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Comparison of the protective effects against chronic doxorubicin cardiotoxicity and the rates of iron (III) displacement reactions of ICRF-187 and other bisdiketopiperazines

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Abstract Histologic and biochemical studies were carried out to compare the protective activity of various bisdiketopiperazines against the cardiac and renal toxicity induced by doxorubicin in spontaneously hypertensive rats (SHR), a well-established animal model of this disorder, with: (1) the rates of hydrolysis of these agents to form the iron-chelating derivatives (which are considered to cause a decrease in the formation of reactive oxygen intermediates) and (2) the ability of these derivatives to bind iron. SHR were given 12 weekly injections of doxorubicin, 1 mg/kg i.v. either alone or 30 min after the administration of ICRF-154, ICRF-187, ICRF-192, ICRF-197, ICRF-198, ICRF-239 and ADR-559. Semiquantitative grading of the severity of the resulting cardiac and renal lesions showed that ICRF-187, ICRF-154 and ADR-559 were the most protective, whereas ICRF-197 and ICRF-239 provided intermediate degrees of protection, and ICRF-192 and ICRF-198 were not protective. Quantitative measurements in vitro revealed only relatively small differences in the rates of opening of the two diketopiperazine rings of the various agents to form the corresponding iron-chelating diacid diamide derivatives, and in the ability of these various derivatives to remove iron from the iron–doxorubicin complex. Such differences showed no relationship with cardioprotective activity. Some bisdiketopiperazines (including ICRF-154 and ICRF-187) with cardioprotective activity also are inhibitors of DNA topoisomerase II; however, the significance of this relationship remains uncertain, since ADR-925, the open-ring derivative of ICRF-187, does not inhibit DNA topoisomerase II.

Key words Doxorubicin cardiotoxicity · $Iron_{(III)}$ displacement · Bisdiketopiperazines

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Introduction

Bisdiketopiperazines were initially found by Creighton et al. [4] to possess cytotoxic and antineoplastic activity. Although these activities have had only very limited clinical applications, renewed interest in these agents developed from the observation that ICRF-159, the racemic mixture of ICRF-187 and ICRF-186 (the (*S*)- and (*R*)-enantiomers, respectively, of ICRF-159), [1, 2-bis (3, 5-dioxopiperazine-1-yl)propane] and ICRF-187 decrease the lethal effects of high doses of daunorubicin in Syrian golden hamsters [16, 17]. A comparative evaluation of the reduction in acute daunorubicin toxicity by a number of other bisdiketopiperazines has revealed that very little alteration can occur in the basic structure of ICRF-187 without a significant loss of protective activity [18]. Other studies [15] have demonstrated that ICRF-187 (also known as ADR-529, dexrazoxane and Zinecard) also causes significant attenuation of the chronic cardiomyopathy induced by doxorubicin and other anthracyclines in a variety of experimental animal models [22] as well as in patients undergoing cancer chemotherapy [29, 31].

The pathogenesis of the chronic cardiotoxicity that ultimately limits the clinical use of doxorubicin is thought to be related to multiple factors, the most important of which appears to be the iron-mediated formation of reactive oxygen intermediates that damage a variety of cellular components [26]. This reaction is mediated by a complex formed by doxorubicin and Fe^{3+}. The current concept of the protective activity of ICRF-187 against doxorubicin-induced cardiomyopathy is that

this agent, because of its nonpolar nature, diffuses rapidly into cells, where it undergoes hydrolysis to an open ring (diacid diamide) derivative (ADR-925) that functions as an effective intracellular chelator of iron [3, 8, 9]. This chelation appears to be associated with removal of the iron from the Fe^{3+}–doxorubicin complex [10, 13]. ICRF-198 (the open-ring hydrolysis product of ICRF-159) also removes iron rapidly from the complex formed between this metal and doxorubicin or other anthracyclines [10, 11, 13]. Other reports have documented the iron-chelating properties of the open-ring hydrolysis products of other bisdiketopiperazines, including ICRF-192, ICRF-193 and ICRF-196 [23, 24]. These findings suggest that other compounds that are structurally related to ICRF-187 also might exert protective activity against doxorubicin cardiotoxicity. This has been the subject of only very limited studies, most of which have been concerned with the acute toxicity resulting from large, single doses of anthracyclines [18]. In long-term toxicity studies, ICRF-186 has been found to be only slightly less protective than ICRF-187 against the chronic cardiomyopathy induced by doxorubicin in spontaneously hypertensive rats (SHR) [33]. ADR-559, the morpholinomethyl derivative of ICRF-159 (also known as MM-159), also has been reported to ameliorate the chronic cardiac toxicity of doxorubicin in beagle dogs [20]. However, detailed comparisons of the effectiveness of these variously substituted bisdiketopiperazines in protecting against the chronic cardiotoxicity of doxorubicin have not been made in any animal model. The structural formulas for these compounds are shown in Fig. 1.

