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Cardiotoxicity and antitumor activity of a copper(II)-doxorubicin chelate

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Summary. The cardiotoxic and cytotoxic effects of the Cu(II)-doxorubicin (DXR) complex $[Cu(DXR)]_n$ are compared with those of the parent drug. It is shown that 10^{-4} *M* $[Cu(DXR)]_n$ has no depressant effects on isolated rat atria, in contrast with an equimolar concentration of the parent drug. No differences were found between the cytotoxic activities of the Cu(II) complex and free DXR on B16 melanoma and HeLa cells. A reduced penetration of the polymeric $[Cu(DXR)]_n$ into the myocardial cells as compared with the free drug was invoked to account for the absence of cardiotoxicity of the DXR complex. On the other hand, the observation that copper-complexation does not affect the cytotoxicity of the drug suggests that extracellular as well as intracellular mechanisms may be involved in the development of its antitumor activity.

Introduction

Since the discovery that superoxide anions and related reactive oxygen species are involved in the cardiotoxic and antitumor effects of doxorubicin (DXR) [2], investigation of the role of transition-metal ions in the mechanism of action of this drug has been undertaken. In fact, several transition-metal complexes can reduce molecular oxygen to superoxide anion [6], and the antitumor anthracyclines can form strong coordinative bonds with transition-metal ions [5].

These properties raised hypotheses about the implications of transition-metal ions for the mechanism of action of anthracyclines:

1. Both structural and electronic properties of copper(II)-anthracycline derivatives have been extensively studied. Although direct e.s.r. (electron spin resonance) evidence of the formation of superoxide anion is lacking, an electron transfer from metal to oxygen has been suggested to be responsible for the stability of Cu(II)-anthracycline derivatives as compared with the Cu(II) hexaquoion [3, 4]. In line with this observation, Wallace [14] reported experimental evidence of Cu(II)DXR-induced, non-enzymatic activation of dioxygen to superoxide anion.

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2. The $Fe(III)(DXR)_3$ complex has also been suggested to induce a non-enzymatic reduction of molecular oxygen, with subsequent production of superoxide anions and related radical species [15]. On the other hand, experimental data seem to suggest that $Fe(III)(DXR)_3$ does not catalyze the NADH dehydrogenase-mediated electron flow from reduced nicotinamide adenine dinucleotide (NADH) to molecular oxygen [1], in contrast with the behaviour displayed by free DXR.

Based on the results mentioned above, two opposite effects may be expected from the chelation of anthracyclines to a transition-metal ion, depending on the involvement of an enzyme-dependent or -independent process. Outside the cells, NADH dehydrogenase is absent; however, the reduction of molecular oxygen could take place by a metal complex-mediated, non-enzymatic mechanism. On the other hand, inside the cell, the metal could inhibit the electron flow from NADH to O_2, thus inactivating the drug. The net effect would then result from the superimposition of two different mechanisms.

Experimental evidence suggests that anthracycline-induced cardiotoxicity is mainly related to effects requiring the presence of the drug in the intracellular environment [9], whereas the antitumor activity may involve both membrane and intracellular sites [8, 11]. Therefore, metal chelation is expected to produce a shift in the therapeutic index of anthracyclines, due to differences in either the electronic properties or the cellular availability of free drugs and complexes.

The present paper reports a study on the cardiotoxic and antitumor effects of the Cu(II)DXR chelate. The choice of DXR arises from both the extensive clinical trials carried out with this agent in several human tumor diseases and the availability of a full chemical characterization of the Cu(II)DXR complex [3, 4].

