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Protective effects of fructose-1,6-diphosphate on acute and chronic doxorubicin cardiotoxicity in rats*

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Summary. The effects of fructose-1,6-diphosphate, an intermediate metabolite of glycolysis, on acute and chronic cardiotoxicity of doxorubicin were investigated in rats. In the acute study, urethane-anaesthetized Wistar female rats treated with 10 mg/kg i.v. doxorubicin developed a widening of the SαT segment, an impairment of $+dP/dt_{max}$, and tachycardia. Pretreatment with 375 and 750 mg/kg i.p. fructose-1,6-diphosphate prevented the SαT segment from widening, whereas only 750 mg/kg i.p. significantly attenuated the heart rate increase. Chronic cardiomyopathy was induced over a 6-week period by weekly doses of 3 mg/kg i.v. doxorubicin, being characterized in vivo by the progressive enlargement of the SαT segment and the occurrence of histological alterations and in vitro by a marked impairment of the inotropic response elicited by adrenaline in isolated hearts from treated rats. Concurrent treatment with 150 and 300 mg/kg i.p. fructose-1,6-diphosphate thrice a week for 6 weeks did not lessen the chronic heart damage, whereas 600 mg/kg given i.p. significantly reduced the widening of the SαT segment and the severity of histological damage in vivo, as well as significantly improving the contractile responses of hearts in vitro. These findings suggest that the administration of fructose-1,6-diphosphate plays a protective role in the acute and chronic cardiotoxicity of doxorubicin in the rat.

Introduction

The reduction of cardiomyopathy represents a major goal in improving the clinical utility of anthracycline antineoplastic drugs. Doxorubicin (DXR) and its derivatives induce an acute and a chronic form of cardiotoxicity [8], the pathogenesis of which has been the object of an exhaustive search. A large body of physico-chemical studies have delineated the complex interaction between the anthracyclines and the molecular milieu of the cells (for a review see [14]). Several in vitro findings have suggested a major role for oxidative stress [11] as well as the impairment of the mitochondrial function and energy metabolism [30] in cardiac injury by DXR, whereas in vivo studies have shown an association between histamine release and heart damage [5, 20]. If the above-mentioned pathogenic mechanisms play a key role in DXR cardiotoxicity, fructose-1,6-diphosphate (FDP) might be suitable for reducing heart injury, since it prevents the cardiac oxidative damage caused by DXR in mice [21], increases the ATP content in myocardial cells in vitro [23], and inhibits DXR-induced histamine release from rat mast cells [33]. For these reasons, the present study was designed to evaluate whether the biochemical properties of the drug offer a real advantage in limiting the cardiotoxicity of DXR in the rat, which has been proven to be a suitable model of experimental DXR cardiomyopathy [35]. The results indicate that the administration of FDP significantly reduces the severity of both acute and chronic DXR cardiotoxicity.

Materials and methods

Experimental animals. Adult female Wistar rats with a mean body weight of 198.4 ± 2.2 g were used. They were fed rat diet and tap water *ad libitum* and were not used for at least 1 week after their delivery to the laboratory. The animals were housed in temperature-controlled rooms on a 12-h light cycle at 22°–24° C and approximately 50%–60% relative humidity. The distribution of animals into groups and the treatment allotted to each group were randomized.

Drugs. Pure samples of FDP disodium and DXR hydrochloride were supplied by Biomedica Foscama-I.R.F.I. (Ferentino, Italy) and Farmitalia-Carlo Erba (Milano, Italy), respectively. Chlorpheniramine maleate (CL) and adrenaline bitartrate were obtained from Essex (Milano, Italy) and Sigma Chemical Co. (St. Louis, Mo, USA), respectively. Aqueous solutions of DXR were freshly prepared immediately before use and protected from light. All other chemicals were of analytical grade.

Acute cardiotoxicity study. Animals were divided into six groups of five rats each and treated as reported in Table 1. Rats were anaesthetized with urethane (1 g/kg i.p.) and the trachea was cannulated to assure a free airway; the animals were allowed to breathe normal room air. At this

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Table 1. Administration schedule for DXR and FDP[a]

Study group	DXR[b]	FDP[c]	Study group	DXR[d]	FDP[e]
Acute:	–	–	Chronic:		
1	–	–	1	–	–
2	–	375	2	–	150
3	–	750	3	–	300
4	10	–	4	–	600
5	10	375	5	3	–
6	10	750	6	3	150
			7	3	300
			8	3	600

[a] Drug doses were reported in the table omitting mg/kg (i.e., FDP 600)
[b] In mg/kg, i.v. infusion over 2 min; the volume injected was 2.0 ml/kg
[c] In mg/kg, given i.p. 30 min before DXR administration
[d] In mg/kg, given i.v. once a week for the first 3 weeks
[e] In mg/kg, given i.p. thrice a week for 6 weeks

dose urethane anaesthesia has been shown to be suitable for cardiovascular investigations [22].

