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Exogenous Doxorubicinol Induces Cardiotoxic Effects in Rats

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Abstract—*An investigation was performed in the rat to assess the cardiotoxic effects of exogenous doxorubicinol compared with those induced by an equimolar dose of its parent drug doxorubicin. Rats received synthetic doxorubicinol or doxorubicin* 3 mg/kg *i.v. weekly for* 3 *weeks and were observed for a further period of* 4 *weeks. Survival, body growth, ECG parameters, and heart histopathology were studied. Doxorubicin markedly affected rat body growth, as well as several ECG parameters such as SαT, RαT, αTP and T-wave. Typical cardiac histological alterations were also induced by doxorubicin. In a similar way, doxorubicinol treatment was associated with a significant inhibition of rat body weight increase, and the appearance of ECG alterations as well as both macro- and microscopic signs of cardiac tissue damage. However these effects were delayed in time and their severity was lower compared with doxorubicin. Overall results indicate that doxorubicinol induces a doxorubicin-like toxic syndrome mainly affecting the heart, although to a lower degree of severity than that caused by the parent drug. It is suggested that the lower toxic potential displayed by doxorubicinol might be due at least in part to its greater polarity and a consequently lower cardiac tissue uptake compared with doxorubicin.*

INTRODUCTION

THE ANTHRACYCLINE antibiotic doxorubicin is one of the most important antitumour agents, and exhibits therapeutic activity against a variety of human neoplasms. In view of the established clinical usefulness of the drug, many studies have been carried out to clarify the mechanisms of its cardiac toxicity. One possible approach to the mechanisms of the pathogenesis of cardiomyopathy induced by doxorubicin and related anthracyclines, is the study of the drug's metabolism in cardiac tissue and the pharmacotoxicological properties of its metabolites [1, 2]. It has been demonstrated that daunorubicin aglycones possess cardiotoxic properties on isolated perfused dog heart [2], and that the cellular metabolic production of daunorubicinol may contribute to the cytotoxicity of daunorubicin [3]. A direct relationship has been suggested between the degree of myocardial toxicity of different anthracyclines and the type and amount of metabolites produced and stored in cardiac tissue itself, especially the C-13 hydroxylated metabolites such as doxorubicinol [1] whose importance cannot be disregarded, since it is the main metabolite found in human plasma after doxorubicin administration [4]. The demonstration of increasing levels of doxorubicinol in heart tissue following repeated doxorubicin doses and the contemporary appearance and progression of cardiotoxicity [5], provided new suggestions about its possible relationship with doxorubicin-induced cardiomyopathy. Recently it has been reported that synthetic doxorubicinol is able to induce the production, both from cardiac sarcosomes and by mitochondrial NADH dehydrogenase, of superoxide anions [6] which appear to be involved in the mechanism of cell damage induced by anthracyclines [7].

Considering the experimental evidence, the present study was undertaken to investigate general and cardiac toxicity of synthetic doxorubicinol after repeated treatment in rats.

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MATERIALS AND METHODS

Animals

Forty-five Sprague–Dawley female rats with a starting weight of 120–150 g were used. They were

