III** *and complex* **IV** *activities.* Table 1 shows the effect of AD 20 on the enzymatic activities of complex **I–III** and **IV** of heart mitochondria isolated from i.p. treated mice. ADM, as already observed in [45], causes a significant decrease of the activities of both complexes which fall respectively to 58% and 56% as compared to control mice. AD 20 injected i.p. 15 min prior to ADM almost completely restores the enzymatic activities of the complexes. No modification of the activities of the complexes is measured in mice treated with AD 20 alone.

Mitochondrial membrane structure modifications. Fluorescence polarization was used to determine the mitochondrial membrane fluidity. The fluorescence polarization P depends on the mobility of a fluorescent marker (1–6 diphenylhexatriene) embedded in the lipid bilayer, hence on the fluidity of the studied membrane. The higher P value measured in ADM treated mice (Table 2), indicates a decreased fluidity. In mice treated with AD 20 + ADM, P is not significantly modified as compared to the control group. In parallel to the evaluation of the mitochondrial membrane fluidity, we measured the lipid peroxidation (Table 3). Lipid peroxidation is increased for mice treated with ADM alone. If AD 20 is added, the lipid peroxidation rate is lower but still more important than in control mice. AD 20 alone has no effect.

DISCUSSION

Numerous studies [15–24] have established the relationship existing between the ADM enhanced free radical production in the heart and the cardiotoxicity of this agent. AD compounds display free radical trapping properties resulting from their capto-dative substituents [51]. They were therefore proposed as a new class of molecules able to overcome ADM cardiotoxicity which is related to the mitochondrial toxicity of ADM in heart cells, as shown by previous works with ADM treated mice [24, 45]. In presence of ADM mitochondria are, with the sarcoplasmic reticulum [49], the major site of production of free radicals. We present here results showing the efficiency of AD 20, one of the most promising AD derivatives, against *in vivo* heart mitochondrial toxicity of ADM. Our preliminary *in vitro* results indicate that the amount of ADM semiquinone radical detected in presence of NADH and mitochondria as measured by ESR, is diminished upon addition of AD 20 in the reaction mixture. This is of importance, considering the property of the semiquinone radical to react with membrane lipids (paper in preparation) and its role in the initiation of the free radical cascade, leading to the production of toxic oxygen radicals $O_2^{\cdot-}$,$OH^{\cdot}$) and H_2O_2. Besides, measurements of complex I and complex **I–III** activities in presence of AD 20 indicate its

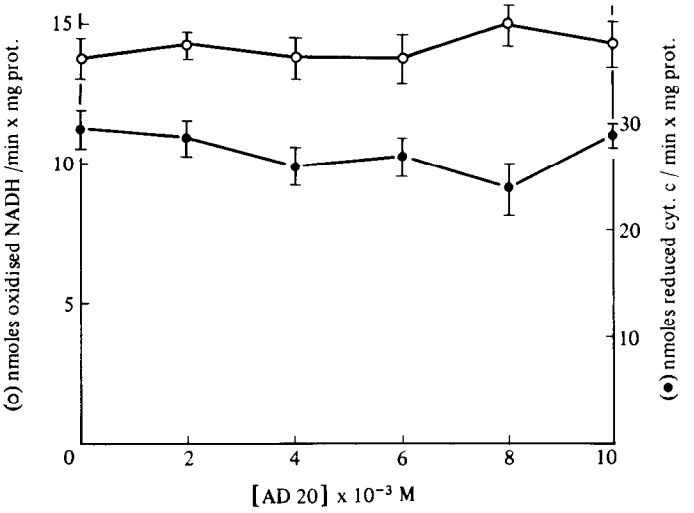


Fig. 4. Effect of increasing concentrations of AD 20 on the activities of NADH dehydrogenase (complex I, ○) and NADH dehydrogenase/cytochrome *c* reductase (complex I–III, ●), of mice heart mitochondria. Experimental conditions are described in Materials and Methods. Each result is the average of three experiments.

Table 1. Enzymatic activity of complex I–III and complex IV of the mitochondrial respiratory chain

	Complex I–III	%	Complex IV	%
No drug (6)	130 ± 18	100	328 ± 44	100
ADM (20 mg/kg) (5)	76 ± 10*	58	184 ± 38*	56
AD 20 (70 mg/kg) (6)	127 ± 18	98	321 ± 41	98
ADM (20 mg/kg) + AD 20 (70 mg/kg) (6)	124 ± 13**	95	286 ± 40**	87

Results are expressed in pmoles of reduced (complex I–III) or oxidized (complex IV) cytochrome *c*/mg protein × min. Percentages of activity as compared with the control group, are also given. The number of animals treated in each group is indicated in ( ). Mitochondria are extracted and enzymatic activities are measured as described in Ref. 24.

