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Cytofluorescence Localization and Disposition of Doxorubicin and Doxorubicinol in Rat Cardiac Tissue

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Abstract—*The localization of cardiac cytofluorescence and tissue levels of doxorubicin (DXR) and doxorubicinol (DXR-ol) were studied in rats treated with a single* (9 mg/kg: 1-*day study*) *or* 3 *weekly doses* (3 mg/kg: 7-*week study*) *of the compounds. A striking orange–red fluorescence was observed in cardiac cell nuclei from DXR* 1-*day rats, whilst heart cells from DXR-ol* 1-*day rats displayed a faint, diffuse fluorescence. Neither cardiac tissue from DXR nor from DXR-ol* 7-*week animals showed any drug-specific fluorescence. HPLC assay showed that in DXR* 1-*day rats the drug was concentrated in the heart, which also contained the endogenously produced DXR-ol. Plasma levels of DXR-ol were initially high in DXR-ol* 1-*day rats but rapidly decreased with time; cardiac levels of DXR-ol remained low. Hearts from DXR* 7-*week rats contained appreciable amounts of DXR and DXR-ol, while very low levels of DXR-ol were found in DXR-ol* 7-*week animals. The data correlated well with the ECG alterations recorded during the study, which were more severe in DXR- than in DXR-ol-treated rats. These results indicate that the lower tissue uptake of exogenously administered DXR-ol might explain its lower toxic cardiac potential compared with DXR.*

INTRODUCTION

PHARMACOKINETIC studies in humans have demonstrated that among the polar metabolites of doxorubicin, the C_{13}-OH derivative doxorubicinol is the most prominent [1]. Greene *et al.* [2] reported that the plasma AUC of doxorubicinol was approximately one-half of the value of doxorubicin in humans with normal liver and renal function, while Chlebowski *et al.* [3] observed doxorubicinol plasma concentrations higher than doxorubicin ones in patients with liver dysfunction. In spite of its large metabolic production, the metabolite has not yet been characterized from a toxicological point of view. In an attempt to evaluate the possible contribution of doxorubicinol to the cardiac toxic effects of doxorubicin, Del Tacca *et al.* [4] found increasing levels of the metabolite in hearts from rats treated with repeated doses of doxorubicin, the increase in doxorubicinol concentration being concomitant with the development of cardiotoxicity. Recently, Danesi *et al.* [5] took this observation one stage further, showing that repeated administration of synthetic doxorubicinol was associated with the development of a doxorubicin-like myocardiopathy, which was characterized by a lesser degree of severity of both ECG and histological alterations. It remained to be demonstrated, however, whether this toxicological profile depends on a different distribution of the exogenously administered metabolite into cardiac tissue, or on a lower toxicity of doxorubicinol compared with doxorubicin.

The aim of the present work was thus to study the subcellular localization and the cardiac levels of both doxorubicin and doxorubicinol after single or repeated treatment in rats; the resulting values were then correlated with the degree of cardiac toxicity. The study of anthracycline localization within cell compartments was performed by fluorescence microscopy [6–8]. The accurate measurement of anthracycline levels in plasma and tissues was achieved by a specific HPLC method [9], while the cardio-toxicological monitoring was carried out by the measurement of the SαT segment of rat ECG [10].

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MATERIALS AND METHODS

Female Sprague–Dawley rats weighing 150 ± 10 (S.D.) g were used in this study. They were housed in groups of three in each cage at an environmental temperature of 22–24°C, with a relative humidity of 55–65%, and a 12 h light–dark cycle was maintained. They were fed laboratory chow (Altromin® pellets, Rieper, Bolzano, Italy) and received tap water *ad libitum*. The animals were observed for 10 days prior to drug administration and baseline ECGs were recorded to document normal basal cardiac activity.