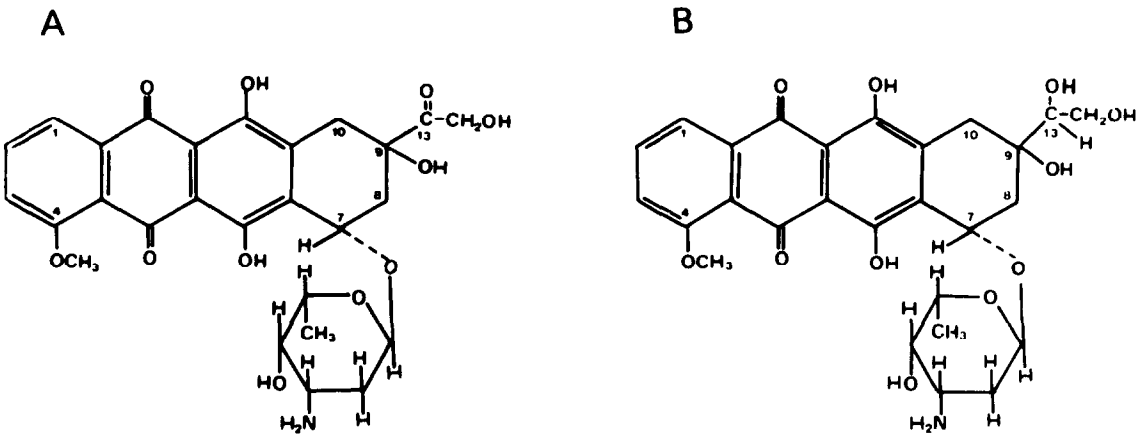

Fig. 1. Chemical structures of doxorubicin (A) and doxorubicinol (B).

housed in groups of 3 in cages at an environmental temperature of 22–24°C, and a relative humidity of 55–65%, and a 12 hr light–dark cycle was maintained. They were fed laboratory chow once daily 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. A weekly recording of body weight was performed during the time of the study (7 weeks); changes in body weight were expressed as Δ weight % which was calculated from the formula:

$$[\text{weight (time } t) - \text{weight (time 0)}] \times 100/\text{weight (time 0)}.$$

Drugs and treatment schedule

Doxorubicin hydrochloride and synthetic doxorubicinol hydrochloride (Fig. 1) were provided by courtesy of Farmitalia—Carlo Erba (Milan, Italy). The drugs were dissolved in an appropriate volume of distilled water to achieve the desired drug concentration (2 mg/ml) and the fluid volume was administered as an i.v. bolus in the caudal vein; drug solutions were freshly made prior to each treatment. Rats were randomly divided into 3 groups of 15 rats each: the first group was control and received isotonic saline i.v.; the second group received doxorubicin 3 mg/kg i.v. once a week for 3 weeks and the third group received doxorubicinol at the same treatment schedule. At the end of the study animals were sacrificed by cervical dislocation and hearts were quickly removed for histological examination.

ECG recording and analysis

ECGs were recorded weekly 24 hr prior to each administration during the treatment period and on the same day of the week during the post-treatment period. Under light ether anaesthesia, needle electrodes were inserted under the skin for the limb lead at position II [8] and ECG recordings were performed in a prone position. To facilitate the accurate measurement of various ECG parameters, a computerized on-line evaluation system was used [9]. ECG monitoring was performed by using a Battaglia Rangoni model ESO 600 polygraph; ECG data, after pre-amplification, were printed out by a Battaglia Rangoni KO 380 analogic recorder and sent to an analog–digital converter (Progetec ADC 8B 100M) directly connected to an Apple IIe Computer, which allowed the recording and the subsequent analysis of ECG signals. This system was used to measure the cardiac frequency (beats/min), the QRS complex, the PR, RαT, and αTP intervals, the SαT segment (msec), and the R- and T-wave voltages (μV) [9].

Histological analysis of cardiac tissue

Histological examination of the myocardium was carried out on preparations from frontal sections of the whole heart, including left and right ventricles, septum and atria. The samples were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.3, processed for paraffin embedding and stained with hematoxylin and eosin. Myocardial lesions were evaluated according to their severity and extension; the product of the severity and the extension gave the total cardiotoxicity score for each animal, from which the mean total score (MTS) for each animal group was calculated [10].