The present study was initiated to determine: (1) whether compounds that are related to ICRF-187 but have structural changes in the central alkyl chain (such as ICRF-154 [desmethyl], ICRF-192 [ethyl] and ICRF-197 [cyclobutyl]) or in the diketopiperazine rings (such as ICRF-198 [diacid diamide] and ICRF-239 [methylimide] or ADR-559 [morpholinomethyl]) have protective effects against the chronic cardiomyopthy induced by doxorubicin in SHR, an animal model that we have characterized extensively in studies of the cardiotoxicity of anthracyclines [19, 21], and (2) whether this protective activity is related either to the rates of hydrolysis of these

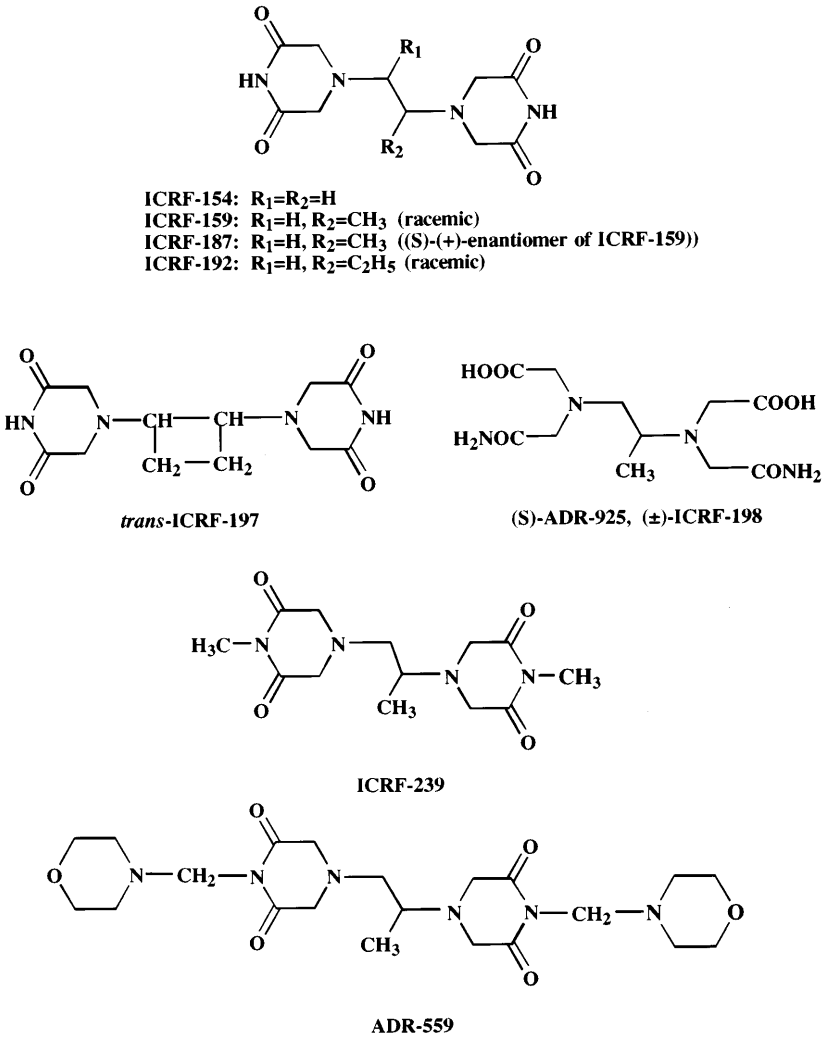

Fig. 1 Structures of the bisdiketopiperazines and their open-ring derivatives

compounds to form the corresponding open-ring derivatives or to the iron-chelating properties of these derivatives, as evaluated by measurements of their ability to remove iron from the Fe^{3+}–doxorubicin complex.

