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ENZYMATIC STUDIES OF THE EFFECT OF Cu (II) ON OXYGEN RADICAL PRODUCTION STIMULATED BY DAUNORUBICIN AND AMETANTRONE

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Abstract—Daunorubicin can bind Cu (II) in Tris–HCl buffer (pH 7.4) and in the presence of NADH (100 μM). The complex is very stable. Cu (II) is not removed from the complex by cytochrome *c* reductase. The complexation of Cu (II) to daunorubicin gives rise to a modification of its redox properties. The complex, unlike the free drug, does not stimulate oxygen radical production. Ametantrone can also form a complex with Cu (II) in the conditions of enzymatic assays. Nevertheless, this complex is not stable in the presence of cytochrome *c* reductase. It dissociates immediately after the addition of the enzyme with releasing the metal ion.

Daunorubicin (DR†) is one of the most promising drugs for the treatment of neoplastic diseases [1]. Unfortunately its effectiveness is severely limited by dose-related cardiotoxicity [2]. Consequently, efforts are being made to understand the molecular basis of the cardiotoxicity and to obtain non-cardiotoxic analogs for use in cancer chemotherapy. This is a very complex problem. Nevertheless two hypotheses are most frequently discussed to explain the mechanism of anthracyclines cardiotoxicity. One group of authors suggests that this effect is correlated with the specific interaction of the drugs with biological membranes, especially with cardiolipin-containing mitochondrial membranes [3]. Nevertheless the majority of investigators attributes cardiotoxic effects of the anthracycline antibiotics to their redox activity [4–6]. It has been demonstrated that daunorubicin was enzymatically activated by reduced flavoproteins (xanthine oxidase, NADPH-cytochrome P-450 reductase, NADH-cytochrome *c* reductase) to semiquinone radicals. These radicals can initiate a free radical cascade, because they are able to transfer a single electron to molecular oxygen and to form reactive oxygen species (O_2, $OH^{\cdot}$, H_2O_2, $^1O_2$). The latter radicals damage nucleic acids and cause lipid peroxidation [7–9]. Cardiac tissue is deficient in the protective enzyme (catalase, superoxide dismutase) and seems unable to resist [10].

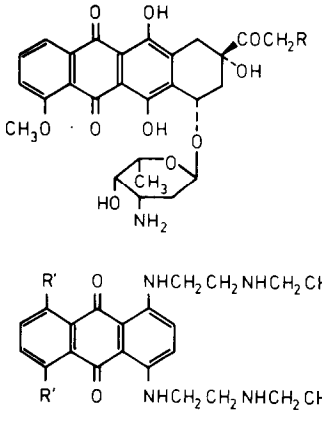

Fig. 1. The structure of daunorubicin (R = H), Adriamycin® (R = OH), ametantrone (R′ = H) and mitoxantrone (R′ = OH).

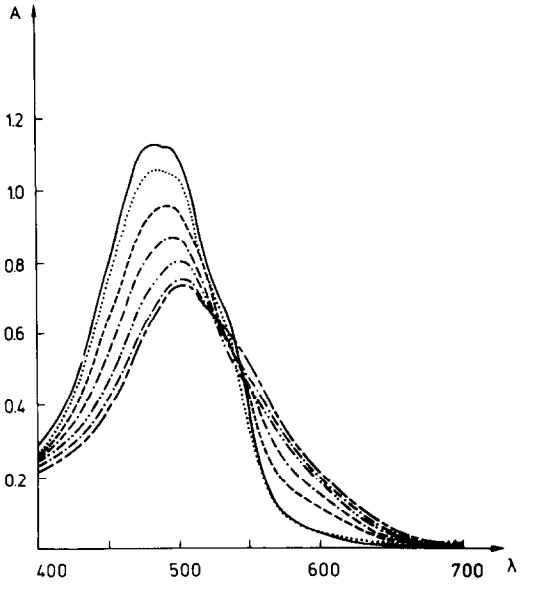

Fig. 2. Absorption spectra of daunorubicin in the presence of various amounts of Cu (II). Experimental conditions: 0.05 M Tris–HCl buffer (pH 7.4), [DR] = 100 μM, [NADH] = 100 μM, the molar ratio of Cu (II) were respectively, 0 (——), 10 μM (····), 25 μM (– – –), 50 μM (– · – · –), 100 μM (— · · —), 150 μM (— · —), 200 μM (— — —).