* $P < 0.05$ as compared to the control group.

** $P < 0.05$ as compared to the ADM group.

Table 2. Membrane fluidity modification of heart mitochondria extracted from treated mice

	P
No drug (6)	0.242 ± 0.004
ADM (20 mg/kg) (5)	0.265 ± 0.005*
AD 20 (70 mg/kg) (6)	0.240 ± 0.008
ADM (20 mg/kg) + AD 20 (70 mg/kg) (6)	0.239 ± 0.007**

P is the fluorescence polarization. The number of treated animals is expressed in ( ). Mitochondria were extracted and measurements were made as described in Ref. 24.

* $P < 0.01$ as compared to the control group.

** $P < 0.05$ as compared to the ADM group.

inability to accept electrons coming from NADH-dehydrogenase and consequently to prevent the direct formation of the semiquinone radical at complex I of the respiratory chain [16], by impairing the reduction of ADM. The molecular mechanism underlying the effect of AD 20 on the *in vitro* ADM semiquinone radical production remains, however, to be clarified; also, the ability of AD 20 to trap directly O_2^- and/or $OH^\cdot$ in the experimental conditions here described should be investigated in further studies.

To estimate *in vivo* mitochondrial damages, parameters defined in previous works [24, 45] were evaluated in ADM treated mice. At a dose of 20 mg/

Table 3. Effect of ADM and AD 20 on lipid peroxidation in mice heart mitochondria *in vivo*

Lipid peroxidation (malonaldehyde equivalents in pmoles/mg protein)	
No drug	415 ± 20
ADM (20 mg/kg)	849 ± 57
AD 20 (70 mg/kg)	446 ± 15
ADM (20 mg/kg) + AD 20 (70 mg/kg)	665 ± 63

In each experiment, three mice were used in order to obtain sufficient lipid concentration. Mice were killed by cervical dislocation and heart mitochondria were 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, considering an ε of 156 $mM^{-1} cm^{-1}$ [50]. Each result is the average of six experiments.

kg i.p. of ADM, serious disturbances of the mitochondrial functions, including lipid peroxidation, membrane rigidification and inhibition of the proper functioning of enzymes in the electron transport chain, are detected. They are overcome by i.p. injection of AD 20 (70 mg/kg), 15 min before giving ADM to the animals. However, lipid peroxidation is still enhanced in the ADM + AD 20 group, though to a lesser extent than in the ADM group. The same observation was made when mice were treated with 4'epi-ADM [24], an ADM analog with reduced cardiotoxicity. A high dose of 4'epi-ADM caused enhanced lipid peroxidation in mice heart but less than a lower dose of ADM. There appears to be a threshold of lipid peroxidation rate in heart mitochondria below which no effect on the mitochondrial membrane (as detected by changes in membrane fluidity) or on the activity of the essential respiratory complexes can be observed. Beyond this threshold, the perturbations become quickly dramatic, with a strong inhibition of the complexes.

These data indicate the efficiency of AD 20 to reduce ADM cardiotoxicity in the *in vivo* model we used and the ADM semiquinone radical production of our *in vitro* investigations. Though encouraging, it must be considered that this study concerned only the subacute toxicity of ADM and that it is essential to collect further data on the ADM detoxification potency of AD agents (especially AD 20) in a chronic treatment. The difficulty lies in the choice of a proper injection schedule. In this work, we decided to inject AD 20 just before ADM, at a dose of 70 mg/kg. We noticed in preliminary studies that this was the most efficient AD 20 concentration, since at lower or higher doses, the mitochondrial detoxification potency of AD 20 is progressively decreased. It can be objected that the interaction between AD 20 and ADM takes place locally (since ADM is injected only 15 min after AD 20) rather than in the heart, reducing ADM transfer into this organ. However, when L1210 tumor bearing mice are treated with AD 20 and ADM in the conditions here described, ADM maintains its therapeutic efficiency while its cardiotoxicity is diminished, resulting in an improved therapeutic index of the drug (submitted). Therefore, a possible local interaction of AD 20 with ADM reducing its cardiotoxicity without affecting its therapeutic action is unlikely. Moreover, this improved therapeutic index is maintained when AD 20 and ADM are injected by different routes (AD 20 i.v. and ADM i.p.) excluding any local interaction of both agents.

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