Materials and methods

Evaluation of the cardioprotective activity of the various bisdiketopiperazines

Adult male SHR (Aoki-Okamoto strain), weighing between 200 and 250 g, were acquired from Charles River Breeding Laboratories, Wellington, Mass. ICRF-154, ICRF-187, ICRF-198 and doxorubicin were provided by the Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, Md., and ICRF-192, ICRF-197 and ICRF-239 were obtained from Dr. Andrew Creighton, Cellular Pharmacology and Antitumor Laboratory, Imperial Cancer Research Fund, Lincolns Inn Field, London, UK. ADR-559 was supplied by Dr. Donald Witiak, Division of Medical Chemistry and Pharmacokinetics, College of Pharmacy, Ohio State University, Columbus, Ohio. The experimental protocol was approved by the Animal Use Committee of the Center for Drug Evaluation and Research of the Food and Drug Administration and was in compliance with the Guidelines for the Use and Care of Laboratory Animals (NIH publication no. 85–23).

A total of 60 SHR were divided into nine groups of 5 to 11 animals each, depending on the availability of the various bisdiketopiperazines. All compounds were dissolved to a final concentration of 10 mg/ml just prior to use. ICRF-187, ICRF-198, ICRF-239 and ADR-559 were dissolved in normal saline. ICRF-192 and ICRF-197 were initially dissolved in 2 ml dimethylsulfoxide (DMSO) and then diluted with 8 ml normal saline, while ICRF-154 was only soluble in 100% DMSO. The doses of the various bisdiketopiperazines and of doxorubicin were selected on the basis of the results of previous studies showing that pretreatment with 50 mg/kg ICRF-187 provides significant protection against the toxicity produced by 1 mg/kg doxorubicin when the two compounds are administered once a week for 12 weeks to SHR [21]. The first eight treatment groups of SHR were pretreated as follows: group 1 (10 animals), 0.2 ml of saline; group 2 (5 animals), 50 mg/kg ICRF-154; group 3 (11 animals), 50 mg/kg ICRF-187; group 4 (10 animals), 50 mg/kg ICRF-192; group 5 (6 animals), 50 mg/kg ICRF-197; group 6 (5 animals), 50 mg/kg ICRF-198; group 7 (6 animals), 50 mg/kg ICRF-239; and group 8 (6 animals), 88 mg/kg ADR-559 (this dose was selected in consideration of the contribution of the two morpholinomethyl groups to the molecular weight of ADR-559). All bisdiketopiperazines were given intraperitoneally (i.p.) 30 min prior to an intravenous (i.v.) injection of 1 mg/kg doxorubicin via the tail vein. The ninth group of SHR (saline controls; 11 animals) received an i.p. injection of saline followed in 30 min by an i.v. injection of saline. All substances were administered once a week for 12 weeks. Animals were weighed prior to each dosing throughout the experimental period.

One week after the last (12th) dosing, when the toxic effects of the final dose of doxorubicin reach a plateau, the animals were euthanized with an overdose of pentobarbital. Blood samples were then collected for hematological and clinical chemistry determinations (Metpath, Baltimore, Md.), and the animals underwent complete necropsies. Animals that died prior to the end of the study also underwent necropsy studies. The heart, kidneys and portions of the liver, lungs and small intestine were fixed in neutral buffered 10% formalin. The hearts were embedded in glycol methacrylate resin, sectioned at a thickness of 1-μ*M* and stained with hematoxylin-eosin and alkaline toluidine blue. The other tissues were embedded in paraffin, sectioned and stained with hematoxylin-eosin. The frequency and severity of doxorubicin-induced cardiac lesions were assessed by light microscopic examination of the plastic sections. The alterations were graded on a scale of 0 to 3 according to the method of Billingham [1]. A scoring scale of 0 to 4 was used to evaluate the severity of renal damage, which was graded as described in detail previously [21].