It was demonstrated that mitoxantrone and ametantrone—two synthetic simplified analogs of anthracycline antibiotics (Fig. 1)—have been significantly

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† Abbreviations used: DR, daunorubicin; AMET, ametantrone.

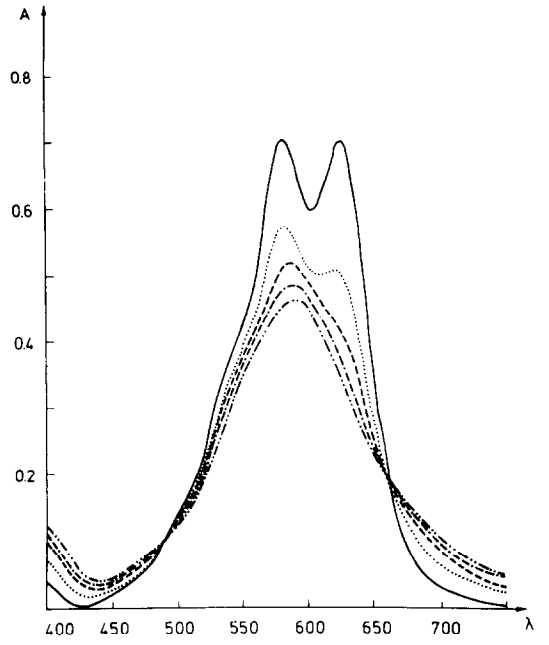

Fig. 3. Absorption spectra of ametantrone in the presence of various amounts of Cu (II). Experimental conditions: 0.05 M Tris–HCl buffer (pH 7.4), [AMET] = 100 μM, [NADH] = 100 μM, the molar ratio of Cu (II) were respectively 0 (——), 50 μM (· · · ·), 100 μM (– – –), 150 μM (– · – · –), 200 μM (— · · —).

Table 1. Effect of Cu (II) on cytochrome *c* reductase activity

$CuCl_2$ added (μM)	Activity cytochrome *c* reductase (units/mL)
0	0.20
100	0.19
200	0.19

The reaction mixture (1.0 mL) contained 0.05 M Tris–HCl buffer (pH 7.4), 50 μM cytochrome *c*, 200 μM NADH, 0.2 units/mL cytochrome *c* reductase and indicated amount of $CuCl_2$.

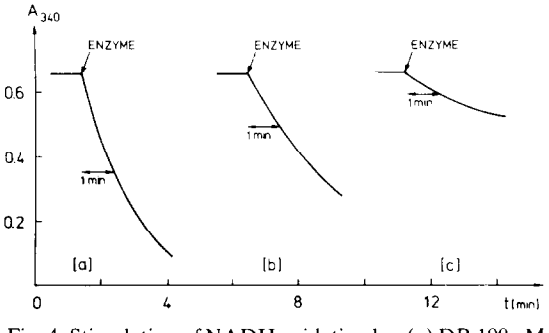

Fig. 4. Stimulation of NADH oxidation by: (a) DR 100 μM; (b) DR 100 μM + Cu (II) 50 μM; (c) DR 100 μM + Cu (II) 200 μM. The 1-mL reaction mixture contained 0.05 M Tris–HCl buffer (pH 7.4), 100 μM DR, indicated amount of Cu (II), 100 μM NADH and 0.2 units/mL cytochrome *c* reductase.

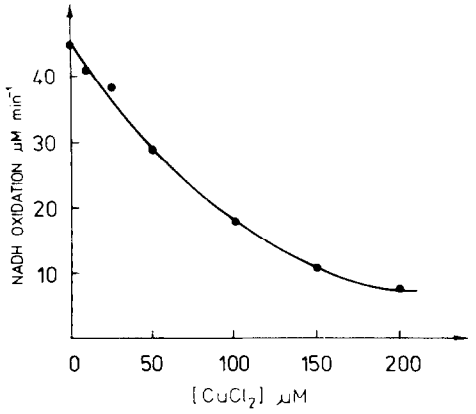

Fig. 5. Effect of Cu (II) on NADH oxidation stimulated by daunorubicin. The reaction mixture (1.0 mL) contained 0.05 M Tris–HCl buffer (pH 7.4), 100 μM DR, 100 μM NADH, indicated amount of Cu (II) and 0.2 units/mL cytochrome *c* reductase.