Systolic and diastolic blood pressures were recorded continuously from a cannulated right common carotid artery [29] via a pressure transducer (Statham model P23ID) connected to the APC channel of a Battaglia-Rangoni ESO-600 polygraph (Battaglia-Rangoni, Bologna, Italy). The heart rate was derived from the pulse wave of the carotid pressure, and the $+dP/dt_{max}$, an indirect but reliable index of left ventricular contractility [7], was measured by differentiating the pulsatile flow signal by means of an analogue device (AO/DP/NS channel, Battaglia-Rangoni, Bologna, Italy). The ECG (lead II) was recorded as previously reported [9]; the SαT segment duration (ms) and T-wave amplitude (μV) were measured as cardiotoxicity endpoints. After a stabilization period of 20 min, the ECG and the haemodynamic parameters were registered before and for 75 min after the end of DXR infusion. To further characterize the effects of histamine on DXR acute cardiotoxicity, a group of 15 rats was treated with 10 mg/kg i.v. CL 30 min before receiving the DXR dose; the animals then received 10 mg/kg i.v. DXR and, 15 min later, 350 or 750 mg/kg i.p. FDP. Control groups received CL and FDP at the doses indicated above.

Chronic cardiotoxicity study. Animals were divided into eight groups of ten animals each and treated as shown in Table 1. Body weight was recorded weekly during the study, which lasted 6 weeks. The ECG (lead II) was recorded weekly, and the heart rate, SαT segment (ms), and T-wave (μV) were also measured [9]. At the end of the study, rats were sacrificed by cervical dislocation and the hearts were randomly separated for isolated organ studies (see below) or histopathology.

Samples of myocardium from the left and right ventricular free wall and septum were collected and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3). Blocks of tissue from each heart were embedded in glycol methacrylate plastic resin and sections (1 μm thick) were prepared and stained with haematoxylin and eosin. Myocardial lesions were evaluated according to their severity and extension, and the degree of cardiac damage was scored on a previously described six-point scale [4]. Sections were evaluated by two investigators who had no prior knowledge of the treatment given to the animals, and a single score was given after evaluation of all three myocardial sections. The light-microscopic examination has successfully been applied to the study of DXR cardiomyopathy in the rat [16].

Isolated heart study. Animals from chronic groups were injected i.p. with 500 IU/kg heparin and then killed by cervical fracture. Hearts were immediately removed and rinsed in Locke phosphate-buffered solution that had the following composition (g/l): NaCl, 9.2; KCl, 0.42; $CaCl_2$, 0.23; glucose, 1.0; $Na_2HPO_4 \cdot 12H_2O$, 0.143; and $NaH_2PO_4 \cdot 2H_2O$, 0.016, oxygenated for at least 10 min with 100% O_2. The aorta was cannulated with a polyethylene catheter (inside diameter, 1.3 mm), and hearts were rapidly transferred to a thermostatically controlled chamber; the entire procedure took less than 1 min. Perfusion was carried out in a non-recirculating system at 37° C with the Locke phosphate buffer oxygenated with 100% O_2 (pH 7.4); perfusion pressure was kept at 60 mm Hg.

Experiments were started after a 20-min perfusion to achieve a rhythmic heart rate and the maximal coronary flow. To obtain a dose-response curve for adrenaline bitartrate, increasing doses of the drug (0.01, 0.1, 1 and 10 μg) were added cumulatively (0.1 ml) to the perfusion medium just above the aortic cannula. Isometric contractile force tracings were recorded as previously described [10]; the DF_{max} (maximal developed force) and $\pm dF/dt_{max}$ (maximal rate of contraction and relaxation) were measured.