Statistical analysis

The Mann-Whitney test was utilized to determine the significance of differences in the severity of cardiomyopathy and nephropathy scores among the various treatment groups. Student's *t*-test and Tukey-Kramer multiple comparisons tests were used to assess differences in body weights and hematological values between the groups

Evaluation of iron binding by the various bisdiketopiperazines

The Fe^{3+}–doxorubicin complex was prepared at a 3:1 drug to Fe^{3+} ratio under slightly acidic conditions, as previously described [3, 9, 10]. A small amount of the Fe^{3+}–doxorubicin complex was added to a thermostatted (25 °C) 1-cm cell containing Tris/KCl buffer (50 m*M*/150 m*M*; pH, 7.4) in a Hamamatsu UV-260 spectrophotometer to give a final Fe^{3+} concentration of 15 μ*M* and a final doxorubicin concentration of 45 μM. The displacement of Fe^{3+} from the Fe^{3+}–doxorubicin complex was followed by recording complete spectra (375 to 650 nm) at fixed time intervals after the addition of the bisdiketopiperazine or its hydrolysis product. The hydrolysis of the bisdiketopiperazines was similarly followed (190 to 330 nm) in NH_3/KCl buffer (50 m*M*/150 m*M*; pH 9.3) at 37 °C at a drug concentration of 50 μ*M*). A pH of 9.3 was chosen in order to solubilize the drugs, which are partly ionized at this pH [11] and to speed up the reaction.

Results

General toxicity and weight changes

Eight of ten SHR given doxorubicin alone died prior to the 13th week of experimentation (Seven after the 12th dose and one after the 10th dose; Table 1). All animals pretreated with ICRF-198 or ICRF-239 died after the 11th or 12th injection of doxorubicin. In contrast, all of the SHR pretreated with ICRF-187 or ICRF-197 survived until the end of the study. Partial protection against the lethal effects of doxorubicin occurred in animals pretreated with ICRF-154, ICRF-192 or ADR-559 (Table 1).

Animals given saline without doxorubicin gained an average of 77 ± 15 g of body weight over the course of the study, whereas the average body weight of the surviving SHR receiving doxorubicin alone did not change (+1 ± 37 g) over the same period. The most significant increase in body weight in the groups pretreated with the various bisdiketopiperazines occurred in those animals receiving ICRF-187 (37 ± 20 g) and ADR-559 (63 ± 52 g). Animals in other pretreatment groups had either minimal increases (ICRF-154, ICRF-197 and ICRF-239) or decreases (ICRF-192 and ICRF-198) in body weight (Table 1).

Gross anatomic changes

Excessive amounts of pericardial and peritoneal fluids were observed in a majority of the animals given

Table 1 Mortality and changes in body weight in SHR treated with bisdiketopiperazines and doxorubicin (DXR). SHR received 12 weekly doses, consisting of 50 mg/kg, i.p. of the bisdiketopiperazines, except for ADR-559, which because of its larger molecular weight was given in a dose of 88 mg/kg. This was followed in 30 min by 1 mg/kg i.v. of doxorubicin

Treatment	Change in body weight (g)	Number SHR dead prior to end of study	Cumulative DXR dose (mg/kg) at time of death
DXR	+1 ± 37*	8/10	10(1), 12(7)
ICRF-154 DXR	+3 ± 33	2/5	9(1), 10(1)
ICRF-187 DXR	+37 ± 33	0/11	–
ICRF-192 DXR	–13 ± 29*	6/11	10(1), 11(4), 12(1)
ICRF-197 DXR	+6 ± 40*	0/6	–
ICRF-198 DXR	–8 ± 61*	5/5	11(5)
ICRF-239 DXR	+16 ± 9*	6/6	11(4), 12(2)
ADR-559 DXR	+63 ± 52	2/6	9(1), 12(1)
Saline	+77 ± 15	0/11	

*$P < 0.05$ vs ICRF-187/DXR group and vs Saline group; Student's *t*-test

doxorubicin alone or pretreated with the various bisdiketopiperazines except ICRF-187. Fluid accumulation was noted in only 1 of 11 SHR pretreated with ICRF-187. The kidneys of SHR given doxorubicin with or without the various bisdiketopiperazines appeared pale compared to those of the saline-treated control animals.