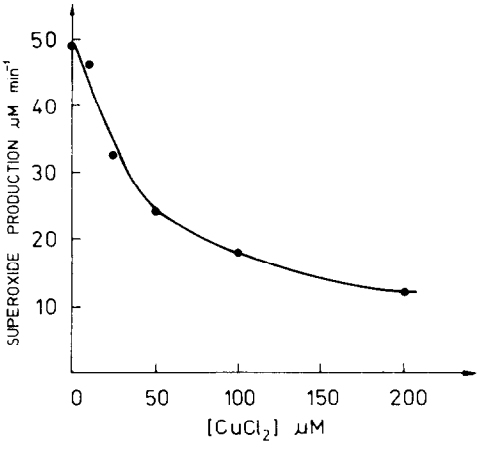

Fig. 6. Effect of Cu (II) on superoxide production stimulated by daunorubicin. The reaction mixture (1.0 mL) contained 0.05 M Tris–HCl buffer (pH 7.4), 100 μM DR, indicated amount of Cu (II), 100 μM NADH, 50 μM cytochrome *c*, SOD 20 μg/mL and 0.2 units/mL cytochrome *c* reductase.

less cardiotoxic while possessing antitumor activity equal to or superior to that of Adriamycin® and daunorubicin [11, 12]. Mitoxantrone and ametantrone are simultaneously less susceptible to enzymatic reduction and less active in stimulating oxygen radical formation than adriamycin and daunorubicin [13].

The capacity of anthracyclines to produce reactive oxygen species depends on their redox properties and on their interaction with enzyme.

In the effort to obtain non-cardiotoxic analogs of the anthracyclines several recent observations have focused attention on the development of a new class of antitumor compounds by complexation of these drugs with metal ions. This drug-to-metal complexation may modify the redox properties of the drugs. It has been shown that anthracycline type compounds such as Adriamycin®, daunorubicin and carminomycin were able to bind strongly metal ions