Statistics. Statistical analysis was carried out with NWA STATPAK software (Northwest Analytical, Inc., Portland, OR, USA) and a Honeywell XP computer. The data presented represent the means ± SE of n observations. Two-way analysis of variance was done to test the effects of time and treatment on each ECG, haemodynamic parameter and body weight measured at different times. Data obtained in the histopathological and isolated heart studies were compared using a one-way analysis of variance. Both analysis were followed by the Tukey test. A P value of <0.05 was considered to be significant.

Results

Acute cardiotoxicity study

During the experiment, haemodynamic and ECG parameters did not significantly change in controls or in rats treated with FDP or CL; the T-wave amplitude and the systolic and diastolic arterial pressures were also not found to be significantly affected in any experimental group. DXR administration induced a progressive and significant widening of the SαT segment that was markedly attenuated by pretreatment with FDP at all doses (Fig. 1). The heart rate increased significantly after DXR infusion and subsequently recovered partially; only 750 mg/kg FDP significantly reduced this effect (Fig. 2).

Pretreatment with CL prevented the tachycardia but did not significantly reduce the SαT segment widening; the subsequent administration of 750 mg/kg FDP produced a significant reduction in the SαT segment dura-

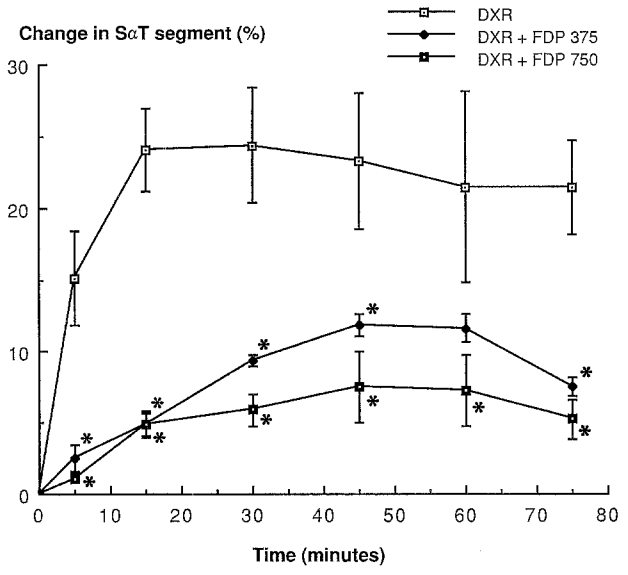

Fig. 1. Enlargement of SαT segment in rats receiving 10 mg/kg DXR alone or in combination with 375 or 750 mg/kg FDP given 30 min before DXR treatment. Time 0 indicates the start of DXR infusion, which lasted 2 min. Results are plotted as the percentage of difference from values before DXR dosing. Data points represent the means ± SE (*vertical bars*) of 5 rats. *, $P < 0.05$, significantly different from corresponding DXR values. The mean ± SE basal values for the SαT segment (ms) were 14.9 ± 0.3 (DXR), 15.1 ± 0.3 (DXR + FDP375), and 14.8 ± 0.2 (DXR ± FDP750)

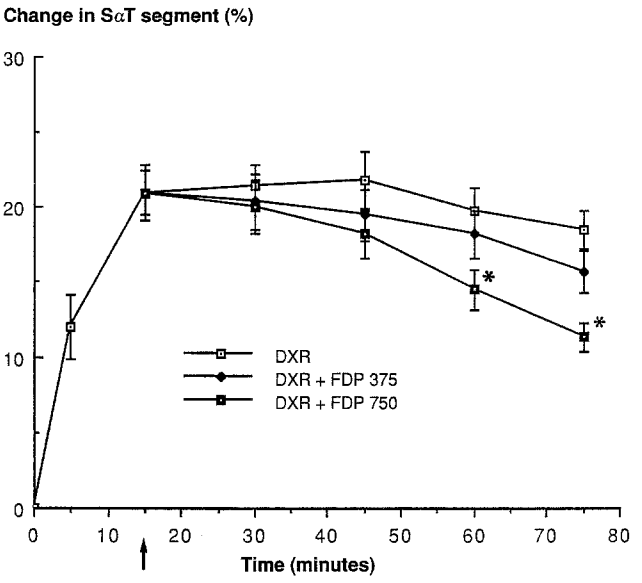

Fig. 3. Enlargement of SαT segment in rats treated i.p. with 10 mg/kg CL 30 min before 10 mg/kg DXR. *Arrow* indicates the administration of 375 or 750 mg/kg FDP. Data points represent the means ± SE (*vertical bars*) of 15 rats up to the 15th min and, thereafter, of 5 rats. Comparisons were made between data points obtained after the administration of FDP. *, $P < 0.05$, significantly different from corresponding DXR values. Other details are identical to those in Fig. 1. The mean ± SE basal values for the SαT segment (ms) were 14.5 ± 0.3 (DXR), 15.0 ± 0.4 (DXR + FDP375), and 14.3 ± 0.4 (DXR + FDP750)

