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Reducing doxorubicin cardiotoxicity in the rat using deferred treatment with ADR-529*, **

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Summary. The purpose of this study was to evaluate the optimal timing of ADR-529 administration to protect rats treated with doxorubicin (DXR) against drug-induced cardiotoxicity. Complete electrocardiographic monitoring (QRS complex, SαT segment and T wave) and the histopathological analysis of cardiac tissue were used to assess the degree of heart damage produced in female rats treated with ten i. v. doses of 1 mg/kg DXR over a period of 15 weeks; body-weight increase and survival were also analyzed to evaluate the toxicity of treatments. Cardiac alterations induced by DXR were compared with those occurring in animals receiving 20 mg/kg i. v. ADR-529 at 30 min prior to DXR administration, starting at the first, third, or sixth DXR dose and given until the end of the study (15th week). Rats treated with DXR were severely cardiomyopathic, showing progressive and irreversible ECG alterations (QRS-complex and SαT-segment widening and T-wave flattening) and marked degeneration of the myocardium (myocyte vacuolation, myofibrillar loss, and endomyocardial fibrosis). The most effective cardiac protection was provided by the administration of ADR-529 beginning with the first or third DXR dose. Delaying treatment with ADR-529 until the sixth DXR dose resulted in a significant reduction in its therapeutic action on heart damage. A significant difference in body-weight increase and survival was observed between the treatment groups: ADR-529 injected prior to the first DXR dose significantly protected animals from DXR toxicity, but this schedule was significantly more toxic than the administration of ADR-529 beginning with the third or sixth DXR dose. Taking into account the degree of cardiac protection and the toxicity of combination treatments, the results of the present study demonstrate the superiority of ADR-529 given prior to the third DXR dose over the other schedules tested. This finding suggests that significant protection against DXR-induced chronic cardiotoxicity in the rat can be obtained using deferred treatment with ADR-529.

Introduction

Among the various attempts thus far undertaken to reduce the severity of doxorubicin (DXR) cardiotoxicity by pharmacological means, the use of the iron chelator ADR-529 [previously known as ICRF-187, (±)-1,2-bis(3,5-dioxopiperazinyl-1-yl)propane] has been proven to be the most successful [8–10]. The rationale for the use of ADR-529 in the prevention of DXR cardiomyopathy arises from the biochemical basis of DXR damage to cardiac tissue. DXR has been shown to form metal complexes with Fe(III) [11], and the drug ligand can reduce the bound Fe(III) to produce a one-electron, oxidized drug radical; furthermore, the Fe(II) can reduce oxygen to hydrogen peroxide and cleave the peroxide to yield the hydroxyl radical. In addition, the DXR-Fe complex can catalyze the transfer of electrons from reduced glutathione to molecular oxygen to yield superoxide, hydrogen peroxide, and hydroxyl radicals. As a consequence of these reactions (for a review, see Myers et al. [13]), the DXR-Fe complex can cleave DNA and cause oxidative damage to cell membranes, which is particularly severe in the relatively unprotected cardiac muscle [15].

ADR-529 likely exerts its action through its ring-opened hydrolysis product, which is similar in structure to ethylenediaminetetraacetic acid (EDTA) and strongly binds metal ions. The protection that ADR-529 provides against the oxidative stress induced by the DXR-Fe(III) complex may be explained by both the displacement of the metal ion from its complex with DXR and the formation of

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** ADR-529 is the proprietary name (Farmitalia Carlo-Erba, Milano, Italy) for ICRF-187

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Table 1. Treatment schedule for DXR and ADR-529[a]

Groups	Weeks of treatment				
	0→1	2→4	5→6	7→11	12→15
ADR-529 + IS	ADR-529 + IS	ADR-529 + IS		ADR-529 + IS	
NaL + DXR	NaL + DXR	NaL + DXR		NaL + DXR	
ADR-529 + DXR	ADR-529 + DXR	ADR-529 + DXR		ADR-529 + DXR	
ADR-529(3) + DXR	NaL + DXR	ADR-529 + DXR		ADR-529 + DXR	
ADR-529(6) + DXR	NaL + DXR	NaL + DXR		ADR-529 + DXR	

