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Doxorubicin-3′-NH-oestrone-17-oxime-ethyl-carbonyl, a doxorubicin-oestrone conjugate that does not redox cycle in rat liver microsomes

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Doxorubicin (Adriamycin®) is a broad spectrum antitumour agent commonly used in the treatment of advanced cancers, either as a single agent or in combination chemotherapy. Unfortunately, a dose-limiting factor in the clinical use of doxorubicin is cardiotoxicity [1], an event that is suggested to be associated with doxorubicin free radical formation [2]. Various approaches have been adopted to decrease this cardiotoxicity through the use of vehicles and formulations predicted to minimize drug uptake and/or damage to heart tissue. These include the use of doxorubicin-entrapped cardiolipin liposomes [3], doxorubicin-*N*-(hydroxypropyl)methylacrylamide copolymers [4] and doxorubicin–iron chelates [5]. An alternative strategy for minimizing doxorubicin-mediated cardiotoxicity is to target this agent to specific tumours. Doxorubicin is one of the preferred treatments for progressive metastatic breast cancer, a tumour type that is sensitive to oestrogen therapy [6]. With this in mind, we have developed a doxorubicin-3′-NH-oestrone-17-oxime-ethyl-carbonyl (Dox-Oes; Fig. 1a) covalent adduct with the aim of targeting doxorubicin (Fig. 1b) to oestrogen receptor positive breast cancer cells. We report here the lack of free radical generation and redox cycling by this conjugate.

Materials and Methods

Drugs and chemicals. Dox-Oes was prepared by reacting the 3′-amino group of doxorubicin with the carboxyl group of 17-carboxyalkyloxime-oestrone [7]. Biochemicals and reagents were purchased from either the Sigma Chemical Co. (Poole, Dorset, U.K.) or Boehringer Mannheim (Lewes, Sussex, U.K.). Bio-Rad protein assay reagent was purchased from Bio-Rad laboratories (D-8000 München, F.R.G.).

Electron spin resonance (esr) studies. ESR studies were carried out using a Varian E109 X-band spectrophotometer at a microwave frequency of 9.5 GHz at ambient temperature. The 1-mL anaerobic incubation mixtures consisted of 250 mM drug, 4 mM NADPH and rat liver microsomal protein (typically 6 mg) prepared as described by [8] in 200 mM phosphate buffer (pH 7.4) containing 5% Tween 20.

NADPH utilization. This was done as described previously [9]. The reaction mixture at 37° consisted of 50 μM drug, 0.1 mM NADPH, 7.5 mM nicotinamide (to inhibit NADPH degradation), 90 μg microsomal protein and buffer (100 mM sodium phosphate/225 mM KCl, 5% Tween 20, pH 7.4). Basal rate NADPH oxidation was determined under identical conditions but without drug.

Superoxide anion formation. Superoxide anion formation was measured at 37° by two methods as previously described [9]. (i) Reduction of acetylated cytochrome *c*. The 1-mL incubation mixture consisted of 1 mM NADPH, 0.07 mM acetylated cytochrome *c*, 19 μg microsomal protein and 50 μM drug in buffer (50 mM Tris/150 mM KCl, 5% Tween 20, pH 7.4). (ii) Oxidation of adrenaline to adrenochrome. The 1-mL incubation mixture consisted of 1 mM NADPH, 2 mM adrenaline bitartrate, 70 μg microsomal protein and 50 μM drug all in buffer (50 mM Tris/150 mM KCl, 1 mM EDTA, 5% Tween 20, pH 7.4). Basal rate superoxide anion formation in liver microsomes was determined as described above without addition of drugs.