tion (Fig. 3). The $+dP/dt_{max}$ was significantly decreased by DXR (basal values, 2,420.3 ± 38.4 mm Hg/s; maximal decrease, −22.6 ± 5.2% at 15 min after DXR), but FDP did not prevent this effect (basal values, 2,020.3 ± 32.5 and 2,190.4 ± 45.2 mm Hg/s; maximal decrease, −20.9 ± 3.2 and −18.2 ± 3.3 for DXR + FDP 375 and 750 mg/kg, respectively).

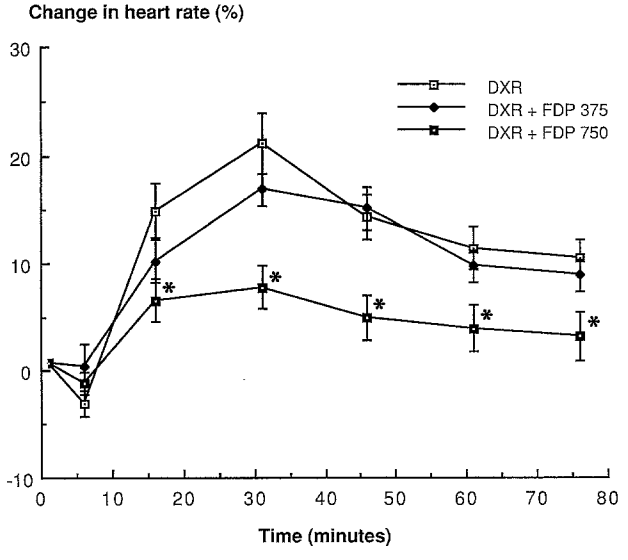

Fig. 2. Change in heart rate in rats receiving 10 mg/kg DXR alone or in combination with 375 or 750 mg/kg FDP given 30 min before DXR treatment. *, $P < 0.05$, significantly different from corresponding DXR values. Other details are identical to those in Fig. 1. The mean ± SE basal values for the heart rate (beats/min) were 383.5 ± 21.4 (DXR), 364.2 ± 18.2 (DXR + FDP375), and 352.2 ± 15.1 (DXR + FDP750)

Chronic cardiotoxicity study

The body growth of animals treated with 150 and 300 mg/kg FDP was not different from that of control rats, whereas the body weight of rats receiving 600 mg/kg FDP was significantly lower than that of controls at the end of the study (Fig. 4). No deaths occurred in rats given FDP at any dose alone or in combination with DXR, whereas two rats treated with DXR alone died during the study. DXR severely affected the body weight increase of animals, and FDP did not significantly lessen this effect (Fig. 4). SαT changes were not recorded in control rats or in animals treated with FDP alone; the T-wave amplitude remained unchanged in all treated groups. The progressive widening of the SαT segment induced by DXR was not modified by treatment with 150 or 300 mg/kg FDP; on the contrary, 600 mg/kg FDP significantly reduced the SαT enlargement (Fig. 5). Controls and animals treated with FDP had normal cardiac histological pictures; severe lesions were detected in hearts from rats given DXR alone (Fig. 6, Table 2) or with 150 mg/kg FDP (Table 2). The DXR + 300 mg/kg FDP regimen did not significantly lower the heart damage (Fig. 7, Table 2), whereas treatment with DXR + 600 mg/kg FDP resulted in a reduced severity of heart lesions (Fig. 8, Table 2).