Drugs and treatment schedule

Doxorubicin–HCl and synthetic doxorubicinol–HCl were provided by courtesy of Fammitalia-Carlo Erba S.p.A. (Milano, Italy). The drugs were dissolved in an appropriate volume of distilled water in order to achieve the desired drug concentration (2 mg/ml) and the solutions were freshly made prior to each administration. Doxorubicin and doxorubicinol were injected into the caudal vein in varying amounts depending on animal body weight. Rats were divided into four groups: the doxorubicin 1-day group, injected once with 9 mg/kg of doxorubicin, and the doxorubicin 7-week group, injected once a week for 3 consecutive weeks with 3 mg/kg of doxorubicin and observed for a further period of 4 weeks; the doxorubicinol 1-day and 7-week groups were treated with the synthetic metabolite at the same treatment schedule as for doxorubicin. 1-Day rats were killed by cervical dislocation 15, 30, 45, 60, 120, 180, 240 and 360 min after drug injection and heart and blood samples were collected; the ECG was recorded just before sacrifice in waking animals placed in a supine position. 7-Week rats were subjected to a weekly ECG recording, 24 h prior to drug administration during the treatment period, and on the same day of the week during the post-treatment period. At the end of the study, the animals were killed by cervical dislocation and heart and blood samples were taken. ECG monitoring and analysis were performed following a method published elsewhere [10]; the heart rate, the QRS complex and SαT segment duration and the T-wave voltage were measured. Arrhythmias, which occurred during the study, were classified as reported by Zbinden [11].

Histofluorescence of rat hearts

Hearts were dissected out 60 min after dosing in 1-day rats and 4 weeks after the last dose of doxorubicin or doxorubicinol in 7-week rats; sample processing was performed as previously reported [7]. Briefly, tissue specimens were frozen in liquid nitrogen-cooled propane, freeze-dried and exposed to formaldehyde vapour (80°C for 1 h), embedded in paraffin wax, sectioned and finally mounted for fluorescence microscopy. Tissue sections were examined with an Ortholux Leitz fluorescence microscope, equipped with an Osram HBO 200 W super pressure mercury lamp light source, a Leitz BG 12 excitation filter and a Leitz K530 barrier filter. The objectives used were Leitz 10× and 25×. Photographs were taken with a Leitz Orthostat and Kodak Ektachrome 400 colour film.

Drug assay in plasma and cardiac tissue

Blood samples (4 ml) were centrifuged after sampling. Both the separated plasma and the heart samples were kept frozen at −20°C in light-protected silanized tubes until analysis. Doxorubicin and doxorubicinol assays were performed by a sensitive HPLC method [9]. Briefly, 1 μg/ml of daunorubicin hydrochloride as the internal standard, 1 ml of phosphate buffer (pH 8.0) and 10 ml of chloroform–heptanol (9:1) were added to plasma samples (1 ml). After centrifugation the organic layer was collected and extracted with 0.5 ml of 0.3 M phosphoric acid. Cardiac tissue samples (1 g) were micronized at −140°C for 2 min; a quantity 8 times their weight of distilled water was added, together with daunorubicin hydrochloride (1 μg/ml) as the internal standard, and then they were processed as in the case of the plasma. Chromatographic analysis was performed with a Varian model 5000 liquid chromatograph equipped with a Perkin–Elmer 650-10 LC fluorescence detector (excitation wavelength: 470 nm; emission 580 nm) and a Supelcosil LC-CN chromatographic column (25 cm × 4.6 mm internal diameter). The sensitivity of the method employed made it possible to detect low drug levels (0.2 ng/ml for plasma; 1 ng/g for tissue). The response was linear in the experimentally observed range of concentrations and the coefficient of variation in repeated analysis was 11% for drug levels <3 ng/ml and 8% for drug levels ≥3 ng/ml.

Statistical analysis

Values presented are means ± S.E. of n observations. Statistical significance was assessed using Student's t-test for paired or unpaired observations. A P value of less than 0.05 was considered significant.