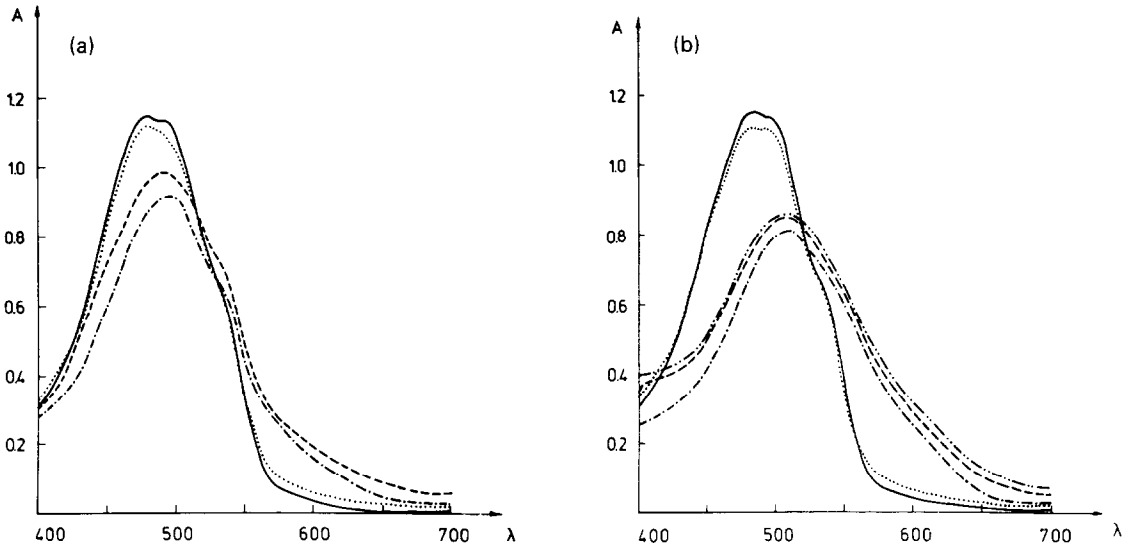

Fig. 7. Absorption spectra of (a) DR 100 μM before (——) and after (· · · ·) enzymatic reaction, DR 100 μM + Cu (II) 50 μM before (—·—) and after (– – –) enzymatic reaction; (b) DR 100 μM before (——) and after (· · · ·) enzymatic reaction, DR 100 μM + Cu (II) 200 μM before (—·—) and after enzymatic reaction (– – –) t_1 = 5 min, (—· ·—) t_2 = 60 min. Conditions for the reaction are described in Materials and Methods.

Table 2. Effect of Cu (II) on NADH oxidation and superoxide production by daunorubicin

$CuCl_2$ added (μM)	NADH oxidation* (μmol/min)	Superoxide production† (μmol/min)
0	45.0	48.9
10	41.8	46.9
25	38.6	32.6
50	28.9	24.5
100	17.7	18.4
200	8.0	12.2

The reaction mixture (1.0 mL) contained *0.05 M Tris–HCl buffer (pH 7.4), 100 μM DR, 100 μM NADH, indicated amount of $CuCl_2$ and 0.2 units/mL cytochrome *c* reductase; †0.05 M Tris–HCl buffer (pH 7.4), 100 μM DR, 100 μM NADH, indicated amount of $CuCl_2$ 50 μM cytochrome *c*, SOD 20 μg/mL and 0.2 units/mL cytochrome *c* reductase.

Table 3. Effect of Cu (II) on NADH oxidation by ametantrone

$CuCl_2$ added (μM)	NADH-oxidation (μmol/min)
0	57.9
100	57.9
150	51.4
200	51.4

The 1-mL reaction mixture contained 0.05 M Tris–HCl buffer (pH 7.4), 100 μM AMET, indicated amount of $CuCl_2$, 100 μM NADH and 2 units/mL cytochrome *c* reductase.

such as Fe (III) and Pd (II) [14–16]. These new agents are not reduced by flavoproteins in contrast to the free drugs and consequently do not produce the reactive oxygen radicals.

Simultaneously, it has been demonstrated that mitoxantrone and ametantrone were also able to complex metal ions/Pd (II) [17].

In the present study we examined in detail the complexation of Cu^{2+} to daunorubicin and to ametantrone and the influence of such parent drug modifications on oxygen radical formation.

MATERIALS AND METHODS

Ametantrone was prepared in our laboratory. Its synthesis, purification and properties have been described previously [18, 19]. Daunorubicin was a gift from Drug Development Branch, National Cancer Institute, Bethesda, MD. Concentrations of drug solutions were estimated spectrophotometrically: at 480 nm (E = 11500 M^{-1} cm^{-1}) for daunorubicin and at 638 nm (E = 7440 M^{-1} cm^{-1}) for ametantrone. Stock solutions were prepared just prior to use. Cytochrome *c* (type VI from horse heart), NADH (grade III), cardiac NADH-cytochrome *c* reductase and superoxide dismutase were obtained from the Sigma Chemical Co. (St Louis, MO). Other chemicals were routine.

Spectroscopic studies. Absorption spectra were recorded on a Beckman Model 360 spectrophotometer. In order to examine the complex formation of quinone drugs with Cu (II), increasing quantities of $CuCl_2$ were added to drug solutions (100 μM) at molar ratio of Cu (II) to quinone varying from 0:1 to 2:1. After a few minutes the absorption spectra were recorded.

Enzymatic assays. Cytochrome *c* reductase activity was determined using cytochrome *c* as the electron acceptor [20]. It was examined by following cyto-