[a] Each treatment was injected i. v. in 2.5 ml/kg fluid volume once a week

IS, Isotonic saline (0.9% NaCl); NaL, 1.87% sodium lactate given 30 min before DXR; ADR-529, 20 mg/kg dissolved in 1.87% sodium lactate given 30 min before IS or DXR; DXR, 1 mg/kg dissolved in 0.9% NaCl

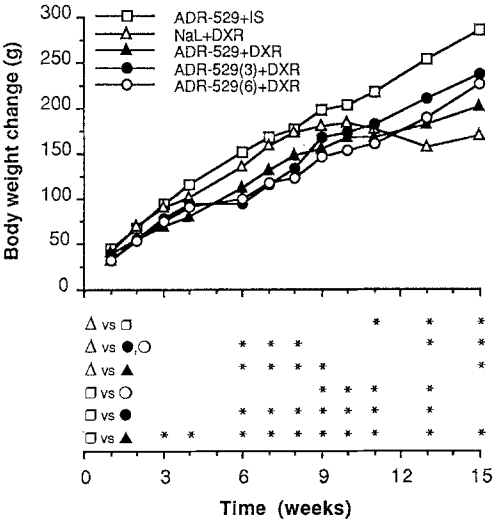

Fig. 1. Effect of DXR and ADR-529 on body weight change expressed as the mean difference between the values obtained ($n = 4-6$) at a given time point and the baseline values (time 0). Error bars are excluded for visual clarity; the SE never exceeded 12%. The statistical comparison among groups is reported by *symbols* between the abscissas; *asterisks* indicate that the difference was statistically significant ($P < 0.05$) at the corresponding week

a less active DXR-Fe(III)-ADR-529 mixed-ligand complex [7]. A significant reduction in cardiac damage has been documented in different animal models and is not associated with a decrease in the antitumor activity of DXR, as demonstrated in clinical studies [18].

ADR-529 has been used in a variety of schedules; however, the optimal dose timing has not yet been established. For this reason, the present study investigated the effect of delaying treatment with ADR-529 on cardiac protection in rats given DXR. The results demonstrate that the administration of ADR-529 beginning at 30 min prior to the third of ten weekly doses of DXR provides significant cardiac protection while minimizing the toxicity of combination treatment.

Materials and methods

Animals and drugs. Adult female Wistar rats (Nossan, Milano, Italy) weighing 180–200 g were used. They were fed a standard rat diet and tap water ad libitum and were not used for at least 1 week following their delivery to the laboratory. The animals were housed at an environmental temperature of 22°–24°C under 50%–60% relative humidity, and a 12-h lighting cycle was maintained. The distribution of animals into groups and the treatment allotted to each group were randomized. ADR-529 and DXR hydrochloride were supplied by Farmitalia-Carlo Erba (Milano, Italy). ADR-529 was reconstituted from lyophilized powder in a sterile, preservative-free 1.87% sodium lactate solution; DXR was dissolved in sterile 0.9% NaCl. Solutions for treatment were freshly prepared immediately before their use and were protected from exposure to light.

Experimental design. Animals were divided into 5 groups of 20 animals each and treated as shown in Table 1. The timing of ADR-529 administration (30 min before DXR) and the dose ratio between ADR-529 and DXR (20:1) represent a well-established treatment schedule that was chosen on the basis of previous reports [6, 9]. Additional animals were treated with either 1.87% sodium lactate or isotonic saline (IS, 0.9% NaCl); since no significant difference was observed between these groups and the ADR-529 + IS group with regard to body weight, ECG parameters, or cardiac histopathology, only the values for the latter treatment were reported and taken as control data for statistical analysis.

Body weight and survival were recorded during the study, which lasted 15 weeks. The ECG (lead II) was monitored and the SαT segment (milliseconds), QRS complex (milliseconds), and T wave (microvolts) were measured [2].