RESULTS

Cytofluorescence of rat hearts

Cardiac tissue sections from doxorubicin 1-day rats fluoresced a striking orange–red (Fig. 1). The greatest drug-specific fluorescence intensity was localized in the nucleus of the cells; it filled also the myocardial cell cytoplasm giving a faint red colour (Fig. 1). This kind of nuclear pattern was not observed in heart slices from doxorubicinol 1-day

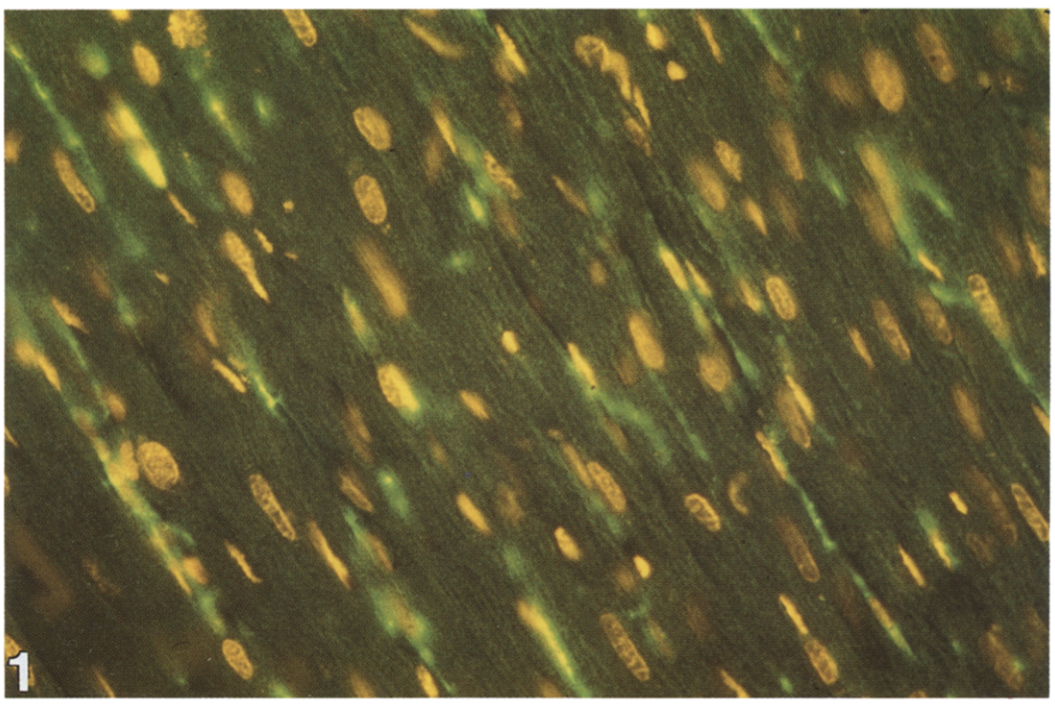

Fig. 1. Longitudinal section of ventricle from a doxorubicin 1-day rat. The nucleus of the myocardial cells appear orange–red coloured while noradrenergic fibres are bright yellow–green in fluorescence. (× 450)