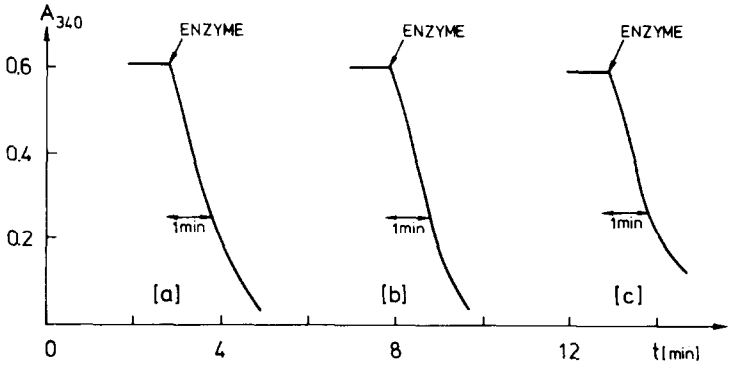

Fig. 8. Stimulation of NADH oxidation by: (a) AMET 100 μM; (b) AMET 100 μM + Cu (II) 100 μM; (c) AMET 100 μM + Cu (II) 200 μM. The 1-mL reaction mixture contained 0.05 M Tris–HCl buffer (pH 7.4) 100 μM AMET, indicated amount of Cu (II), 100 μM NADH and 2 units/mL cytochrome *c* reductase.

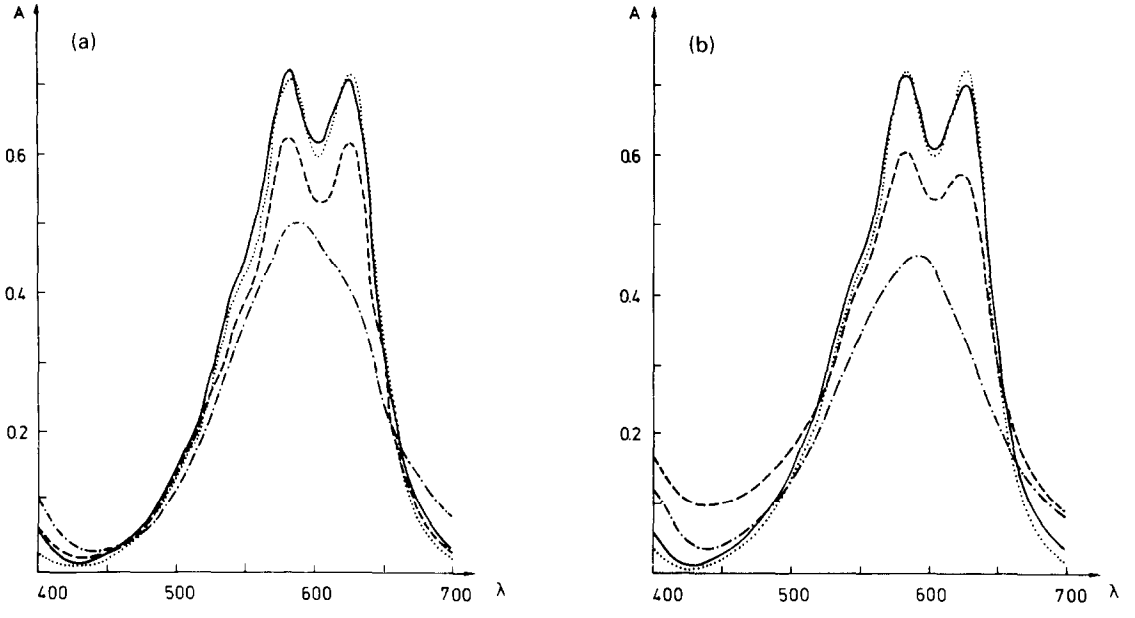

Fig. 9. Absorption spectra of (a) AMET 100 μM before (——) and after (· · · ·) enzymatic reaction, AMET 100 μM + Cu (II) 100 μM before (— · —) and after (– – –) enzymatic reaction; (b) AMET 100 μM before (——) and after (· · · ·) enzymatic reaction, AMET 100 μM + Cu (II) 200 μM before (— · —) and after (– – –) enzymatic reaction. Conditions for the reaction are described in Materials and Methods.

chrome *c* reduction at 550 nm using an extinction coefficient for cytochrome *c* (reduced minus oxidized) of 19600. The reaction mixture (1.0 mL) contained 0.05 M Tris–HCl buffer (pH 7.4), 50 μM cytochrome *c*, 200 μM NADH and ~0.2 units/mL cytochrome *c* reductase.