Myocardial pathology

Doxorubicin caused myocyte alterations consisting of cytoplasmic vacuolization and loss of myofibrils. These changes were identical to those reported previously in SHR given 1 mg/kg doxorubicin weekly over a 12-week period [19, 21, 32]. Both types of alterations often occurred in the same cell and involved greater numbers of myocytes as the lesions increased in severity. Damaged cells were diffusely distributed throughout the myocardium. Data on the incidence and severity of the myocardial lesions in the various treatment groups are presented in Table 2.

The SHR treated with doxorubicin alone had severe lesions (Table 2), as did those treated with doxorubicin plus ICRF-192 and ICRF-198. The severity of myocardial lesions was significantly reduced in animals pretreated with ICRF-154, ICRF-187, ICRF-197, ICRF-239 and ADR-559 ($P < 0.05$, Table 2). Lesion scores of 1.0 to 1.5 were observed in SHR pretreated with ICRF-154, ICRF-187 and ADR-559. The protective effects of these three compounds were significantly greater than those of ICRF-197 and ICRF-239 ($P < 0.05$).

Pathology of noncardiac tissues

Doxorubicin induced severe renal alterations, including vacuolization and sclerosis of the glomeruli, dilatation of the tubules with intraluminal protein casts and interstitial lymphocytic infiltration. The renal lesion scores in animals given doxorubicin alone ranged from mild to severe (Table 3). All SHR, except in one instance, pretreated with the various bisdiketopiperazines prior to doxorubicin developed renal alterations (Table 3). However, none of the lesions was severe (lesion score of 4) in these animals. A significant decrease in the severity of the renal lesions occurred in SHR pretreated with ICRF-187, ICRF-197, ICRF-198, ICRF-239 and ADR-559, compared to the animals receiving doxorubicin alone ($P < 0.05$, Table 3). However, there was no significant difference in the degree of renal protective activity among these five compounds. The kidneys from control animals receiving saline had normal morphology.

Hematologic studies

Treatment of SHR with doxorubicin caused significant decreases in red blood cell count, hemoglobin concentration and hematocrit, in comparison with the values obtained from the animals receiving only saline ($P < 0.05$, Table 4). Furthermore, significant decreases in these three hematologic values were also observed in surviving animals pretreated with ICRF-154, ICRF-187, ICRF-192, ICRF-197 and ADR-559 versus the control

Table 2 Cardiomyopathy scores in SHR treated with bisdiketopiperazines and doxorubicin (DXR).Treatment groups are as described in Table 1

Treatment	No. of animals	Myocardial lesions score					
		0	1	1.5	2.0	2.5	3.0
DXR	10	0	0	0	1	2	7
ICRF-154/DXR*	5	0	2	3	0	0	0
ICRF-187/DXR*	11	0	8	3	0	0	0
ICRF-192/DXR	10	0	0	0	1	4	5
ICRF-197/DXR*	6	0	0	0	4	1	1
ICRF-198/DXR	5	0	0	0	1	1	3
ICRF-239/DXR*	6	0	0	0	6	0	0
ADR-559/DXR*	6	0	3	2	0	0	0
Saline	11	11	0	0	0	0	0

*$P < 0.05$ vs DXR only group; Mann-Whitney test

Table 3 Nephropathy scores in SHR treated with bisdiketopiperazines and doxorubicin (DXR). Treatment groups are as described in Table 1

Treatment	No. of animals	Renal lesion score				
		0	1	2	3	4
DXR	10	0	0	3	3	3
ICRF-154/DXR	5	0	1	1	3	0
ICRF-187/DXR*	11	0	4	4	3	0
ICRF-192/DXR	11	0	1	4	6	0
ICRF-197/DXR*	6	0	3	3	0	0
ICRF-198/DXR*	5	0	1	3	1	0
ICRF-239/DXR*	6	0	3	1	2	0
ADR-559/DXR*	6	1	2	1	1	0
Saline	11	11	0	0	0	0