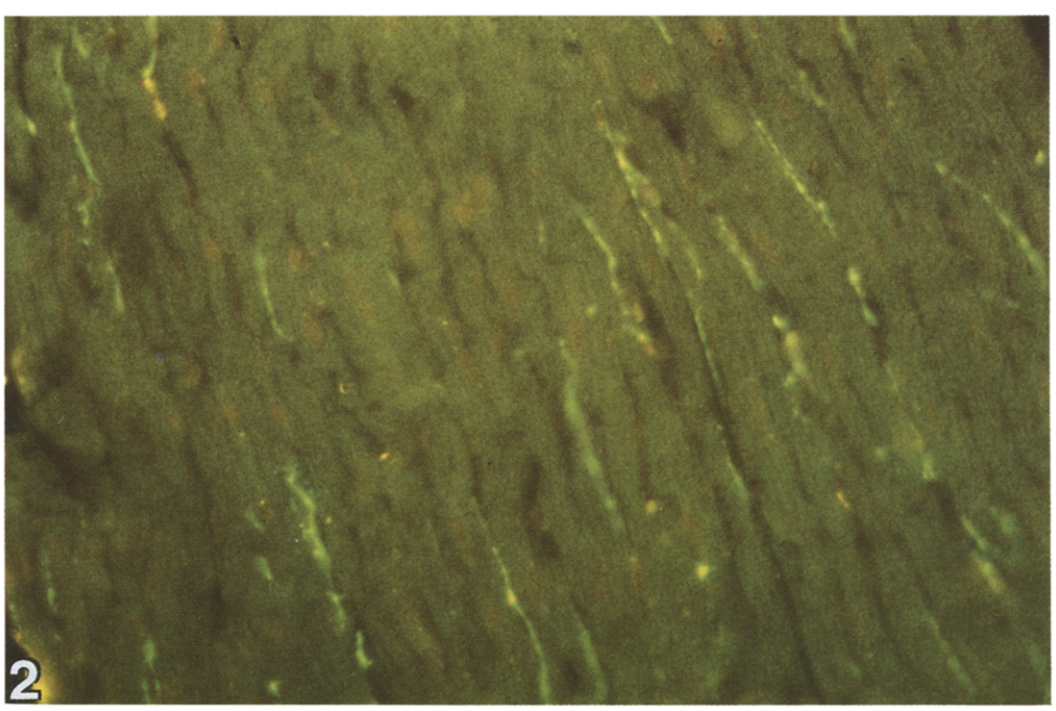

Fig. 2. Longitudinal section of ventricle from a doxorubicinol 1-day rat. Both the nucleus and the cytoplasm of the myocardial cells are faintly orange–red fluorescent. Noradrenergic yellow–green-fluorescing fibres are evident along myocardial cell bundles. (× 450)

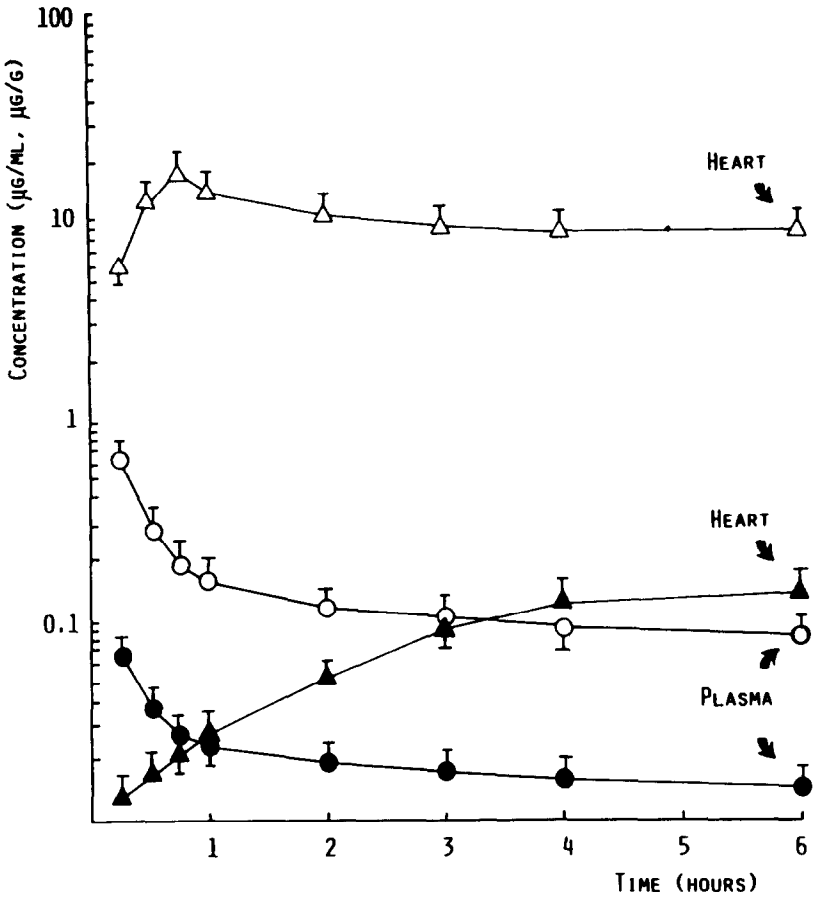

Fig. 3. Doxorubicin (open symbols) and doxorubicinol (closed symbols) concentrations in plasma and heart of doxorubicin 1-day rats after the administration of a single 9 mg/kg *i.v. dose. Each point represents the mean of eight separate determinations ± S.E. (vertical bars).*