Materials and methods

Preparation of the Cu(II)DXR complex. DXR was kindly supplied by Farmitalia-C. Erba S.p.A., Milano. The purity of the drug was assessed by reverse-phase HPLC (HP 1090) on Microbore columns (100 × 2.1 mm) thermostated at 40° C (H_2O phosphate buffer 0.1 *M*, pH 3.5; CH_3CN). Since anthracycline solutions are light-sensitive, fresh stock solutions were prepared shortly before use. Standard Cu(II) solutions (ca. 10^{-3} *M*) were prepared from reagent-grade $CuCl_2 \cdot 2\,H_2O$ (Carlo Erba) and mixed

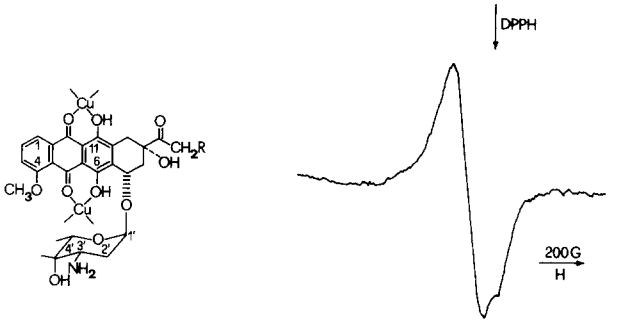

Fig. 1. The e.s.r. spectra, recorded in aqueous solution at −150° C, and molecular scheme of $[Cu(DXR)]_n$

with a double volume of an equimolar anthracycline solution. The pH value was adjusted to 6.5 with a 0.01 *N* NaOH solution.

Evaluation of cardiac inotropism. The effects on cardiac inotropism were evaluated in spontaneously beating rat atria. In fact, DXR has been shown to produce a dose-dependent inhibition of the contractile performance of these preparations [13]; a parallel has been established between the cardiotoxic effects of DXR as measured according to this experimental model and the acute signs of cardiotoxicity in humans [12].

Atria were isolated from female Sprague-Dawley rats (Charles River, Calco, Italy) weighing approximately 125–140 g. Preparations were quickly transferred to an organ bath filled with modified Tyrode's solution of the following composition: 11 m*M* glucose, 137 m*M* NaCl, 5.37 m*M*, KCl, 0.51 m*M*, $MgCl_2$, 12 m*M* $NaHCO_3$, 1.8 m*M* $CaCl_2$, and 0.46 m*M* NaH_2PO_4 (pH 7.2). The solution was maintained at 37° C and bubbled with a mixture of O_2 and CO_2 (95%: 5% by vol.). The atria were subjected to a load of 1 g and allowed to equilibrate for 45 min. At the end of this period, DXR or its Cu(II) complex was added to the incubation medium to a final concentration of 10^{-4} *M*. The contractile response developed by the preparations was expressed as the maximal rate of tension development (dF/dt). The observation period was discontinued 60 min after addition of the drug. Differences in the inotropic effects of DXR and its complex at the end of the observation period were evaluated by analysis of variance using Scheffe's test for multiple comparisons.

Evaluation of cytotoxicity. B16 melanoma cells (8×10^4/ml) were seeded in RPMI 1640 (Flow Laboratories, Irvine, UK) + 10% foetal calf serum (Flow Laboratories) in 6-well tissue-culture clusters (Coster, Cambridge, Mass.). After 24 h, the medium was removed and cells were incubated for 60 min in Hanks' balanced salt solution containing the drug at concentrations ranging from 1.6×10^{-8} to 10^{-5} *M*. At the end of the treatment, cells were washed with saline or medium (see *Results*) and incubated in complete culture medium for 72 h. At this time, cells were trypsinized and counted in a Coulter counter ZBI (Coulter Electronics, Luton, UK). Cell viability was determined by trypan blue dye exclusion. An identical procedure was adopted for HeLa cells.

Assessment of the stability of the Cu(II) complex in the cardiac intracellular environment. The stability of Cu(II)DXR in the cardiac intracellular environment was assessed by e.s.r. spectroscopy in preparations obtained as follows. Whole hearts excised from female Sprague-Dawley rats were homogenized in ice-cold incubation medium (1:2 w/v) with an Ultra-Turrax (IKA-Werk, Staufen, FRG) homogenizer. The homogenate was subsequently centrifuged at 4,000 *g* for 20 min; the pellet was discarded and the supernatant was recentrifuged at 10,000 *g* for 30 min. The supernatant was further centrifuged for 90 min at 40,000 *g*. The resulting supernatant corresponds to the cytosolic and microsomal fractions, but for the sake of simplicity it is referred to as "cytosol" throughout this paper. Cu(II)DXR complex was diluted to 10^{-4} *M* in the cytosol; this concentration corresponds to the theoretical situation in which the drug complex is present inside the cells at the same concentration as in the extracellular fluid.