Enzyme activity has been expressed in units, where 1 unit of activity is that amount of cytochrome *c* reductase capable of reducing 1 μM of cytochrome *c* per min at pH 7.2 and 25°.

NADH oxidation was measured at 340 nm using an extinction coefficient of 6.22 $mM^{-1}\,cm^{-1}$. The 1-mL reaction mixture contained 0.05 M Tris–HCl buffer (pH 7.4), 100 μM quinone drug, indicated amount of $CuCl_2$, 100 μM NADH 0.2–2 units/mL cytochrome *c* reductase.

Superoxide anion production was determined by the rate of SOD-inhibitable cytochrome *c* reduction at 550 nm [21]. The reaction mixture (1.0 mL) contained 0.05 M Tris–HCl buffer (pH 7.4), 100 μM quinone drug, indicated amount of $CuCl_2$, 100 μM NADH, 50 μM cytochrome *c*, SOD 20 μg/mL and 0.2 units/mL cytochrome *c* reductase. All reactions were initiated by the addition of the enzyme.

RESULTS

Daunorubicin–Cu (II) and ametantrone–Cu (II) systems: spectroscopic studies

The formation of Cu (II)–daunorubicin and Cu (II)–ametantrone complexes has been followed by visible absorption. The spectra have been recorded in the conditions of enzymatic assays. The 1-mL reaction mixture contained 0.05 M Tris–HCl buffer (pH 7.4), 100 μM NADH, 100 μM quinone drug and the indicated amount of $CuCl_2$:

The addition of Cu (II) to a solution of daunorubicin (Fig. 2) and ametantrone (Fig. 3) caused striking modifications of the parent drug absorption spectra. The addition of increasing amounts of Cu (II) to a solution of daunorubicin gave rise to a shift to higher wavelength of the absorption band in the visible region.

In the case of ametantrone the addition of Cu (II) to its solution gave rise to a decrease of the intensity of the absorption in the visible region and to a disappearance of the absorption band at 627 nm.

These results strongly suggested that the complexes of Cu (II) with daunorubicin and with ametantrone have been formed at pH 7.4 (0.05 M Tris–HCl buffer) and that the presence of NADH did not prevent their formation. The conditional stability constants for these complexes (calculated by a computer program described in Ref. 22) were respectively: $\lg\beta_{CuDR_2} = 8.7 \pm 0.3$ and $\lg\beta_{Cu(AMET)_2} = 8.0 \pm 0.2$.

Effect of complex formation on NADH oxidation and superoxide production

Firstly, in order to verify if the presence of $CuCl_2$ influences the cytochrome *c* reductase activity, we have examined the effect on the addition of $CuCl_2$ on the activity of this flavoprotein. Our results (Table 1) demonstrate that the presence of Cu (II) does not change the enzyme activity. Cu (II) alone affects neither the NADH oxidation nor the superoxide production (data not presented).

As presented in Figs 4a, 5 and 6, daunorubicin was very effective in stimulating NADH oxidation and superoxide production catalysed by cytochrome *c* reductase. The gradual addition of Cu (II) to a solution of daunorubicin caused an increase of the complex quantities and consequently diminished NADH oxidation and superoxide production.

Thus, the formation of Cu (II)–daunorubicin complex reduced the drug ability to produce oxygen radicals.

The absorption spectra of DR + Cu (II) systems were recorded before and after the enzymatic reaction stopped by the total consumption of 100 μM NADH (Fig. 7). Our results suggested that the complex of Cu (II) with daunorubicin was very stable. It did not dissociate after the injection of the enzyme. As shown in Figs 4–6, the complex was significantly less effective in stimulating NADH oxidation and superoxide formation. Ametantrone was not susceptible to undergo rapidly the enzymatic reduction while activity of cytochrome *c* reductase was equal to 0.2 units/mL. Nevertheless at the increased activity of the enzyme (2.0 units/mL) the drug stimulated NADH oxidation to the extent comparable to the stimulation of NADH oxidation by anthracyclines (Table 3).

In contrast to daunorubicin, the addition of Cu (II) to a solution of ametantrone did not decrease NADH oxidation (Fig. 8, Table 3). Absorption spectra of AMET + Cu (II) systems recorded before and after enzymatic reaction (Fig. 9) indicated that the complex of Cu (II) with ametantrone was not stable under enzymatic reaction conditions. The addition of cytochrome *c* reductase caused its rapid dissociation on the metal ion and on the free drug.