rats. In this case a faint red fluorescence was observed in the nucleus and cytoplasm of myocardial cells (Fig. 2). Noradrenergic axons appeared as yellow–green fluorescent fibres and were observed among myocells and surrounding cardiac vessels (Figs. 1 and 2). No appreciable drug-specific fluorescence was observed in cardiac tissue sections from either doxorubicin or doxorubicinol 7-week groups.

Doxorubicin and doxorubicinol levels in plasma and cardiac tissue

Plasma and cardiac levels of the anthracyclines in doxorubicin and doxorubicinol 1-day rats are shown in Figs. 3 and 4 respectively. Compared with that observed for doxorubicin 1-day rats (Fig. 3), doxorubicinol plasma levels were higher, but heart concentrations remained low in doxorubicinol 1-day rats (Fig. 4), indicating a reduced tissue uptake of the polar metabolite. Doxorubicin and doxorubicinol were not detected in the plasma of either doxorubicin or doxorubicinol 7-week rats. Appreciable amounts of doxorubicin and its metabolite were measured in the heart of doxorubicin 7-week animals (Fig. 5), indicating the cardiac metabolism of the parent drug. Very low cardiac levels of doxorubicinol were measured in doxorubicinol 7-week rats (Fig. 5). Doxorubicin was not detected in either plasma or heart after the administration of doxorubicinol, indicating that the metabolite is not re-converted into the parent drug.

ECG findings

Table 1 shows the ECG parameters measured in doxorubicin and doxorubicinol 1-day rats over a period of 360 min after dosing. The only alteration observed in both groups was a significant widening of the SαT segment, which occurred at the 60th min post-dose and completely reversed to predose values in doxorubicinol-treated rats. ECG findings from doxorubicin and doxorubicinol 7-week rats are presented in Table 2. The major ECG sign of cardiotoxicity was the SαT segment enlargement, which was more pronounced in doxorubicin 7-week rats, indicating more severe cardiac damage than that induced by doxorubicinol treatment. A significant reduction in the heart rate was observed only in doxorubicinol-treated rats (Table 2). Cardiac arrhythmias occurred in doxorubicin and doxorubicinol 7-week rats, but the incidence was remarkably higher in the latter group (Table 3).