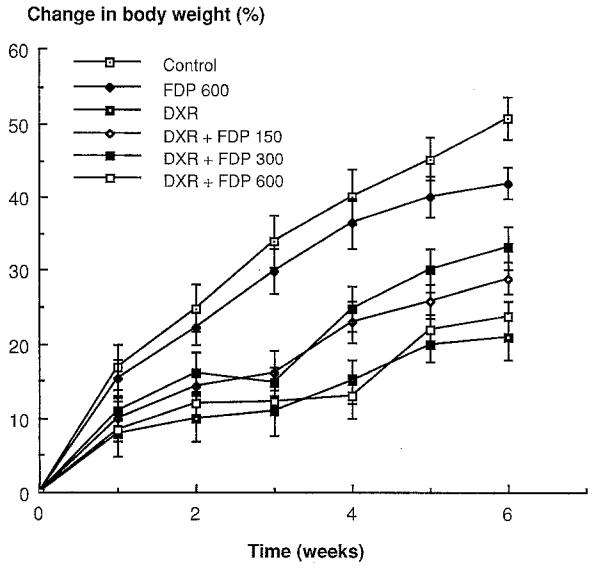

Fig. 4. Growth rate of controls and rats receiving FDP, DXR, or FDP+DXR. Results are plotted as the percentage of difference from values at the beginning of treatment (time 0). Data points represent the means ± SE (*vertical bars*) of 8–10 rats. To avoid the superimposition of symbols, the statistical significance was not indicated. Animals treated with 600 mg/kg FDP had a body weight significantly lower than that of controls at the 6th week, whereas rats receiving DXR or DXR+FDP at any dose had a body growth significantly lower than that of controls after the 2nd week. The mean ± SE basal values for the body weight (g) were 202.0 ± 3.4 (control), 203.2 ± 4.2 (FDP 600), 198.3 ± 2.8 (DXR), 199.2 ± 3.0 (DXR+FDP 150), 196.7 ± 2.7 (DXR+FDP 300) and 190.2 ± 3.1 (DXR+FDP 600)

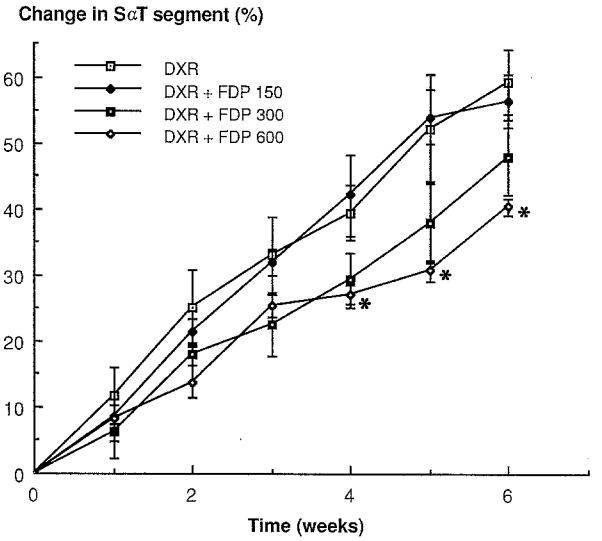

Fig. 5. Enlargement of SαT segment in rats receiving DXR or FDP+DXR. *, $P < 0.05$, significantly different from corresponding DXR values. Other details are identical to those in Fig. 4. The mean ± SE basal values for the SαT segment (ms) were 15.7±0.4 (DXR), 16.5±0.5 (DXR+FDP 150), 16.4±0.5 (DXR+FDP 300) and 16.1±0.4 (DXR+FDP 600)

Isolated heart study

No significant differences were observed in any experimental group with respect to the heart's wet weight and the basal contractility. Adrenaline gave rise to a dose-dependent increase in DF_{max} and $\pm dF/dt_{max}$, with no differences between controls and groups given FDP at any dose. Hearts from rats treated with DXR alone, DXR+150 mg/kg FDP, DXR+300 mg/kg FDP and DXR+600 mg/kg FDP displayed a significantly lower response with respect to controls; however, a contractile response significantly higher than that of animals given DXR was found in hearts from rats receiving DXR+600 mg/kg FDP (Table 3).

Discussion

The present results demonstrate that FDP provides significant protection against acute cardiotoxicity induced by DXR in the rat, whereas in the chronic study only high doses of the drug significantly lessen the severity of the cardiomyopathy. Our data regarding the effects of FDP on acute DXR cardiotoxicity are in line with those observed in other experimental models of acute cardiac injury, including the isolated rat heart exposed to DXR [2].

