Chelation of Copper(II) by Daunomycin and 5-Iminodaunomycin and Interaction of the Complexes with Mononucleotides: an ESR Study

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Abstract

Coordination of copper(II) ions by daunomycin and 5-iminodaunomycin has been studied by electron spin resonance spectroscopy, at various values of pH and r, the anthracycline-to-Cu(II) molar ratio. At r = 1–5, polymeric complexes are formed in the case of daunomycin. At $r = 5$, a mononuclear complex is predominant and at $r = 10$, this is the only one formed with the ^{63}Cu and ^{65}Cu hyperfine interaction being clearly defined in the $g_{\parallel}$ region ($g_{\parallel} = 2.26$, $^{63}A_{\parallel} = 175$; $^{65}A_{\parallel} = 190$ G). For 5-iminodaunomycin both chelation sites are involved in the coordination and a polymeric structure (in which exchange interactions between Cu(II) centers operate) is stable in the range $r = 1–3$. At $r = 3$, the triplet state of a dinuclear Cu(II) complex is observed and 5-iminodaunomycin behaves as both a bridging and a terminal ligand. For $r = 5–10$, the dinuclear complex coexists with the mononuclear one. In the presence of mononucleotides dGMP, dAMP, dCMP and thymidine, no ternary complex such as mononucleotide/Cu(II)/anthracycline was observed.

Introduction

ESR spectroscopy has been shown to be a useful technique for the investigation of the chelation properties of antitumor anthracyclines of paramagnetic transition metal ions. The results of our previous studies [1–4] have shown that Cu(II) ions act as a probe and reveal: (a) minor structural modifications on the sugar moiety (doxorubicin *versus* 4′-epidoxorubicin); (b) the different substitution of the anthraquinone moiety of semi-synthetic anthracycline analogues; (c) the self-association of different anthracyclines at high drug/Cu(II) molar ratios.

The above-mentioned structural features seem also to be responsible for a different distribution of the electrons between the copper ion and the drug ligand, as is clearly shown by the values of the magnetic tensor components. The results reported in our studies provide a rationale for the stabilization, by molecular oxygen, of the complexes of some of the anthracyclines having specific structural requirements.

Very recently, Wallace [5] has suggested that copper(II) is involved in the mechanism of action of the antitumor anthracyclines. Apparently a 'non-enzymatic' oxygen reduction is catalyzed by the copper(II)/anthracycline chelates. Similar behavior has been found with the Fe(III)/anthracycline complexes [6] for which the kinetics of the dioxygen reduction seem to be also affected by the structural features of the daunosamine sugar moiety. However, details of the above-mentioned interaction are not clearly defined as yet, and we felt that an in-depth analysis of the electronic structure of the Cu(II) complexes was warranted in view of an understanding of the role of the activated oxygen species (·OH, ·OOH, $O_2^{\cdot-}$) in the cytotoxicity and cardiotoxicity of the anthracyclines.

The present paper reports on an ESR investigation of Cu(II) complexes of daunomycin, a drug used in the treatment of acute leukemia, and 5-iminodaunomycin, a compound which has been found to be active on several commonly used tumor models and human xenographs [7–9] and which, at variance with daunomycin, is almost devoid of cardiotoxicity [10–12] and is much less mutagenic [13] (Scheme 1). The ESR investigation has also been extended to cover the interaction of Cu(II)/anthracycline complexes with the DNA bases to ascertain whether a ternary mononucleotide/Cu(II)/anthracycline complex is formed which might have a bearing on the cytotoxic action of this class of drugs.

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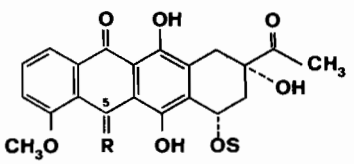

R = O daunomycin (1)

R = NH 5-iminodaunomycin (2)

S = L-daunosamine

Scheme 1.