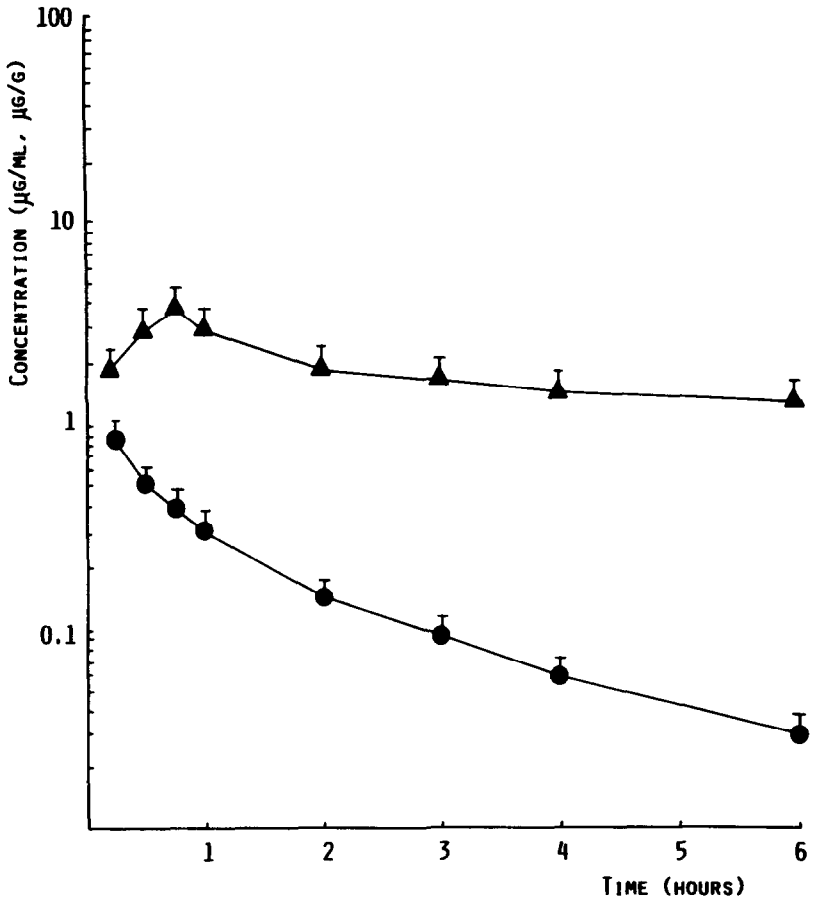

Fig. 4. Doxorubicinol concentrations in plasma (●) and heart (▲) of doxorubicinol 1-day rats after the administration of 9 mg/kg *i.v. single dose. Each point represents the mean of eight separate determinations ± S.E. (vertical bars).*

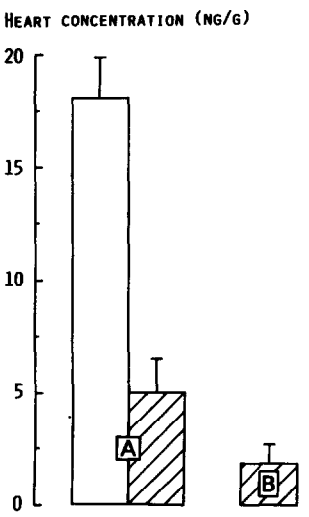

Fig. 5. Doxorubicin (open bars) and doxorubicinol (striped bars) levels in cardiac tissue of doxorubicin (A) and doxorubicinol (B) 7-week rats 4 weeks after the last of three weekly doses of either drug. Each value represents the mean of 12–14 *observations ± S.E. (vertical bars).*

DISCUSSION

The fluorescence of anthracycline antibiotics is one of the most striking physico-chemical features of this class of antineoplastic agents. This property is currently utilized for sensitive assays of anthracyclines in tissues and biological fluids [12, 13]. Previous reports have employed the characteristic orange–red fluorescence of doxorubicin and daunorubicin to investigate the localization of these compounds in tissues; cytofluorescence studies have shown them to be restricted to the cell nucleus of various tissues [6–8, 14] including heart tissue [7]. In the present study, cytofluorescence microscopy showed that doxorubicin localizes almost entirely in the nucleus of heart cells; the cardiac tissue concentration of doxorubicin, as measured by the HPLC technique, correlates well with the intensity of fluorescence localized at the nucleus. It appears that the nucleus of the cell can influence both the intracellular and the tissue distribution of the drug. One possible explanation of this phenomenon is that doxorubicin possesses a high affinity for binding to nucleic acids either by intercalation with the double helix or through covalent or ionic linkages [15], becoming physically immobilized within the nucleus [16]. The kinetic profile of doxorubicin in plasma is characterized by a long terminal phase half-life [17] of the same order as the slow efflux of the drug from the cells, suggesting that its elimination may also be determined by a slow release of the nuclear-bound drug from the tissues. Our findings demonstrate also a

Table 1. *ECG parameters measured in doxorubicin (DXR) and doxorubicinol (DXR-ol) 1-day rats*†*