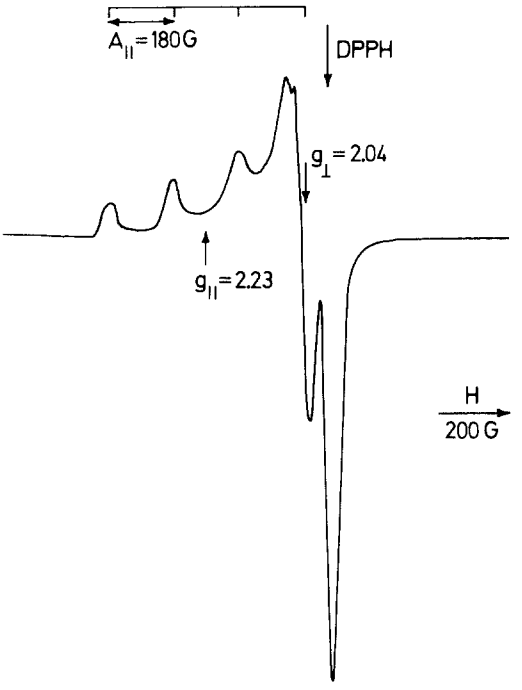

Fig. 2. The e.s.r. spectrum of $[Cu(DXR)]_n$, recorded in cytosol at 150° C

The e.s.r. spectra of these samples were recorded on a Varian E-109 spectrometer provided with a variable temperature apparatus to check the stability of the Cu(II) complex in cytosol; DPPH (1,2-diphenyl-2-picrylhydrazyl) was used for field calibration. The following e.s.r. operating conditions were adopted: microwave frequency, 8.95 GHz; microwave power, 5 mW; modulation amplitude, 16 G; receiver gain, 1×10^3; time constant, 0.064 s; scan range, 4,000 G; scan time, 4 min. The stability of the Cu(II) complex in the tumor-cell culture medium was assessed by an analogous procedure.

Results

Characterization of the Cu(II)DXR complex

The e.s.r. spectra of Cu(II)DXR aqueous solution, obtained from 10^{-4} *M* $CuCl_2$ and anthracycline solutions, showed the formation of a polymeric Cu(II) derivative. The stability and electronic properties of the complex have

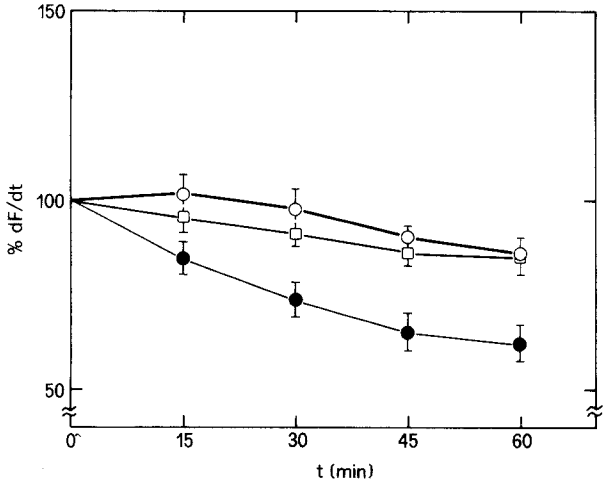

Fig. 3. The effect of doxorubicin (DXR) and Cu(II)-DXR on the contractile performance of isolated rat atria (mean ± SE of 6–10 preparations). ■, controls; ●, DXR; ○, Cu(II)DXR