Experimental

5-Iminodaunomycin was prepared as according to Tong *et al.* [14]. The purity of the anthracyclines was checked by reverse-phase HPLC (HP 1090) on a Microbore column (100 × 2.1 mm) thermostated at 40 °C, by elution with phosphate buffer solution (pH = 3.5) and acetonitrile, with the organic solvent gradient changing from 4 to 65% in 25 min at a flow rate of 0.5 ml/min. Because of the sensitivity of the anthracycline solutions to light, stock solutions were prepared just before use. Standard Cu(II) solutions (*ca.* 10^{-3} M) were prepared from Reagent grade $CuCl_2 \cdot 2H_2O$ (Carlo Erba, Milan) and mixed with the appropriate volume of either daunomycin or 5-iminodaunomycin aqueous solutions to reach the desired r value (*i.e.* the anthracycline-to-copper(II) molar ratio). The pH adjustments were made with 0.01 N NaOH or HCl solutions and the values determined with a Gibertini DP-100NE pH meter at room temperature. A small amount of ethylene glycol was added in order to obtain glassing solutions for the ESR measurements at −150 °C. The ESR spectra were recorded on a Varian E109 spectrometer, equipped with a variable temperature accessory, and DPPH was used for the field calibration. The mononucleotides, 2′-deoxyguanosine, 2′-deoxyadenosine, 2′-deoxycytosine 5′-monophosphate (dGMP, dAMP, dCMP) and thymidine were purchased from Sigma Chemical Co. and used as received.

Results

Cu(II)/Daunomycin Chelates

We have previously reported [2] that for anthracycline/copper(II) ratios $r = 5-10$, a magnetically diluted copper complex is formed in which the metal ion is chelated by 'stacked-up' drug molecules and does not experience spin exchange with other Cu(II) ions. The value of r at which the stacking of the ligand occurs varies with the structure of the anthracycline. For high r values (e.g. 5 to 10), the electronic structure of the Cu(II) complex does not depend on the ligand structure and, because of the absence of spin–spin interaction, good resolution of the ESR signals is obtained. When $r = 1$, two epimeric structures such as doxorubicin and 4′-epidoxorubicin lead to very different ESR spectra [1]. The chelating properties of daunomycin at $r = 1$ parallel those observed in the case of doxorubicin. Figure 1 shows