*$P < 0.05$ vs DXR only group; Mann-Whitney test

Table 4 White blood cell and red blood cell counts, hemoglobin concentrations, and hematocrit values in SHR treated with bisdiketopiperazines and doxorubicin (DXR). Treatment groups are as described in Table 1. Values are mean ± SD from 4 to 10 animals

Treatment	White blood cells ($\times 10^3$)	Red blood, cells ($\times 10^3$)	Hemoglobin (g/100 ml)	Hematocrit (%)
DXR	2.9 ± 0.4	3.9 ± 0.2**	7.5 ± 0.5**	22.7 ± 1.3**
ICRF-154/DXR	3.3 ± 0.3	4.0 ± 0.8**	8.2 ± 1.1**	21.1 ± 5.2**
ICRF-187/DXR	6.5 ± 2.4	6.4 ± 1.5*,**	11.9 ± 2.1*,**	33.4 ± 7.6*,**
ICRF-192/DXR	5.3 ± 0.7	4.8 ± 1.1**	8.0 ± 1.1**	24.2 ± 4.8**
ICRF-197/DXR	6.7 ± 2.4	6.6 ± 0.9*,**	12.8 ± 1.7*,**	37.2 ± 3.2*
ADR-559/DXR	11.8 ± 5.8*,**	5.7 ± 1.3**	10.7 ± 2.0**	32.6 ± 5.7**
Saline control	4.3 ± 1.5	9.8 ± 0.15	15.7 ± 0.4	45.3 ± 2.1

*$P < 0.05$ vs DXR only group; **$P < 0.05$ vs Saline group; Tukey-kramer multiple comparisons test

group ($P < 0.05$, Table 4). White blood cell counts were not significantly altered in any of the doxorubicin-treated groups except in animals pretreated with ADR-559. In this group, the white blood cell counts were significantly higher than those found in the groups receiving saline only, doxorubicin alone or the other ICRF compounds ($P < 0.05$, Table 4). Moreover, the red blood cell count, the hemoglobin concentration and the hematocrit were significantly higher in SHR pretreated with ICRF-187 and ICRF-197 than in those receiving only doxorubicin ($P < 0.05$, Table 4).

Ring-opening hydrolysis of the bisdiketopiperazines

We have previously shown [10, 11] that the ring-opening hydrolysis of ICRF-187 to its diacid diamide results in UV spectral changes that allow the reaction to be followed as a function of time. The drugs listed in Table 5 showed spectral changes that were very similar to those observed with ICRF-187 [11]. The decrease in absorbance at 227 nm was used to follow the ring-opening hydrolysis reaction. As described previously [11], the absorbance-time data were fitted to a three-parameter exponential decay equation to yield the first order rate constant k_{obs}. In the case of ICRF-187, the opening of each of the two diketopiperazine rings occurs with the same rate constant [13]. The good exponential fits that were observed indicate that this was occurring with the analogues as well.

Table 5 First-order rate constants for the ring-opening hydrolysis of various bisdiketopiperazines measured spectrophotometrically at a wavelength of 227 nm in NH_3/KCl buffer (pH 9.3) at 37 °C. The first-order rate constants were obtained by fitting the absorbance-time data to three-parameter exponential decay equation. The errors are fitting errors only from the nonlinear least squares

Drug	k_{obs} (min^{-1})	$t_{1/2}$ (min)
ICRF-154	1.60 ± 0.02	43
ICRF-187	2.11 ± 0.01	33
ICRF-192	2.01 ± 0.01	34
ICRF-197	1.93 ± 0.01	36
ADR-559	1.81 ± 0.01	38

The $t_{1/2}$ for the ICRF-187 reaction was 0.55 h (Table 5) at pH 9.3 compared to a $t_{1/2}$ of 16.3 h at pH 7.4 also at 37 °C [11]. Since the hydrolysis is largely base-catalyzed at pH 7.4 [11], the first-order rate constants obtained at pH 9.3 (Table 5) should be nearly in proportion to those at pH 7.4. The results shown in Table 5 indicate that, in spite of significant differences in the central chains of the bisdiketopiperazines, the rate constants for hydrolysis of these compounds do not vary significantly. Even the N-substituted morpholinomethyl derivative (ADR-559) undergoes hydrolysis at a rate similar to that of the other analogues. These results suggest that under these basic conditions ADR-559 undergoes rapid hydrolysis to ICRF-159 (the racemic form of ICRF-187), which then undergoes ring-opening hydrolysis.