Statistical analysis

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

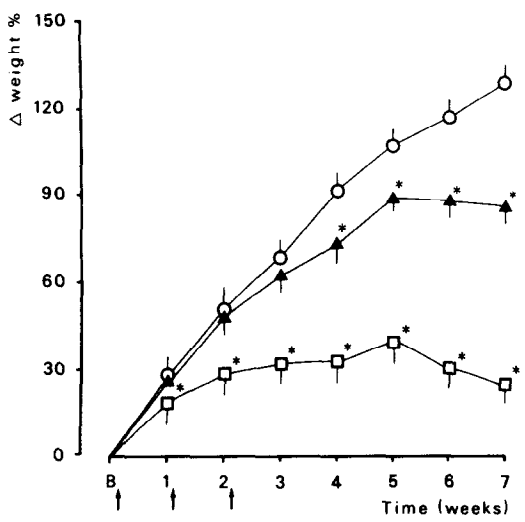

Fig. 2. Percentage change of rats' body weight during repeated treatment with doxorubicin (□) 3 mg/kg *i.v. once a week for* 3 *weeks or doxorubicinol* (▲) *at the same treatment schedule; control rats:* ○. *Arrows indicate drug administration. Each value represents the mean of* 13–15 *observations* ± *S.E. (vertical bars). Student's t-test for unpaired observations:* *$P < 0.05$ *vs. controls at the same time.*

RESULTS

General toxicity and weight changes

During the fourth and fifth weeks of the experiment, 2 animals treated with doxorubicin and 1 treated with doxorubicinol died. The body weight changes of animals from each experimental group are shown in Fig. 2. The body growth of animals receiving doxorubicin was severely affected; the increase observed between the start of treatment and the fifth week was followed by a clear drop during the following 2 weeks and the body weight recorded at the end of treatment was significantly lower than controls. The body growth of animals treated with doxorubicinol was significantly lower than that of controls from the fourth week on, although it was significantly higher than that of doxorubicin-treated animals from the second week on. The body growth-curve of doxorubicinol-treated animals exhibited a trend similar to that of doxorubicin-treated rats, with an increase between the start of treatment and the fifth week, followed by a slight drop during the following 2 weeks (Fig. 2).

Gross anatomical changes

The autopsy of doxorubicin-treated animals showed ascites and pleural effusions in 5 rats (including 2 rats that died during the study); pale, flabby and hypotrophic hearts were observed in all treated animals, but cardiac enlargement was not apparent. The kidneys and the liver from the majority of doxorubicin-treated animals appeared to be edematous and pale. Haemorrhagic lesions in the small bowel were observed in 5 rats, including 2 rats that died during the experiment. In doxorubicinol-treated rats, ascites and pleural effusions were noted in 2 rats (including 1 rat that died during the experiment). Almost all the hearts of doxorubicinol-treated animals appeared to be hypotrophic and flabby, but to a lesser degree compared with doxorubicin-treated animals; cardiac enlargement was not apparent. The kidneys and the liver from the majority of doxorubicinol-treated animals appeared to be slightly edematous. Haemorrhagic lesions in the small bowel were observed in 2 rats, including 1 rat that died during the study. No gross anatomical alterations were observed in control rats. Diffuse hair loss, especially involving the neck, was observed in almost all doxorubicin-treated animals; in doxorubicinol-treated ones, a lesser degree of hair loss was observed compared with doxorubicin-treated rats.

ECG analysis

During the experiment, the ECGs of saline-treated rats remained unchanged (Table 1). In doxorubicin-treated rats, no changes were observed in heart rate, PR interval or R-wave amplitude. The earliest and most substantial change observed during the experiment was a progressive, irreversible widening of the SαT segment; this effect became significant during the 1st week of treatment and continued to increase throughout the experiment. SαT enlargement was accompanied by a consequent reduction in the αTP interval, significant at the 2nd week; this reduction became more pronounced as time passed (Table 1). A slowly developing flattening of the T-wave was also observed; this effect became significant at the 2nd week and was found to increase slightly throughout the experiment. A significant QRS complex widening occurred at the 2nd week, with a further increase during the following week (Table 1). From this point on, it decreased progressively and reached pre-drug levels at the 5th week. The widening of the RαT became significant at the 2nd week, and subsequently increased throughout the remaining time of the study (Table 1). In doxorubicinol-treated rats, no alterations occurred in the PR interval or R-wave. The evaluation of ECGs from rats receiving doxorubicinol disclosed a progressive, irreversible widening of the SαT segment, which became significant 2 weeks later than in doxorubicin-treated animals. SαT enlargement was associated with a reduction in the αTP interval, significant at the fifth week. A slowly developing flattening of the T-wave was also observed; compared with pre-drug values, it became significant at the fourth week; no further changes were observed during the following weeks (Table 1). In addition to this, a significant QRS complex widening was found at the fourth week; this change reversed to pre-drug levels during the following week. A significant RαT widening was