FDP may be beneficial to the damaged heart, since it significantly enhances energy production by acting as a metabolic substrate and activating several of the enzymes involved in glycolysis, particularly the rate-limiting enzyme phosphofructokinase [19]. The administration of FDP to dogs with acute cardiac ischaemia [23] and haemorrhagic shock [25] has improved the haemodynamics, reduced the ECG alterations, and increased the cardiac levels of creatine phosphate and ATP. A fall in high-energy phosphate production has also been demonstrated in cultured heart cells exposed to DXR, and this effect has been related to the acute toxicity of the drug [28]. Although the damage to the energy supply by DXR is dependent on the interaction of the drug at the mitochondrial level, which resulted in an impaired oxidative metabolism [30], FDP might exert its protective effect by bypassing the mitochondrial damage and improving cellular energy production through an increase in anaerobic metabolism.

A peculiar feature of cardiac cell injury by DXR resides in the ability of the drug to impair the main routes for reducing the sarcoplasmic free Ca^{2+}, since DXR inhibits the Na^+-Ca^{2+} exchange of heart sacrolemmal vescicles [6] as well as the Mg^{2+}-dependent Ca^{2+}-ATPase of the sarcoplasmic reticulum [3] and increases the Ca^{2+} influx via stimulation of the slow channels [1], thus leading to cellular Ca^{2+} overload. Furthermore, the drug impairs the Na^+-K^+-ATPase activity of rat heart cytomembranes [26]. The loss of ion homeostasis in cardiac cells might lead to the prolongation of both the action potential duration [18] and the repolarization phase of the ECG [9]. The impairment of ion translocation across the sarcolemma could be a target for the action of FDP, which may restore these abnormalities. The drug seems to possess this property; it has been shown that FDP induces an uptake of K^+ ions against the gradient in rat erythrocytes in vitro [12] and markedly reduces the K^+ loss and increases the ATP content in the ischaemic canine myocardium [24]. The increase in cellular ATP levels through the glycolytic pathway might be relevant, due to the fact that a preservation of the membrane function, including Ca^{2+} homeostasis, may be achieved in this way [15]. A further study has demonstrated

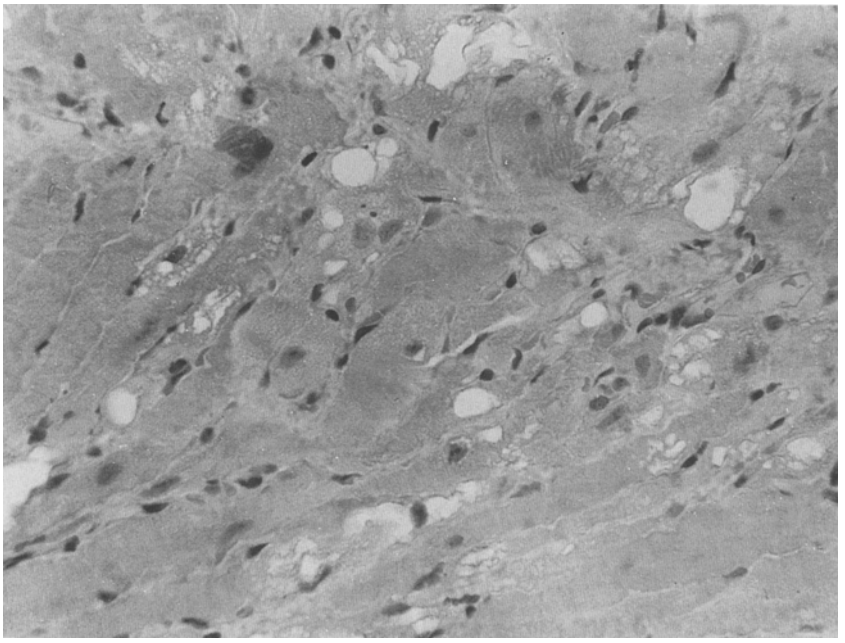

Fig. 6. Thin section (1 μm) of myocardium from the left ventricle of a rat 4 weeks after treatment with three weekly doses of 3 mg/kg DXR. Severe morphological alterations are evident, consisting of vacuolation affecting nearly every myocyte. Haematoxylin and eosin, ×450

Table 2. Effect of FDP on DXR cardiomyopathy[a]

	Cardiomyopathy score[b]	Hearts (*n*)	Significance
DXR	2.38 ± 0.24	4	
DXR + FDP 150	2.30 ± 0.12	5	NS
DXR + FDP 300	1.90 ± 0.10	5	NS
DXR + FDP 600	1.40 ± 0.10	5	$P < 0.05$

[a] For treatment groups see Table 1
[b] Grading system according to Bristow et al. [4]; values represent the mean ± SE; NS, not significant

that FDP activates the Mg^{2+}-dependent Ca^{2+}-ATPase of the human erythrocyte membrane and significantly reduces myocardial Ca^{2+} uptake in the isolated heart (Galzigna et al., data to be published).