At the 7th, 13th, and 15th week of the study, animals from each group were killed by cervical dislocation and their hearts were removed for histopathology; samples of myocardium from the left ventricle and septum were 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 1.8-μm-thick sections were stained with toluidine blue. Myocardial damage was evaluated by light microscopy by investigators who had no prior knowledge of the treatment given to the animals; the product of two different scores for the extent and the severity of lesions gave the total cardiotoxicity score for each animal, from which the mean total score (MTS) for each group was calculated [17].

Data analysis. Except for the MTS, data represent the mean difference ± SE between n values at a given time point and the baseline values (time 0). To evaluate the effect of treatment on body weight, ECG parameters, and cardiac histopathology, we used one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls (SNK) test for multiple comparisons. The SNK test is performed as is Tukey's test, with one exception: the difference in the SNK procedure lies in the determination of the critical value, which is $q_{\alpha,v,p}$, where p represents the number of means within the range being tested [21]. Probability levels of less than 0.05 were considered to be significant.

Results

Animals injected with ADR-529 + IS were active, showing normal weight gain until the time they were killed. In contrast, rats injected with NaL + DXR showed inactivity, reduction in food consumption, hair loss, and a significant reduction in body-weight gain starting from the 11th week of treatment as compared with animals treated with

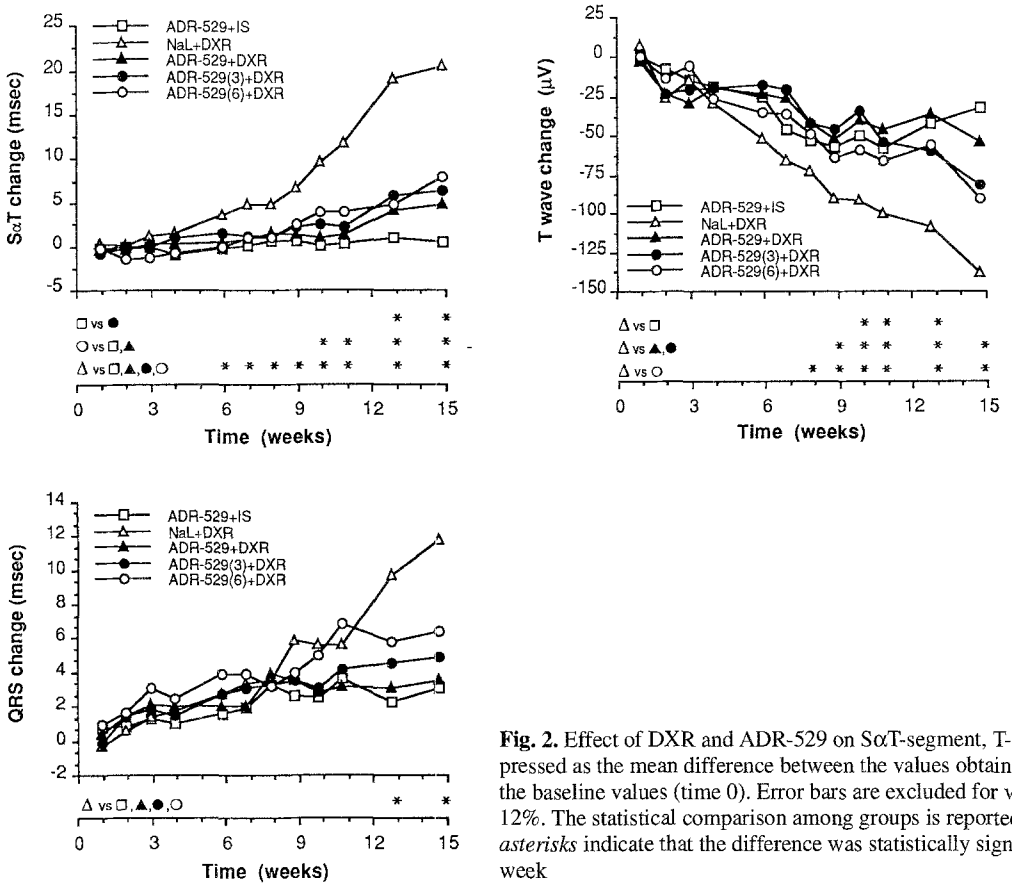

Fig. 2. Effect of DXR and ADR-529 on SαT-segment, T-wave and QRS-complex changes expressed as the mean difference between the values obtained ($n = 4-6$) at a given time point and the baseline values (time 0). Error bars are excluded for visual clarity; the SE never exceeded 12%. The statistical comparison among groups is reported by *symbols* between the abscissas; *asterisks* indicate that the difference was statistically significant ($P < 0.05$) at the corresponding week