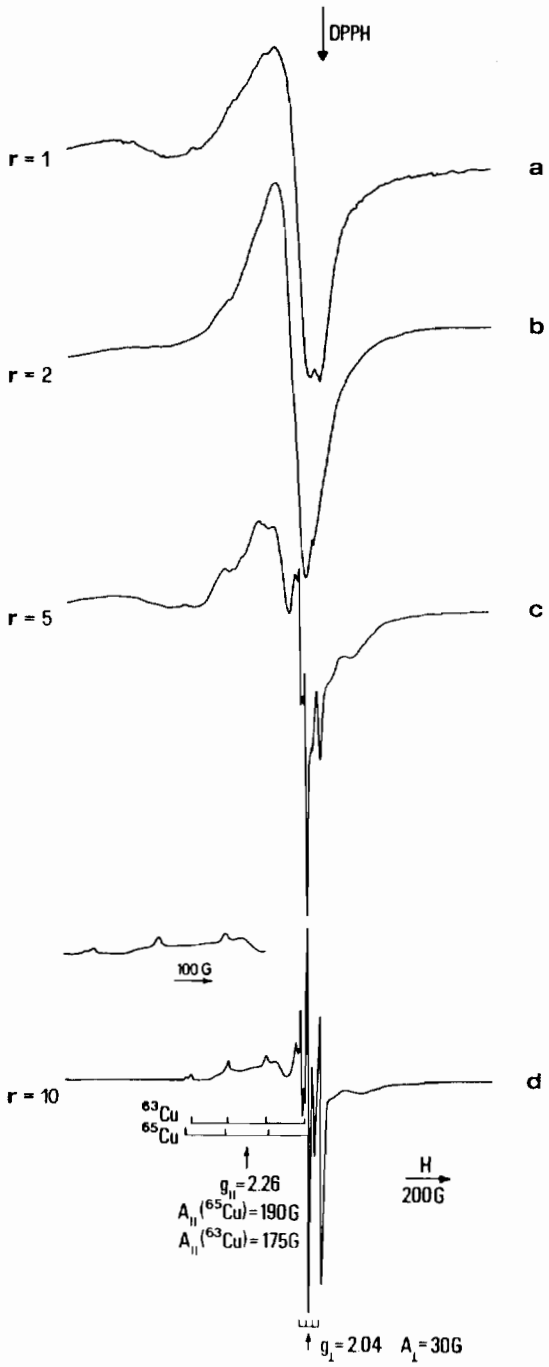

Fig. 1. ESR spectra of 10^{-3} M frozen (−150 °C) aqueous solutions of $CuCl_2$/daunomycin, pH = 6.5, at different daunomycin/Cu(II) ratios (r).

the ESR spectra of the daunomycin/Cu(II) chelates for r values ranging from 1 to 10.

For $r = 1-2$, only polymeric complexes are formed, whereas at $r = 5$ the ESR spectrum is attributed to a mononuclear complex similar to that already reported for doxorubicin and 4′-epidoxorubicin [1, 2], although some residual polymeric structure is still observable. However, at $r = 10$, the monomeric adduct is the only species present and the exceptionally good resolution of the spectrum allows one to clearly distinguish the two resonance regions: *i.e.* the parallel one, at lower field ($g_{\parallel} = 2.26$) for which the hyperfine coupling interactions with ^{65}Cu and ^{63}Cu are clearly defined and characterized by $A_{\parallel} = 190$ and 175 G, respectively; and the perpendicular resonance region ($g_{\perp} = 2.04$) for which the hyperfine coupling with the two Cu isotopes cannot be distinguished ($A_{\perp} = 30$ G). The complex is already formed at pH = 4 and is assigned the same structure already reported for doxorubicin at the same r value. In the latter case, the spectral resolution was not as good, probably because of the presence of residual polymeric structures in the system.

Cu(II)/Daunomycin/Mononucleotides

The excellent resolution of the ESR spectra of the Cu(II)/daunomycin complex at $r = 10$ prompted us to investigate the ternary interaction Cu(II)/daunomycin/nucleotide at this r value. Several investigations on the interaction of DNA/anthracyclines in the presence of Cu(II) ions have been previously reported [15, 16] but no firm conclusion on the formation of a ternary complex has been reached. The difficulty in the structural assignments stems from the lack of a direct characterization of the Cu(II) ligand field in the presence of the above-mentioned ligands. In our previous investigations the poor resolution in the parallel region of the ESR spectra of Cu(II) complexes of either doxorubicin or 4′-epidoxorubicin, both at low and high r values, prevented the achievement of direct evidence for the ligand-induced perturbation of the copper center.

New ESR experiments were carried out on the ternary mixture of Cu(II)/daunomycin/mononucleotide in the ratio 1/10/10. Under the conditions of drug–mononucleotide equimolarity, the Cu(II) ion should have the same probability of interacting with either the drug or the mononucleotide. At the same time, since the relative ratio of the drug-to-metal ion was 10, we expected, as above reported, good resolution and a better chance of detecting the perturbation induced by the presence of the other ligand, *i.e.* the mononucleotide. The spectra of the ternary mixtures Cu(II)/daunomycin/mononucleotide were compared with those of the Cu(II)/mononucleotide systems in which the nucleotide was present in a ten-fold excess.