Results and Discussion

Table 1 shows that doxorubicin stimulated basal rate NADPH oxidation 3-fold and superoxide anion generation 2-fold in rat liver microsomes. This tissue fraction is a rich source of cytochrome P-450 reductase, an enzyme known to participate in the activation of doxorubicin. The results are consistent with a doxorubicin redox cycle that results in reactive oxygen generation at the expense of cellular reducing equivalents as has been previously described [2, 10]. Detection of metabolically generated doxorubicin free radicals in the absence of oxygen (Fig. 2a) further supports this since under these conditions NADPH reduces doxorubicin to a semiquinone (free radical) which is detectable by esr. The asymmetry of the doxorubicin spectrum is consistent with previous results [11] and is suggested to be a consequence of immobilization of the doxorubicin semiquinone generated in a microsomal environment. In contrast, Table 1 shows that Dox-Oes does not redox cycle, as indicated by no stimulation of basal rate NADPH oxidation or superoxide anion generation in the presence of this conjugate. In support of this, Dox-Oes does not generate an esr-detectable free radical intermediate (Fig. 2b). Furthermore, Dox-Oes actually inhibits doxorubicin free radical formation (Fig. 2c). The concentrations of doxorubicin, Dox-Oes and microsomal protein used for the esr studies was considerably greater than that used for measuring the redox activity of these compounds (see Materials and Methods). This was to ensure that a doxorub-

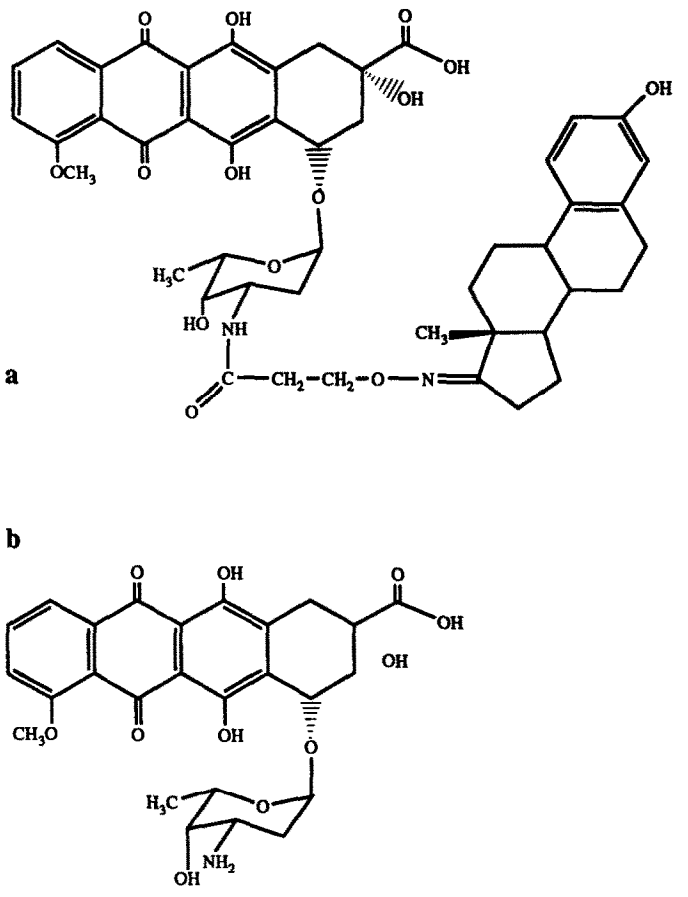

Fig. 1. Structure of doxorubicin-3′-NH-oestrone-17-oxime-ethyl-carbonyl (a), and doxorubicin (b).

icin-free radical could be detected in this system and also to ensure that the lack of an esr signal for Dox-Oes was not merely a consequence of the insensitivity of esr spectroscopy in the detection of free radicals.

It is possible that these results can be explained by an interaction of the steroid moiety of the Dox-Oes conjugate with flavin adenine dinucleotide (FAD) and/or flavin mononucleotide (FMN). These coenzymes are involved in electron transfer by cytochrome P-450 reductase and other flavoproteins [12]. Doxorubicin is known to bind to flavin components of microsomal reductases with high affinity [13] and hence an interaction of Dox-Oes with these sites might prevent electron transfer to doxorubicin. This would explain the inhibition of doxorubicin-free radical formation in the presence of Dox-Oes (Fig. 2). The interaction of the steroid component of Dox-Oes with the flavin sites would

Table 1. Effect of doxorubicin and doxorubicin–oestrogen conjugate on superoxide anion generation and NADPH oxidation by rat liver microsomes

Compound	Superoxide anion generation*		NADPH* oxidation
	Acetylated cytochrome *c* reduction	Adrenochrome formation	
None	19.5 ± 3.3	30.2 ± 1.8	10.7 ± 1.5
Doxorubicin†	42.3 ± 0.5‡	65.7 ± 2.0‡	33.8 ± 1.2
Dox-Oes†	19.5 ± 3.3	38.5 ± 2.0	10.7 ± 1.5

Results are expressed as mean ± S.D. of at least three determinations.
* nmol/min/mg microsomal protein.
† Concentration: 50 μM.
‡ Inhibited by superoxide dismutase (320 μg/mL).