Table 2. Cardiomyopathy scores in rats treated with DXR and ADR-529

Groups	Weeks of treatment		
	7th	13th	15th
ADR-529 + IS	–	–	0 (6)
NaL + DXR	2.55 ±0.45 (5)	6.5 ±0.29 (5)	7.56 ±0.29 (5)
ADR-529 + DXR	1.2 ±0.25 (4)	1.6 ±0.4* (4)	4.0 ±0.67* (4)
ADR-529(3) + DXR	1.67 + 0.21 (6)	3.0 ±0.45* (6)	4.4 ±0.4* (5)
ADR-529(6) + DXR	3.0 ±0.45 (6)	3.5 ±0.5** (4)	4.8 ±0.5** (5)

Data represent the mean MTS values ± SE for each group; the number of hearts evaluated are shown in parentheses
* $P < 0.05$ vs NaL + DXR
** $P < 0.05$ vs ADR-529 + DXR

ADR-529 + IS (Fig. 1), which served as the control group. At the end of the study (15th week), the survival and body growth of animals treated with DXR were significantly improved by ADR-529, particularly in the ADR-529(3) + DXR group (Fig. 1), as compared with animals receiving NaL + DXR. However, a transitory but significant reduction in body-weight gain during the mid-course of the study was observed in rats treated with DXR + ADR-529 on any schedule. Combination treatment with ADR-529 and DXR resulted in a significant impairment of body-weight increase as compared with controls; this effect was more severe and of longer duration in the ADR-529 + DXR group than in the ADR-529(3) + DXR and ADR-529(6) + DXR groups (Fig. 1), indicating the development of a modest dose-dependent toxicity.

The analysis of the survival of treated animals revealed a difference between the three ADR-529 + DXR schedules and the NaL + DXR protocol: mortality was higher in animals treated with NaL + DXR (25%) as compared with ADR-529 + DXR (20%), ADR-529(6) + DXR (15%), and ADR-529(3) + DXR (5%), indicating that the latter schedule was the most effective regimen in reducing DXR-induced deaths. Thus, the finding of high mortality in the group given ADR-529 + DXR, which proved to be the most effective in terms of cardiac protection (see below), indicated that the deaths were not dependent on cardiac toxicity or failure.

The ECG signs of cardiotoxicity were severe and progressive in the NaL + DXR group and consisted of a significant enlargement of the SαT segment and the QRS complex and a T-wave flattening (Fig. 2). ADR-529 given on any schedule significantly protected hearts from DXR damage; this effect was documented by the significant reduction in the severity of the QRS-complex, SαT-segment, and T-wave changes as compared with the values obtained for rats receiving NaL + DXR (Fig. 2). Significant differences in the degree of protection were observed among the groups treated with ADR-529: on the basis of the SαT segment and the T wave changes, ADR-529 + DXR and ADR-529(3) + DXR were the most effective schedules (Fig. 2); ADR-529(6) + DXR significantly reduced the severity of the cardiotoxicity induced by NaL + DXR but did not offer the degree of protection provided by ADR-529 + DXR or ADR-529(3) + DXR. For other comparisons, see Fig. 2.

The results of the histopathology studies on heart samples from treated animals are summarized in Table 2. Animals receiving ADR-529 + IS had a normal cardiac picture; the myocardial lesions observed in the left ventricle of animals treated with NaL + DXR were typical of those described for a variety of animal species and consisted of cytoplasmic vacuolization and myofibrillar loss, which in many instances occurred in the same cells. These alterations became more severe with time and were associated with high MTS scores (Table 2). ADR-529(3) + DXR and ADR-529 + DXR were highly effective in reducing the severity of DXR-induced cardiotoxicity (Table 2), whereas the ADR-529(6) + DXR schedule was significantly less effective (Table 2).