With the exception of 2′-deoxyguanosine 5′-monophosphate, the remaining nucleotides do not seem to appreciably interact with Cu(II) ions in the presence of anthracyclines and therefore, for them, only the Cu(II)/daunomycin diadduct was formed in the ternary system at pH = 4. At pH = 5, the daunomycin substitutes for dGMP and only the daunomycin/Cu(II) complex is present in solution. These results seem to rule out the presence, at physiological pH, of a ternary nucleotide/Cu(II)/anthracycline complex in which both ligands are directly bound to Cu(II); however, if the other possible complex (Cu(II)/anthracycline/nucleotide) were formed, the drug–nucleotide interaction might not be strong enough to appreciably perturb the copper ligand field and therefore would not be detected by ESR. It appears that the drug is characterized by a higher affinity for the Cu(II) ions and this is also borne out when a comparison of the ESR parameters of the Cu(II)/nucleotides and Cu(II)/drug complexes is made; this must, in our opinion, derive from the better chelating properties of the dihydroxyanthraquinone structure of the anthracyclines. When doxorubicin and 4′-epidoxorubicin were considered, no evidence was found for the formation of a ternary complex. We feel that the reported suggestion [16] of the formation of ternary DNA/Cu(II)/anthracycline complexes in which the copper ion bridges the biomolecule and the drug must be treated with caution.

Cu(II)/5-Iminodaunomycin Chelates

The recent report [17] on the chelating properties of Cu(II) ions on 5-iminodaunomycin led us to reinvestigate this system in the light of the results we have obtained with other anthracycline analogs. Furthermore, as this compound is claimed to be a poor catalyst in the anthracycline-mediated enzymic activation of molecular oxygen to cytotoxic and cardiotoxic species (such as $O_2^{\cdot-}$, ·OH, ·OOH, etc. [18]), it was interesting to define the ability of its copper complex to bind molecular oxygen.

Figure 2 shows the spectral modifications undergone by the Cu(II) ion resonances at different ratios (r) of 5-iminodaunomycin/Cu(II) and they can be summarized as follows.

$r = 1$

The metal–drug complex exhibits a spectrum similar to that of the Cu(II)/daunomycin adduct, in that a polymeric structure can be assigned. Both chelating sites of the chromophore (*i.e.* C5=NH, C6–OH; C12=O, C11–OH) are involved in the chelation and a spin-exchange mechanism between Cu(II) centers is operative. The complex begins to form at pH = 5 and at pH = 7 its resonances are the only ones present.