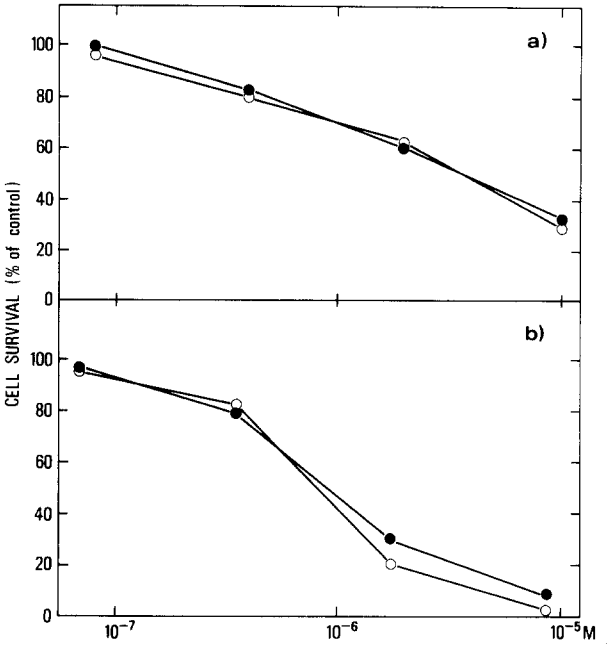

Fig. 4. The cytotoxicity of doxorubicin (DXR) and Cu(II)-DXR complex on **a** B16 and **b** HeLa cells. Data represent the means of six different experiments, with two replicates per experiment (SE < 10%). ●, DXR; ○, Cu(II)DXR

been discussed in previous reports [3, 4]; its polymeric structure was established on the basis of the strong Cu-Cu exchange interaction observed in the e.s.r. spectrum (Fig. 1). These e.s.r. studies also indicate that the complex obtained in aqueous solution from DXR and $CuCl_2$ in a 2:1 ratio must actually be formulated as $[Cu(DXR)]_n$; in other words, the drug:metal ratio r does not correspond to the nominal value r = 2 but is actually r = 1. For this reason, 50% of the DXR should be detected in the reaction mixture as free drug; surprisingly, we found that the free drug was undetectable in the visible region near 500 nm (λ_{max} = 480 nm). Free DXR seemed to be unavailable for biological purposes as well. In fact, $5 \times 10^{-5} M$ free DXR, corresponding to 50% of the full DXR concentration adopted in this study and to the theoretical free drug concentration in the complex solution, was found to reduce atrial contractility to 75.90% ± 9.84% of the baseline value after 60 min; in contrast, Cu(II)DXR complex was found to be devoid of significant cardiotoxic effects (see Fig. 3 and following section). A likely explanation might be that the free ligand interacts with the polymeric complex and/or is adsorbed on its surface. No residual hexaquoion $[Cu(H_2O)_6]^{2+}$ was observed by e.s.r. at this drug: metal ratio, the complexation equilibrium being favoured by the excess of drug [3, 4].

The same results were obtained by mixing $CuCl_2$ and $10^{-4} M$ DXR with a 2:1 drug:metal ratio at pH 6.5 in Hanks' or Tyrode's solution. On the other hand, when e.s.r. spectra were recorded in cytosol instead of the aqueous environment, they showed dramatic changes: in spite of the presence of DXR, the signals (Fig. 2) were identical to those of $CuCl_2$ in cytosol, and the g-tensor values corresponded with those of the Cu(II) complexes with protein ligands [7]. These observations suggest that the Cu-anthracycline derivative is unstable inside the cells. The spectrum obtained for the complex in tumor-cell culture medium was identical to that obtained for $CuCl_2$ in cytosol, suggesting that a dissociation of the complex occurs.

Cardiac effects

Figure 3 shows that in control preparations the contractile performance undergoes a steady decrease with time, reaching 85.17% ± 5.32% of the baseline value after 60 min. In this experimental model, $10^{-4} M$ DXR was found to induce a time-dependent impairment of atrial contractility, with a dF/dt reduction to 62.08% ± 4.75% of the baseline value by the end of the observation period. This reduction is statistically significant ($P < 0.05$) as compared with the control values. In contrast, the contractile force developed by Cu(II)DXR-treated preparations did not differ from control values, reaching 85.53% ± 2.88% of the baseline value in 60 min.