Time (min)	0	60	120	180	240	360
DXR						
QRS	13.6 ± 2.4‡	13.5 ± 1.8	13.0 ± 2.2	13.6 ± 3.1	13.5 ± 2.1	13.7 ± 2.0
SαT	18.0 ± 1.8	19.9 ± 0.9§	19.2 ± 1.0§	19.1 ± 1.2§	19.2 ± 2.1§	18.9 ± 1.1§
T	225.9 ± 16.7	230.2 ± 18.0	231.1 ± 17.9	235.5 ± 19.9	229.7 ± 18.8	227.5 ± 14.5
HR	402.8 ± 27.5	408.1 ± 30.2	409.2 ± 31.1	412.3 ± 30.2	410.7 ± 24.9	416.6 ± 23.3
DXR-ol						
QRS	13.2 ± 2.9	13.4 ± 2.6	13.1 ± 3.4	13.7 ± 3.1	13.7 ± 2.0	13.6 ± 1.9
SαT	17.5 ± 2.2	18.8 ± 1.7§	18.1 ± 2.2	18.0 ± 2.3	18.1 ± 1.9	17.9 ± 1.4
T	229.9 ± 24.1	230.5 ± 20.2	231.1 ± 22.2	237.4 ± 21.9	226.7 ± 23.8	239.6 ± 25.5
HR	389.5 ± 37.1	395.5 ± 38.2	399.3 ± 37.5	398.8 ± 32.5	402.3 ± 25.5	391.2 ± 28.9

*Rats were injected with DXR or DXR-ol 9.0 mg/kg as a single i.v. dose; time 0 indicates pre-treatment values.
†QRS and SαT: ms; T (T-wave): μV; HR (heart rate): beats/min.
‡Values are the mean ± S.E. of eight observations.
§Student's *t*-test for unpaired data: $P < 0.05$ vs. basal values (time 0).

Table 2. *ECG parameters measured in doxorubicin (DXR) and doxorubicinol (DXR-ol) 7-week rats*†*

Time (weeks)	0	1	3	5	7
DXR					
QRS	14.0 ± 0.5‡	14.8 ± 0.7	17.5 ± 0.9§	14.9 ± 1.2	14.8 ± 0.9
SαT	17.9 ± 0.9	22.4 ± 1.0§	27.3 ± 1.2§	32.1 ± 1.8§	35.4 ± 1.9§
T	245.0 ± 9.9	252.9 ± 12.0	220.4 ± 10.1§	209.2 ± 7.6§	203.2 ± 9.8§
HR	460.2 ± 15.4	450.9 ± 18.2	448.8 ± 16.5	440.2 ± 19.2	452.2 ± 18.1
DXR-ol					
QRS	13.8 ± 0.9	14.2 ± 1.0	15.3 ± 0.8	15.1 ± 1.2	14.3 ± 0.8
SαT	18.3 ± 1.1	19.2 ± 1.1	21.1 ± 0.9§	23.3 ± 1.2§	26.3 ± 1.1§
T	235.4 ± 12.6	250.1 ± 19.7	236.4 ± 16.5	219.4 ± 12.3§	215.2 ± 10.1§
HR	441.1 ± 13.8	444.9 ± 10.0	450.1 ± 10.6	425.3 ± 10.2§	409.9 ± 9.9§

*Rats were injected with DXR or DXR-ol 3.0 mg/kg once a week for 3 consecutive weeks and observed for a further period of 4 weeks; time 0 indicates pre-treatment values.
†QRS and SαT: ms; T (T-wave): μV; HR (heart rate): beats/min.
‡Values are the mean ± S.E. of 12–14 observations.
§Student's *t*-test for paired data: $P < 0.05$ vs. basal values (time 0).