DISCUSSION

Many investigators suggest that cardiotoxicity of anthracyclines is correlated with their ability to undergo a redox cyclic reaction and consequently to initiate a free radical cascade.

Adriamycin® and daunorubicin significantly increase both the rate of NADH oxidation and superoxide production over control level. Activation of anthracyclines to semiquinone radicals is attributed to the presence of a quinone group which is very susceptible to reduction.

Ametantrone has also a quinone moiety in its structure. We demonstrated that this compound stimulated also NADH oxidation, however at a higher activity of cytochrome *c* reductase equal to 2 units/mL.

The ability of anthracyclines and anthracenodiones to produce reactive oxygen species can be limited by their structural modifications. The compounds can form metal complexes. The site of complexation of metal ions [Fe (III), Pd (II)] to anthracyclines involves the C_{12}-carbonyl oxygen atom and C_{11}-phenolate group. On the other hand it has been demonstrated that these complexes did not increase superoxide formation over control level [14–16].

Thus, the interaction of Adriamycin® and daunorubicin with Fe (III) and Pd (II) modify the redox properties of the drugs. It has been also demonstrated [23, 24] that anthracyclines formed two well-defined species with Cu (II), one involving two molecules of drug per Cu (II) ion and the other, one molecule of drug per Cu (II) ion.

In fact, to be sure that daunorubicin can form a complex with Cu (II) in the conditions of enzymatic assays, we examined the complex formation of the drug with Cu (II) in Tris–HCl buffer (pH 7.4) and in the presence of NADH (100 μM). Our results (Figs 2 and 7) demonstrate that daunorubicin can bind Cu (II) under enzymatic reaction conditions. The complex is very stable. Cu (II) is not removed from the complex by cytochrome *c* reductase. Daunorubicin is chelated to Cu (II) through the carbonyl oxygen on C_{12} and the phenolate oxygen on C_{11}. It thus appears that the complexation of Cu (II) at the quinone moiety gives rise to a modification of the redox properties, because the quinone group which is the most susceptible to reduction is blocked by chelation of metal ion and is inaccessible to the enzyme. Thus, the addition of increasing amounts of $CuCl_2$ to daunorubicin solution causes a progressive increase of the complex quantities and consequently a gradual diminution of NADH oxidation and superoxide anion production in comparison to free daunorubicin.

Ametantrone is also able to form a complex with Cu (II) in the conditions of enzymatic assays. The complexation of Cu (II) to the drug gives rise to a decrease of the intensity of the absorption in the visible region and to a disappearance of the absorption band at 627 nm (Fig. 3), but does not give rise to a shift of the absorption band as it is observed in the case of complexation of Cu (II) to daunorubicin (Fig. 2). Kolodziejczyk and Garner-Suillerot [17] suggest that the four nitrogen atoms of the side chains of ametantrone are the most probable candidates for

the binding to metal ions. The type of complexation does not block the quinone function in the molecule of the drug.

On the other hand the complex of Cu (II) with ametantrone is not stable in the presence of the enzyme. Its interaction with cytochrome *c* reductase causes the release of metal ion. Thus, the addition of increasing amounts of $CuCl_2$ to ametantrone solution does not yield a decrease of NADH oxidation and superoxide production in comparison to free ametantrone. In conclusion:

—Because daunorubicin is chelated to Cu (II) through the carbonyl oxygen on C_{12} and the phenolate oxygen on C_{11} [23, 24], so the complex formation with Cu (II) at the quinone moiety of the drug gives rise to a modification of its redox properties.

—Ametantrone can also form complex with Cu (II). Nevertheless, this complex is not stable in the presence of cytochrome *c* reductase. It dissociates immediately after the addition of the enzyme with releasing the metal ion.

—The Cu (II)–daunorubicin complex, unlike the free drug is not able to catalyse the electron transfer from NADH to molecular oxygen and consequently to initiate a free radical cascade.

—The complex formation of daunorubicin with metal ions can be a promising route to get its therapeutically interesting non-cardiotoxic analogs.

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