Table 1. Rat ECG parameters measured in control group, in doxorubicin (Dxr)- and doxorubicinol (Dxr-ol)-treated groups

Time (weeks)		0	1	2	3	4	5	6	7
QRS	Control	13.1±0.2	13.1±0.2	13.3±0.2	13.2±0.2	13.1±0.3	13.2±0.2	13.2±0.3	13.3±0.5
	Dxr	13.2±0.2	13.0±0.2	14.9±0.7*	16.2±1.4*	14.8±1.0*	13.3±0.8	13.4±1.4	13.5±0.9
	Dxr-ol	13.8±0.8	13.7±0.6	14.2±0.5	14.7±0.5	16.1±0.6*	14.9±0.8	14.7±0.7	14.0±0.9
SαT	Control	18.5±0.4	18.8±0.8	18.5±0.6	18.6±0.8	18.7±0.8	18.5±0.8	18.7±0.7	18.9±0.9
	Dxr	18.2±0.4	20.7±0.7*	22.5±0.9*	24.9±1.1*	26.8±1.4*	30.9±1.7*	36.8±2.8*	42.2±3.0*
	Dxr-ol	18.5±0.5	19.2±0.7	19.4±0.7	21.8±0.8*	24.3±1.1*	25.6±1.2*	29.3±1.5*	31.2±1.9*
RαT	Control	30.8±0.6	30.9±0.6	31.3±0.6	30.6±0.5	30.9±0.4	31.2±0.6	31.4±0.8	30.8±0.9
	Dxr	30.3±0.5	31.5±0.9	34.5±1.1*	38.6±1.2*	38.2±2.2*	40.2±3.6*	41.4±4.0*	42.9±4.5*
	Dxr-ol	30.9±0.9	31.0±0.8	31.9±1.1	32.2±0.8	34.1±0.7*	35.6±1.0*	36.4±1.9*	37.8±2.2*
αTP	Control	55.4±2.0	55.9±5.0	56.8±3.4	54.8+4.9	55.5±5.5	54.8±6.7	55.9±7.3	56.9±7.9
	Dxr	53.4±3.1	52.0±4.5	48.1±1.9*	45.6±2.0*	45.3±1.9*	44.1±2.3*	43.4±2.5*	42.1±4.3*
	Dxr-ol	52.6±2.5	54.2±1.7	54.8±3.0	53.3±2.9	52.5±5.4	48.0±2.0*	46.1±3.2*	45.0±4.1*
T-wave	Control	245.6±6.2	253.5±5.8	248.3±5.7	251.2±6.0	250.2±5.7	252.4±5.0	249.7±5.3	253.9±7.5
	Dxr	247.2±5.0	251.5±7.3	230.7±7.5*	227.3±6.1*	210.2±6.8*	209.9±6.9*	210.3±7.2*	208.8±9.3*
	Dxr-ol	239.9±8.8	243.7±8.9	239.7±8.6	239.6±9.1	220.1±8.9*	218.3±8.3*	221.5±9.2*	218.4±10.1*
Freq.	Control	449.9±7.8	447.9±8.3	445.5±8.7	451.2±6.7	447.9±6.4	449.9±6.6	447.6±6.6	457.3±8.9
	Dxr	457.9±7.8	458.1±9.8	456.7±9.9	461.5±6.7	454.5±5.8	456.4±6.5	453.5±7.4	462.8±8.6
	Dxr-ol	448.9±8.6	450.3±9.7	446.9±7.9	450.1±9.9	445.3±12.2	422.2±8.9*	419.8±10.8*	408.8±11.2*