Table 3. *Types and incidence of arrhythmias in doxorubicin (DXR) and doxorubicinol (DXR-ol) 7-week rats**

Drug	Types of arrhythmias	Incidence†
DXR	Atrial fibrillation	21.4%
	Arial flutter	14.3%
	Nodal rhythm	21.4%
DXR-ol	Atrial flutter	42.8%
	Nodal rhythm	42.8%
	Atrioventricular block (type II)	57.1%

*Rats were treated as reported in Table 2.
†No. of survivors with arrhythmias/no. of survivors at the end of the study × 100.

good correlation between the faint red fluorescence of hearts from doxorubicinol 1-day rats, and the low cardiac tissue concentration of the synthetic metabolite, indicating a reduced tissue uptake of doxorubicinol. The metabolite appears to be confined to the plasma compartment, from which it is rapidly cleared, in agreement with the findings of other authors [17, 18]. The cardiac cytofluorescence and tissue concentrations found in the present study confirm the observations of Del Tacca *et al.*

[4] and Danesi *et al.* [5], who suggested that the kinetic behaviour of doxorubicinol is due to the presence of the OH-group on the C-13 carbonyl, making the molecule more polar than doxorubicin. Fluorescence microscopy failed to detect any anthracycline-specific fluorescence associated with cardiac tissue sections from either doxorubicin or doxorubicinol 7-week rats. These findings are not surprising in view of the very low level of both drugs, which were detectable only by HPLC assay. The difference between the cardiac tissue levels of doxorubicin and doxorubicinol after single or repeated treatment correlates well with the different severity of drug-induced cardiac tissue damage revealed by ECG alterations. The quench of doxorubicin to cell nuclei, demonstrated by cytofluorescence is characteristic of this compound, whose cardiotoxicity might be a direct consequence of the interaction with DNA [19]. On the contrary, the reduced cardiac alterations induced by doxorubicinol in both groups of rats, appear to depend on the very low cardiac tissue levels reached after treatment. This fact is supported by the observation that the toxic side-effects of anthracyclines depend to a large extent on the concentration reached in tissues [20]. *In vitro* studies have shown that both doxorubicin and doxorubicinol stimulate superoxide anion formation in cardiac sarcosomes and by mitochondrial NADH dehydrogenase [21], and induce lipid peroxidation of cell membranes (Gervasi, personal communication) in a very similar manner. The role of oxygen radical formation and oxidative stress as well as lipid peroxidation in doxorubicin-induced cardiomyopathy has been clearly established [13, 22, 23]. From this point of view, the lower cardiotoxic potential of doxorubicinol found in the present study appears to be associated with its very low tissue uptake. ECG monitoring revealed a high incidence of arrhythmias and a significant reduction in the heart rate in doxorubicinol 7-week rats. Although the pathogenesis of doxorubicinol-induced arrhythmias has not been examined in the present work, we suggest that arrhythmias might depend on the drug's polarity, leading to the preferential disposition of the exogenously administered metabolite on cell membranes. This view is supported by the following considerations: firstly, biochemical studies [24, 25] have demonstrated that the cell interaction of polar anthracyclines is characterized by drug absorption by surface structures (most probably phospholipids of cell membranes). The following phase of membrane permeation and intracellular penetration is very limited, as also demonstrated by cytofluorescence and HPLC assays in the present study. Secondly, *in vitro* studies have shown that doxorubicinol stimulates superoxide anion production by cardiac sarcosomes [21], and oxyradical-mediated membrane lipid peroxidation in rat hearts (Gervasi, personal communication), phenomena which are both implicated in cell membrane damage. Doxorubicinol-induced arrhythmias were recorded only in doxorubicinol 7-week rats and occurred at least 3 weeks after the start of treatment; this indicates that a repeated cell impact of the exogenously administered metabolite is necessary to induce a critical alteration responsible for the following onset of arrhythmias. Whatever the mechanism may be, its importance is evident, since arrhythmias occurring during doxorubicin treatment are potentially life-threatening.

Finally, in the light of the data reported in the present study, it appears that C-13 hydroxylation does not represent, at least in the case of doxorubicin, a metabolic route leading to less toxic compounds. Our data underline the necessity of a toxicological characterization of anthracycline metabolites, particularly C-13 hydroxylated ones.

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