Table 6 Reaction of the Fe^{3+}–doxorubicin complex with the hydrolysis products and the parent compounds of various bisdiketopiperazines. The measurements were made spectrophotometrically at a wavelength of 600 nm in Tris/KCl buffer (pH 7.4) at 25 °C. The first-order rate constants (k_{obs}) were obtained by fitting the absorbance-time data to a three-parameter exponential decay equation. The errors are fitting errors from the nonlinear least squares analysis. *na* not applicable

Drug	Reaction with Hydrolysis product		Reaction with Parent drug	
	$t_{1/2}$ (min)	% Fe^{3+} removed	$k_{obs} \times 10^2$ (min^{-1})	% Fe^{3+} removed
ICRF-154	6.5	83	2.42 ± 0.06	79[a]
ICRF-187	6.6	82	3.51 ± 0.06[a]	83
ICRF-192	8.1	88	2.47 ± 0.04	82
ADR-559	8.3	71	0.34 ± 0.01	28[b]
ICRF-198	6.6	83	na	na
EDTA	5.0	87	na	na

[a]In 8% DMSO to increase the solubility
[b]At 110 min, as the reaction was incomplete

Removal of Fe^{3+} from the Fe^{3+}–doxorubicin complex by the open-ring hydrolysis products of the bisdiketopiperazines

We have previously demonstrated that ADR-925 is able to quickly and efficiently displace Fe^{3+} from the Fe^{3+}–doxorubicin complex [3, 9, 10]. The open-ring derivatives of the analogues were also examined to determine the rate at which they displace Fe^{3+} from the Fe^{3+}–doxorubicin complex and to evaluate the correlation between this rate of removal and the cardioprotective activity of each parent compound. The open-ring derivatives of the ICRF compounds were prepared by hydrolyzing the drugs in NH_3/KCl buffer for 4.5 h at 37 °C. This corresponds to about eight half-lives (Table 5), which is sufficient to cause nearly 100% hydrolysis. The spectral changes observed upon the addition of 100 μ*M* hydrolysis product to the Fe^{3+}–doxorubicin complex were very similar to those found for ICRF-198 [3, 9]. The removal of Fe^{3+} from the Fe^{3+}–doxorubicin complex was followed at 600 nm, where the complex has an absorption band. A fast initial drop in absorbance was observed during the first minute, followed by a slower process that occurred over several minutes. The $t_{1/2}$ values given in Table 6 were calculated for this second slower process from the absorbance-time data at times longer than 2.4 min. The percentage of Fe^{3+} removed from the Fe^{3+}–doxorubicin complex was estimated from the absorbance at 600 nm at 40 min at the completion of the reaction relative to the absorbances of the Fe^{3+}–doxorubicin complex (A^{600} 0.138) and uncomplexed doxorubicin (A^{600} 0.007). The hydrolysis products of the bisdiketopiperazines were able to efficiently displace Fe^{3+} from the Fe^{3+}–doxorubicin complex, as were EDTA and ICRF-198. The hydrolysis products of ICRF-154, ICRF-192 and ADR-559 were about as effective as that of ICRF-187 in displacing Fe^{3+} from the Fe^{3+}–doxorubicin complex (Table 6).

Removal of Fe^{3+} from the Fe^{3+}–doxorubicin complex by the bisdiketopiperazines

We have previously shown [3, 10] that the Fe^{3+}–doxorubicin complex promotes the hydrolysis of ICRF-187, thereby resulting in removal of Fe^{3+} from the Fe^{3+}–doxorubicin complex. The spectral changes that were observed upon mixing 300 μ*M* of the drug with Fe^{3+}–doxorubicin at pH 7.4 were consistent with the removal of Fe^{3+} from the Fe^{3+}–doxorubicin complex. The first order rate constants k_{obs} for this reaction (Table 6) were determined by fitting the absorbance-time data at 600 nm as described previously [3, 11]. Except for ADR-559, the rate constants varied by a factor of less than two. The final percentage of Fe^{3+} removed was similar to that found for the hydrolyzed forms of the drugs. The slower rate of reaction of ADR-559 probably reflects the time necessary for the morpholinomethyl group to be hydrolyzed to produce ICRF-159, which then reacts with Fe^{3+}–doxorubicin.