Each value represents the mean of 13–15 observations ± S.E. Student's *t*-test for paired data: $*P < 0.05$ vs. basal values (time 0). QRS, SαT, RαT, and αTP values: msec; T-wave values: μV; frequency (Freq.): beats/minute.

recorded during the fourth week; subsequently it increased throughout the remaining time of the study (Table 1). Unlike doxorubicin, doxorubicinol treatment was associated with the appearance of bradycardia in the animals examined; the reduction of heart rate was significant at the fifth week and remained unchanged until the end of the study. At various times, almost all the animals receiving doxorubicinol developed cardiac arrhythmias, consisting of atrial fibrillation, atrial and ventricular premature beats and/or intraventricular blocks; in the doxorubicin-treated group, only 3 out of 15 animals developed cardiac arrhythmias, in particular atrial fibrillation.

Cardiac histopathology

The evaluation of cardiac tissue from control rats showed no histological alterations (Table 2). The examination of myocardial tissue from doxorubicin-treated animals revealed the presence of severe lesions, typical of anthracyclines, consisting of sarcoplasmatic macrovacuolation, interstitial or cellular edema, atrophy, and necrosis and/or fibrosis in the majority of heart sections observed, with high MTS values (Table 2). Myocardial alterations were also observed in cardiac tissue from doxorubicinol-treated rats; the degree of severity, however, was lower than that of doxorubicin-treated rats, as shown by the low MTS values (Table 2). Lesions consisted of myofibril degeneration and sarcoplasmatic microvacuolation in single scattered myocytes. Both in doxorubicin- and in doxorubicinol-treated rats, the degree of severity of histological alterations was more pronounced in the left atrium and ventricle than in the right ones; the right atrium of doxorubicinol-treated animals was actually unaffected (Table 2).

*Table 2. Histological results expressed in MTS obtained from rat cardiac tissues analyzed at the end of the experiment in control group and in doxorubicin (Dxr)- and doxorubicinol (Dxr-ol)-treated groups**

	LA	LV+S	RV	RA
Control	0	0	0	0
Dxr	6	6	3.5	2.6
Dxr-ol	1.4	1.7	0.3	0.08

*See Materials and Methods section. Each value represents the MTS of cardiac lesions from 13 to 15 animals. LA: left atrium; LV+S: left ventricle and septum; RV: right ventricle; RA: right atrium.

DISCUSSION

The results of the present study provide evidence that the administration of synthetic doxorubicinol in rats is associated with the appearance and development of a toxic syndrome, in which a doxorubicin-like cardiomyopathy represents the most important aspect. The data obtained show that the degree of cardiac toxicity induced by exogenous doxorubicinol is lower than that induced by equimolar doses of doxorubicin. These results are not unexpected on the basis of available data indicating that doxorubic-