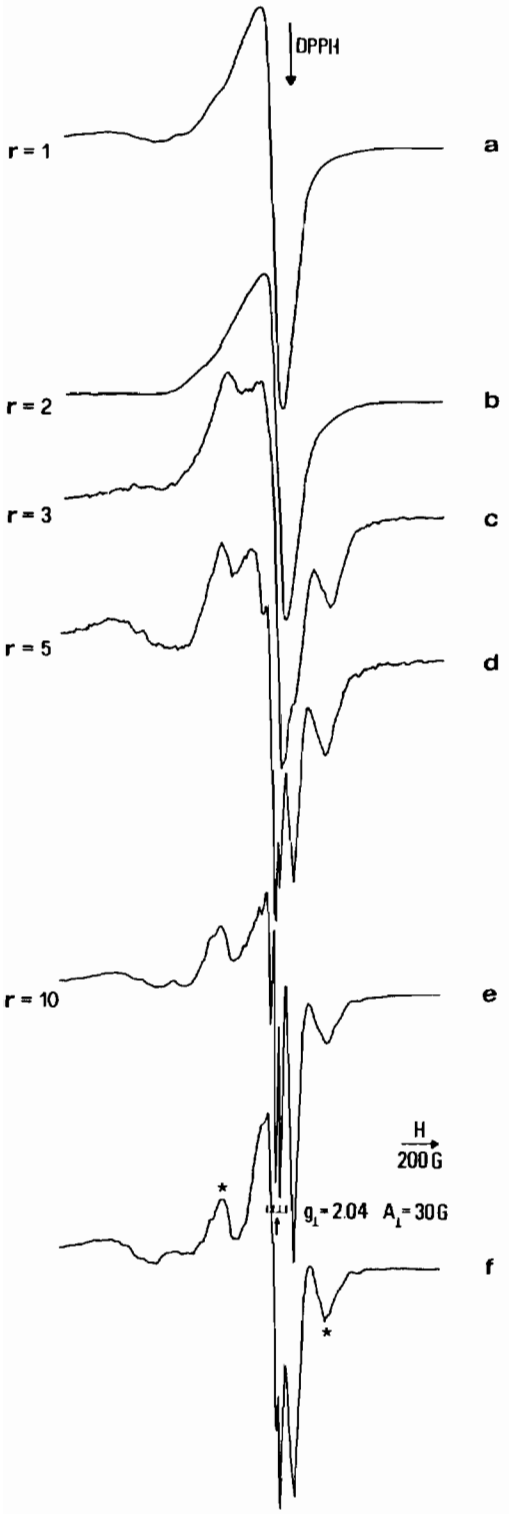

Fig. 2. ESR spectra of 10^{-3} M frozen (−150 °C) aqueous solution of $CuCl_2$/5-iminodaunomycin at different drug-to-copper ratios (r) and at pH = 6.5. The starred signals are attributed to triplet Cu(II) centers.

r = 3

Starting at this r value and at pH = 4, new resonance lines develop beside those observed at $r = 1$. The resonance fields are indicative of exchange interaction between two Cu(II) centers and may be rationalized in terms of a Cu(II) triplet spin-state. The closeness of the additional resonances to $g = 2$ suggests a small value (0.05–0.1 cm^{-1}) for the zero-field splitting parameter and is consistent with the lack of intensity for the $\Delta M_s = 2$ transition [19]. Resonance lines of the polymeric Cu(II) adduct found at $r = 1$ are also present. The anthracycline could in principle behave as both a bridging and a terminal ligand. We suggest that both types of behavior are active in the dinuclear Cu(II) derivative in which one daunomycin bridges two Cu ions which, in turn, chelate another daunomycin molecule. This property of stabilizing dinuclear derivatives is unique in the coordination chemistry of Cu(II) with anthracyclines, with the exception of aclacinomycin which, as suggested by Garnier-Suillerot [20], forms hydroxo-bridged Cu(II) complexes. In our case, such a type of coordination can be ruled out on the basis of the small exchange coupling we observed.

r = 5 to 10

The lines of the dinuclear derivative overlap those of the Cu(II) mononuclear complex, whose resonances are well defined in the perpendicular region of the spectrum. The mononuclear adduct has a spectrum reminiscent ($g_\perp = 2.04$; $A_\perp = 30$ G) of those of the Cu(II) complexes for which the anthracycline is 'stacked-up' [2]. This complex coexists with the dinuclear species. The relative amount of the mononuclear species is higher at $r = 10$ and decreases when dGMP is present in the system with the metal/anthracycline/nucleotide ratio being 1/10/10.

Conclusions

The results reported in this paper reveal some significant peculiarities of two anthracyclines (*i.e.* daunomycin and 5-iminodaunomycin) with respect to doxorubicin and 4′-epidoxorubicin, when the ESR spectral features of their Cu(II) complexes are compared. The spin–spin exchange interaction between Cu(II) centers, for high r values, is more easily removed in the case of daunomycin than in that of either doxorubicin or its epimer 4′-epidoxorubicin. Therefore the 'stacking-up' of the ligand, that results in the formation of mononuclear adducts from the polymeric complex, is a more efficient mechanism in the case of daunomycin.

The strongest exchange interaction is verified in the case of the 5-iminodaunomycin complex whose dinuclear form is found to be stable over a wide range of pH and r values. 5-Iminodaunomycin is outstand-

ing in this respect among all other anthracyclines studied.

No ternary interaction of the type nucleotide/Cu(II)/anthracycline has been observed, which is an indication of the higher affinity of Cu(II) ions for the dihydroxyanthraquinone ligand. Although, at present, no data are available on the activity of the complexes studied as compared to that of the free ligand, the spectral differences of the corresponding Cu(II) complexes of daunomycin and 5-iminodaunomycin point to a substantially different behavior. Similar differences have already been reported by us in our time-resolved fluorescence studies of the interaction of daunomycin and 5-iminodaunomycin with DNA and cardiolipin [21, 22].

Studies on the antitumor activity and cardiotoxicity of the complexes reported in this paper are in progress.

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