The minimal attenuation of SαT segment widening and the prevention of tachycardia by pretreatment with CL provides evidence that DXR-induced histamine release plays a role in the acute cardiovascular effects of DXR in rats, as shown in previous works [5, 13, 20]. The significant reduction by FDP of acute and chronic cardiotoxicity might be due, at least in part, to the ability of the drug to prevent histamine release, as previously demonstrated in rat mast cells [33]. However, in the present study FDP did not attenuate the impairment of $+dP/dt_{max}$ by DXR, which is a typical effect of anthracyclines [17], and a sig-

Table 3. Effects of incremental doses of adrenaline on isometrically contracting heart preparations obtained from control, DXR, and DXR + FDP-treated rats

		Adrenaline (μg):			
		0.01	0.1	1	10
Controls ($n=5$):					
DF_{max}	(1.6 ± 0.2 g)	66.9 ± 2.2	175.3 ± 9.3	218.8 ± 14.2	266.6 ± 7.4
$+dF/dt_{max}$	(77.3 ± 5.2 g/s)	53.1 ± 5.1	130.0 ± 5.7	142.2 ± 8.5	161.1 ± 10.7
$-dF/dt_{max}$	(33.2 ± 5.8 g/s)	−109.9 ± 11.4	−248.8 ± 18.8	−297.9 ± 19.9	−380.4 ± 22.3
DXR ($n=4$):					
DF_{max}	(1.5 ± 0.2 g)	31.9 ± 7.1*	72.3 ± 9.9*	117.2 ± 13.0*	124.8 ± 13.6*
$+dF/dt_{max}$	(72.9 ± 6.5 g/s)	25.2 ± 6.1*	50.8 ± 8.7*	100.5 ± 9.0*	127.2 ± 10.2*
$-dF/dt_{max}$	(35.7 ± 4.1 g/s)	− 17.1 ± 3.9*	− 97.6 ± 9.8*	−125.4 ± 12.5*	−139.1 ± 18.3*
DXR + FDP 600 ($n=5$):					
DF_{max}	(1.6 ± 0.3 g)	35.9 ± 6.9	77.9 ± 8.7*	122.5 ± 12.7*	170.4 ± 8.4*, **
$+dF/dt_{max}$	(72.3 ± 1.7 g/s)	35.7 ± 8.2	90.6 ± 7.7*, **	143.9 ± 8.7**	167.2 ± 9.6
$-dF/dt_{max}$	(40.1 ± 4.0 g/s)	− 28.4 ± 2.2	−101.9 ± 8.0*	−168.4 ± 9.3*, **	−186.5 ± 8.9*, **

Results represent the means ± SE of the percentage of change from values calculated before adrenaline (reported in brackets)
*$P<0.05$, comparing DXR or DXR + FDP 600 with controls
**$P<0.05$, comparing DXR + FDP 600 with DXR