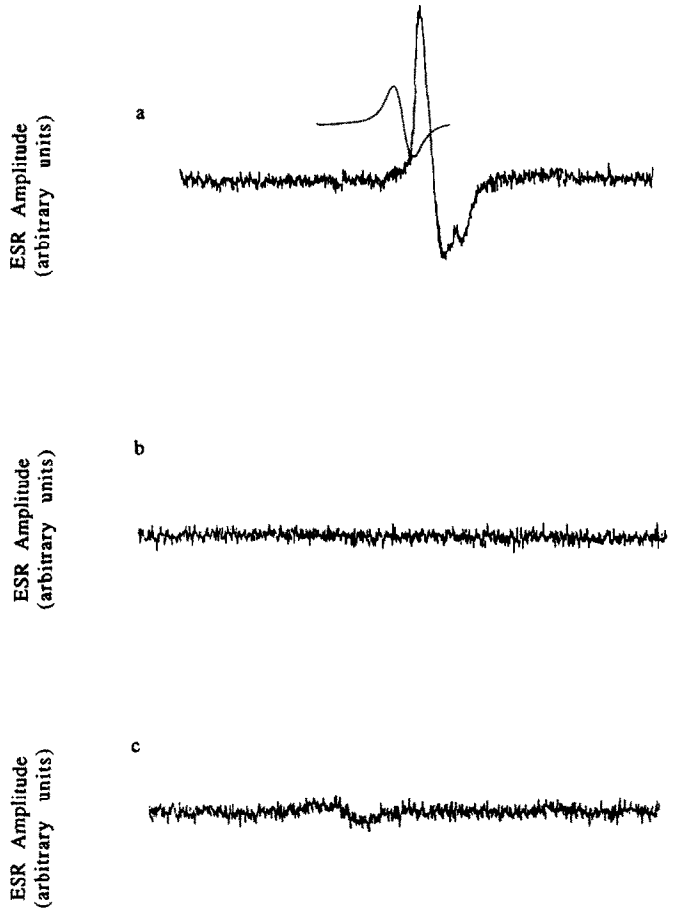

Fig. 2. Electron spin resonance spectroscopy of (a) doxorubicin (250 mM) g = 2.0032, and the peak-to-peak line-width is 0.2 G, (b) doxorubicin–oestrone conjugate (250 mM), (c) doxorubicin (250 mM) + doxorubicin–oestrone conjugate (250 mM) when incubated at 22–23° with NADPH supplemented rat liver microsomes in the absence of air. Esr operating conditions: field-set 3550 G, microwave frequency 9.5 GHz, and scan range of 50 G, using a time constant of 0.4 sec. The small spectrum in (a) is that of the calibration standard dipicrylphenylhydrazyl free radical.

also explain the inhibition of electron transfer to and hence lack of free radical formation by the covalently linked doxorubicin moiety. It would appear that this proposed steroid interaction does not interfere with electron transfer by the flavoprotein since no inhibition of basal rate NADPH oxidation or superoxide generation by Dox-Oes is evident (see Table 1). We have found that oestradiol itself does not inhibit doxorubicin-free radical generation (results not shown) and thus conclude that it is the covalent aminoglycosidic Dox-Oes adduct that causes an inhibition of doxorubicin free radical formation.

In summary doxorubicin-3′-NH-oestrone-17-oximeethyl-carbonyl (Dox-Oes) is a covalent adduct of the anthracycline antitumor agent doxorubicin and oestrogen. Dox-Oes does not generate free radicals in rat liver microsomes as detected by electron spin resonance spectroscopy or redox cycle as shown by lack of superoxide anion formation and NADPH oxidation. Furthermore Dox-Oes actually inhibits free radical formation by doxorubicin used in equimolar amounts. The lack of free radical formation by doxorubicin when covalently linked to oestrone supports the development of Dox-Oes as a non-cardiotoxic derivative whilst potentially improving its targeting to oestrogen positive breast tumour cells.

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