Discussion

Cardiotoxicity has long been recognized as the complicating factor of cancer chemotherapy with anthracyclines, particularly DXR. For this reason, a variety of efforts have been made to reduce the cardiotoxicity of DXR without compromising its antitumor activity. The favorable effect of ADR-529 treatment on DXR cardiotoxicity has been proven in several studies. Herman and Ferrans [8] demonstrated that ADR-529 not only delays the onset of cardiotoxicity but also provides long-term protection; anthracycline-induced cardiac lesions did not develop in rabbits even at 3 months after the termination of treatment with both daunorubicin and ADR-529. Furthermore, ADR-529 exerted significant protection when given with very large cumulative doses of DXR over a prolonged period of time, enabling the administration of tolerable doses of DXR to dogs that otherwise would have been lethal [10]. In addition, ADR-529 treatment was highly effective in reducing the severity of cardiomyopathy and was associated with a moderate reduction in the nephropathy induced by epirubicin in spontaneously hypertensive rats, suggesting its possible use under conditions characterized by an increased risk for the development of DXR toxicity [4].

In the present study, animals treated with DXR manifested the toxic syndrome typical of anthracycline administration: weight-gain impairment, decrease in survival, prolongation of the QRS complex and the SαT segment, flattening of the T wave, and severe myocyte damage. A QRS-complex widening reflects a negative dromotropic effect of DXR [9], whereas an increased SαT-segment duration and a reduction in T-wave voltage have been associated with a prolongation of cardiac repolarization [2]. As a consequence of the alterations in myocardial contractility, an impairment of cardiac compliance and relaxation can occur after either acute [5] or chronic [19] exposure to anthracyclines. The present study demonstrates that ADR-529 significantly improves the body growth and survival of animals treated with DXR while reducing the severity of DXR-induced cardiac toxicity. Among the treatment schedules tested, ADR-529(3) + DXR was the most effective since it was associated with minimal toxicity and maximal cardiac protection as compared with ADR-529 + DXR and ADR-529(6) + DXR.

As pointed out by many authors, there are several hypotheses to explain anthracycline cardiotoxicity, but none of them adequately integrates the existing clinical and experimental data (for a review, see Olson and Mushlin [14]). Differences in drug distribution, metabolism, and elimination [3] and the ability of DXR to produce a wide array of biochemical alterations, including (a) intercalation with DNA, (b) interactions with iron in the cell and thus the generation of free radicals, (c) interference with oxidative phosphorylation, (d) inhibition of the uptake of nutrients, and (e) depletion of cells of glutathione and adenosine triphosphate, have clouded the picture regarding DXR toxicity and the structural requirements of anthracyclines for the production of toxic effects [1, 16].

However, albeit with some limitations [14], the free-radical generation resulting from drug-iron chemistry [12, 13] remains a popular hypothesis that could conceivably account for the time-related nature of anthracycline cardiotoxicity. Free radicals, which can be generated "site-specifically" by the drug-metal complexes bound to the target biomolecule [13], could acutely damage nucleic acids without causing overt cardiac disfunction until the myocytes are no longer capable of repairing the damage to biomolecules. If anthracyclines or their metabolites are not rapidly cleared from the myocytes, they could serve as chronic generators of free radicals, which continuously damage macromolecules until the reserve is depleted, repair is inefficient, and cardiac disfunction becomes apparent. Treatment with ADR-529 can be effective in preventing the evolution of DXR cardiotoxicity, provided that its administration is started at a point at which cardiac reserve is sufficient, as in the case of animals treated with ADR-529 beginning with the third but not the sixth dose of DXR. Noteworthy are the Ca^{2+}-chelating properties of ADR-529, which suggest that an alternative mechanism of ion chelation might be operative [20].

In conclusion, the results presented herein highlight some of the advantages of modifying the timing of ADR-529 administration and suggest that treatment with this drug can be further optimized by deferring its administration after the beginning of the DXR treatment as in the case of the ADR-529(3) + DXR schedule. This observation may have clinical implications, since the possible toxicity of the two drugs may be reduced and ADR-529 could be given only to patients who obtain benefit from DXR treatment.

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