inol possesses cytotoxic properties. In particular, the metabolite inhibits nucleic acid synthesis [11], stimulates superoxide anion production, and increases the rate of NADPH and NADH consumption by cardiac sarcosomal fractions and by mitochondrial NADH dehydrogenase *in vitro*, in a very similar way to doxorubicin [6]. Since it has been proved that these biochemical events may be associated with cardiotoxicity [12], it appears that the C-13 hydroxylation of doxorubicin does not modify its subcellular toxicity but, probably, alters the processes of cellular uptake and distribution on which the effects of *in vivo* treatment depend. In previous reports it has been stressed that the transport of anthracycline antibiotics through the cell membrane plays a crucial role in their cytotoxicity, both in normal and in cancer cells [13, 14]. At present conclusive evidence is still lacking, but there are agreeing data suggesting that the chemical properties of doxorubicin, and structurally-related anthracyclines, make it likely that they would move across cell membranes by passive diffusion [14, 15], although other mechanisms of transport cannot be excluded [13]. Assuming passive diffusion to be the most important mechanism of cellular uptake of anthracyclines, the degree of cell penetration is an inverse function of drug polarity and hydrophobicity. The reduction of the C-13 carbonyl of doxorubicin leads to the formation of the more polar analog doxorubicinol [4, 16], whose lower penetration into cardiac cells, compared with doxorubicin [17], is in agreement with the hypothesis of passive diffusion. In addition, Bachur *et al.* [16] estimated the polarity of both doxorubicin and doxorubicinol by 1-butanol-phosphate buffer partitioning and its relationship to drug accumulation by cells; these studies demonstrated that the metabolite possesses higher polarity and lower cellular uptake compared with doxorubicin. A possible explanation for the pathogenesis of cell damage induced by doxorubicinol is suggested by the findings of Skovsgaard and Nissen [13]: the cell interaction of polar anthracyclines (e.g. daunorubicinol and doxorubicinol) is characterized by drug absorption by surface structures, most probably phospholipids in the cell membranes, with which anthracyclines may form electrostatic complexes. The following phase of membrane permeation and intracellular penetration appears to be very limited, unlike less polar anthracyclines (e.g. daunorubicin and doxorubicin) [13]. These data are in agreement with previous findings [17], indicating lower cardiac tissue levels of doxorubicinol compared with the levels found for doxorubicin after repeated doxorubicinol or doxorubicin treatment respectively. Based on these results, the cardiotoxic effects of doxorubicinol might be due to its interaction with the cardiac cell surface. Doxorubicin and related anthracyclines have been demonstrated to be membrane-active molecules [18]; these drugs inhibit the transplasma membrane redox system, which may act as a modulator of the heart cell membrane charge, by controlling the flux of several ions, including Ca^{2+}, in or out of the cells [19, 20]. The alterations induced by doxorubicin to this system are probably involved in the reduction of resting potential and the increase of automaticity [21], in the prolongation of the action potential duration in isolated dog ventricular muscle and Purkinje fibers exposed to doxorubicin [22], as well as in the increased duration of action potential which has been found in heart cells from rats chronically treated with doxorubicin [23]. These effects have been related to ECG signs of anthracycline cardiotoxicity, consisting of arrhythmias and QRS widening [24], T-wave flattening [23] and SαT prolongation [9] in animal models. Definitive conclusions about the different influences of doxorubicin and doxorubicinol treatments on ECG parameters cannot be drawn on the basis of the present results. However, the high rate of arrhythmias and the reduction in the heart rate observed in doxorubicinol-treated rats, might be related to the postulated cell surface interaction of the exogenously-administered metabolite, since drugs which modify cardiac cell polarity by interacting with the sarcolemma, are able to induce arrhythmias [25–29]. Undoubtedly, the high intracardiac levels reached by doxorubicin in treated rats [5] make it possible to exploit the full toxic potential of the drug; however, it is well-recognized that doxorubicin may be actively cytotoxic without entering the cells, and that cell surface damage is associated with a number of cell alterations including change of membrane fluidity [30], alterations of several sarcolemmal enzyme activities [31], inhibition of plasma membrane NADH dehydrogenase [19, 32], or cytomembrane phospholipid peroxidation [33, 34]. Exogenously administered doxorubicinol does not achieve high intracardiac levels [17]; this fact might explain, at least in part, the lower general and cardiac toxicity of doxorubicinol treatment compared with doxorubicin.

Finally, the high intracellular levels of doxorubicinol observed in cardiac tissue after repeated treatment with doxorubicin in rats [5] and the features of toxicity associated with doxorubicinol treatment demonstrated in the present study, could lead to the following conclusions: (1) the polarity of the hydroxylated metabolite doxorubicinol appears to be associated with a low membrane permeability [16]; for this reason the cellular efflux of doxorubicinol, produced by the cytoplasmatic metabolism of doxorubicin, is reduced, and it concentrates intracellularly [5, 35]; (2) by reaching appreciable intracellular levels after doxorubicin treatment, doxorubicinol might contribute to several toxic

effects induced by doxorubicin [5].

Additional experiments are needed to investigate other aspects of the cell surface interaction of doxorubicinol.

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