Antitumor activity

The antitumor activity of DXR and its Cu(II) complex was evaluated in B16 melanoma and HeLa cells. Due to the instability of the Cu(II) complex in culture medium, the cells were incubated for 1 h in Hanks' solution containing the drugs; to remove the drugs from outside the cells, these were subsequently washed with either saline or RPMI medium prior to the evaluation of cell viability. The two alternative washing procedures were adopted to check the possibility that the amino acid-containing RPMI medium might dissociate the membrane-bound complex, thus enabling the penetration of free drug into the cells. However, this hypothesis can be ruled out, since similar results were obtained when the cells were washed with either media; therefore, data obtained with both experimental procedures have been pooled.

Figure 4 shows the results of six experiments carried out in duplicate in B16 melanoma cells. The ID_{50} value of DXR was $3.3 \times 10^{-6} M$; the antitumor activity of the Cu complex was similar to that of the parent drug ($ID_{50} = 3.4 \times 10^{-6} M$) (Fig. 4a). As in B16 melanoma cells, no differences were found between the cytotoxic ef-

fects of Cu(II)DXR complex and parent drug in HeLa cells (Fig. 4b). The ID_{50} value of DXR was 1×10^{-6} *M*; the antitumor activity of the Cu complex did not differ from that of the parent drug.

Discussion

The present investigations were carried out with a DXR-Cu complex of polymeric structure, where free DXR was not present. The experiments show that complexation with Cu(II) does not affect the antitumor effect of DXR as measured in a murine as well as a human cell line, whereas it abolishes the cardioinhibitory effects of DXR in isolated rat atria. Under the hypothesis that anthracycline-induced cardiotoxicity is mainly related to the intracellular generation of free radicals [2] and due to the fact that Cu(II)DXR complex is unstable in the intracellular environment, it is impossible to attribute the absence of cardiotoxic effects of the Cu(II) complex to its electronic properties, since it is not present as such within the cells.

A possible explanation for the lack of cardiotoxicity displayed by DXR complexed to Cu(II) might be that the Cu(II)DXR complex is prevented from reaching the extravascular space due to the large size of its polymeric particles. To test this hypothesis, a comparison was made between the binding of [^{14}C]-DXR and that of Cu(II)-[^{14}C]-DXR by myocardial cells after exhaustive washing of the extracellular spaces. The results obtained in these experiments unambiguously demonstrate that this hypothesis should be discarded, as no significant differences were observed (data not shown). However, these data do not enable us to establish whether the drug is bound at the cellular surface or at intracellular sites.

The e.s.r. experiments carried out in cytosol showed that, should it penetrate into myocardial cells, the Cu(II)DXR complex would break down to free DXR, which in turn would produce the expected contractile impairment. Therefore, the different inotropic effects of Cu(II)DXR vs DXR are most likely due to the fact that the polymeric complex $[Cu(DXR)]_n$ does not reach the myocardial intracellular environment, whereas the parent drug does.

The present experiments cannot provide an explanation for the different behaviour displayed by Cu(II)DXR in myocardial vs tumor cells. It was proposed that, in contrast with the cardiotoxic effects, the antitumor activity of DXR may involve both intracellular (drug-DNA interactions) and extracellular sites. This last possibility is supported by the observations reported by Tritton et al. [11], showing that the cytotoxicity of "immobilized" DXR is equal to, or even greater than, that of the free drug in some cell lines; further support is provided by the results reported by Sinha et al [10], showing that DXR can induce free-radical production in both the intra- and extracellular compartments of tumor cells.

However, based on the data obtained in the present study, it is not possible to conclude whether the different biological behaviour displayed by Cu(II)DXR is attributable to the differing ability of myocardial and tumor cells to handle the complex (e.g. endocytosis of the complex; dissociation of the complex and subsequent uptake of the free drug) or to the fact that the Cu(II)DXR complex behaves like an "immobilized" form of DXR; the two mechanisms might contribute to the overall effect. Whatever the mechanistic interpretation, the present investigations show that Cu(II) chelation provides a way for the dissociation of the cardiotoxic and cytotoxic properties of DXR, without modification of the chemical structure of the anthracycline.

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