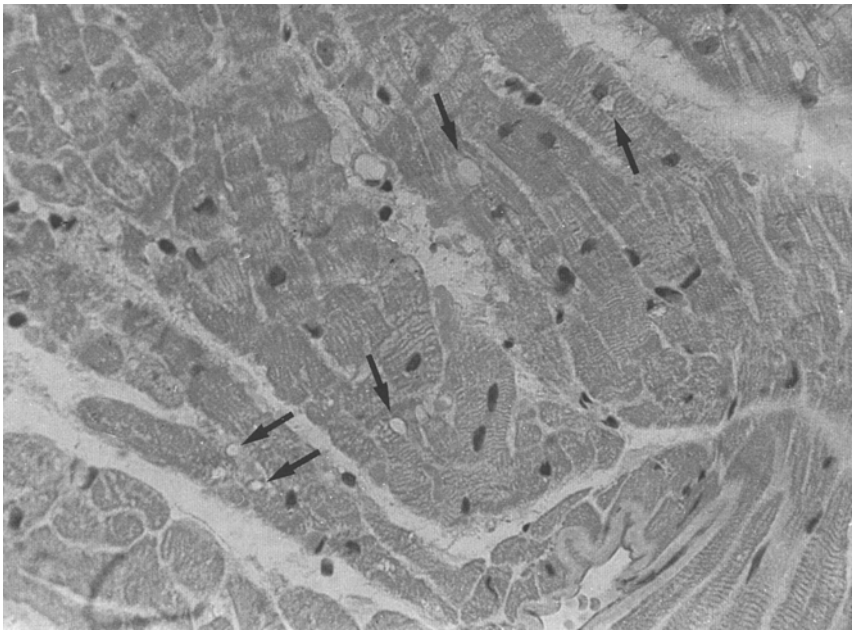

Fig. 7. Thin section (1 μm) of myocardium from the left ventricle of a rat treated with three weekly doses of 3 mg/kg DXR as well as 300 mg/kg FDP every 2 days for 6 weeks. A few affected cells with cytoplasmic vacuolation (*arrows*) surrounded by unaffected myocytes can be seen. Haematoxylin and eosin, ×450

nificant protection against chronic DXR cardiotoxicity, which is the major toxic effect of DXR, was achieved only by high doses of FDP.

Potential explanations for the failure of FDP to achieve full protection against DXR cardiotoxicity include the participation of other factors in the tissue damage induced by anthracyclines, such as semiquinone free-radical production [14], lipid peroxidation [27], and interaction with DNA [34], with the inhibition of both DNA and RNA synthesis [31]. FDP inhibits superoxide production in human neutrophils stimulated by phorbol-ester by acting on the $O_2^{\cdot}$ generating system or by directly influencing cell activation by phorbol-ester [32]. Although these results cannot be directly ascribed to cardiac toxicity by DXR, it would be worthwhile to investigate the activity of FDP on superoxide anion generation by DXR. In this connection, a significant reduction by FDP of lipid peroxidation and catalase activity in heart tissue from mice treated with DXR [21] has recently been demonstrated, providing evidence of an inhibition by FDP of DXR-induced oxidative stress.

Finally, the beneficial effects of FDP against DXR acute cardiotoxicity suggest that a short-term control of this drug-induced tissue damage may be achieved by this therapeutic approach. The protective effects of FDP on chronic cardiomyopathy found in the present study demonstrate that, at least in the rat, cardiotoxicity results from multifactorial events that are partially affected by FDP.

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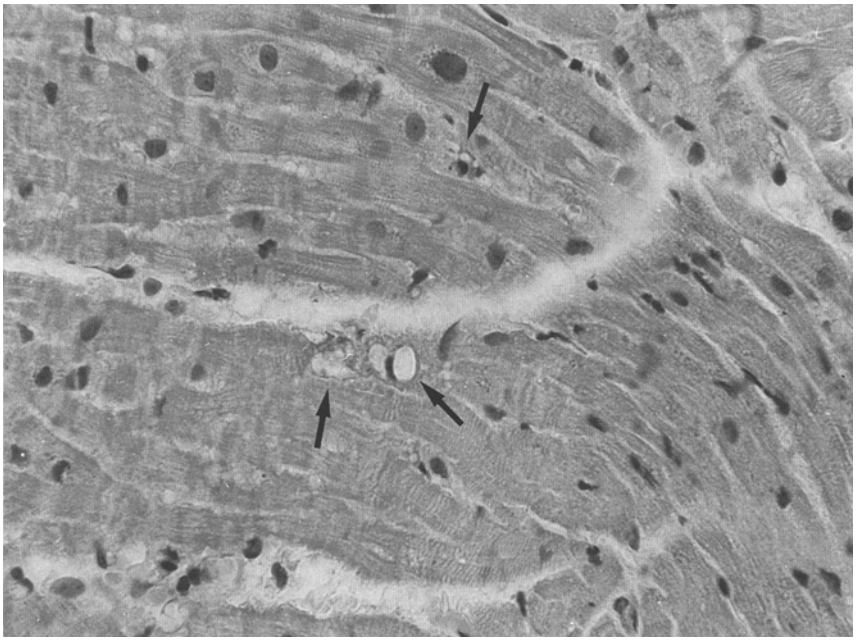

Fig. 8. Thin section (1 μm) of myocardium from the left ventricle of a rat treated with three weekly doses of 3 mg/kg DXR as well as 600 mg/kg FDP every 2 days for 6 weeks. Isolated myocytes showing sarcoplasmic vacuoles (*arrows*) are visible. Haematoxylin and eosin, ×450

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