Discussion

The results of the present study provide the first detailed documentation of the protective effects of a number of bisdiketopiperazines against the cardiotoxicity and nephrotoxicity induced in SHR by the chronic administration of doxorubicin. Among the compounds tested, only ICRF-154 and ADR-559 provided a degree of cardioprotection (lesion scores of 1 or 1.5) similar to that observed with ICRF-187, the prototype drug of this class of agents. ICRF-197 and ICRF-239 provided significant but less effective protection (majority of lesion scores 2.0), whereas ICRF-192 and ICRF-198 were not cardioprotective under the conditions of the present study. The patterns of protection against the cardiac and the renal toxicity of doxorubicin were different in some respects. ICRF-187, ICRF-197 and ADR-559 were most protective against the nephrotoxicity, whereas ICRF-154, ICRF-192 and ICRF-198 did not significantly alter

this toxicity. It should be noted that the renal toxicity of doxorubicin and other anthracyclines occurs in rodents but not in humans [2]. The reason for this difference is not known. ICRF-187, ICRF-197 and ADR-559 were most effective in ameliorating the decreases induced by doxorubicin in red blood cell counts, hemoglobin and hematocrit.

Mechanisms of protection by bisdiketopiperazines

Our understanding of the mechanisms of protection by bisdiketopiperazines is limited. In the case of ICRF-187, it is generally accepted that cardioprotection is mediated through intracellular iron binding, an action which decreases the site-specific formation of reactive oxygen intermediates [8, 9]. We have recently shown that ICRF-187 also inhibits the apoptosis (and also the necrosis) induced by doxorubicin in epithelial cells of the intestine and renal tubules [34]. However, we have also shown that this effect is not operational in myocardium, since doxorubicin does not induce apoptosis in cardiac myocytes in SHR under conditions identical to those employed in the present study [34]. Although these differences remain unexplained, they clearly indicate that different mechanisms contribute to the toxicity induced by doxorubicin in various organ systems.

The following characteristics of the various bisdiketopiperazines must be taken into consideration in interpreting the results of the present study: (1) aqueous solubility; (2) ability to penetrate into cells by passing in a nonpolar form through the plasma membrane; (3) rate of conversion to an open-ring iron-chelating derivative, and (4) affinity of this derivative for iron (III), particularly as demonstrated by its ability to displace iron from the Fe^{3+}–doxorubicin complex. In addition, consideration must be given to the relative ability of the bisdiketopiperazines and their open-ring derivatives to inhibit DNA topoisomerase II. This effect, which appears to be unrelated to iron chelation, is considered to be an important determinant of the antineoplastic effect of the bisdiketopiperazines [25, 30]; however, its relevance to the cardioprotective effects of these agents remains to be fully evaluated.

Solubility of bisdiketopiperazines

The solubilities of different bisdiketopiperazines vary considerably. Both ICRF-186 and ICRF-187 are much more water-soluble than ICRF-159, the corresponding racemic compound [27]. Both agents exerted significant cardiac and renal protective activity. ADR-559, which also was highly protective against cardiotoxicity and nephrotoxicity, also is highly soluble. It is necessary to point out that the two morpholinomethyl groups are readily hydrolyzed from the ADR-559 molecule, so that this compound becomes ICRF-159, the open-ring derivative of which is ICRF-198. ICRF-239, another relatively soluble compound, did not protect against doxorubicin-induced lethality but did attenuate the cardiac and renal damage. ICRF-192 and ICRF-197 are less soluble and had to be prepared in DMSO and saline. ICRF-197 attenuated both the cardiac and renal toxicity, but ICRF-192 did not alter either toxicity. ICRF-154 is very insoluble and had to be dissolved in DMSO. Although this limited solubility constitutes a significant practical problem, ICRF-154 was very highly protective against the cardiac toxicity of doxorubicin. Nevertheless, this agent was not protective against doxorubicin nephrotoxicity, and the reasons for this discrepancy are not known. It is evident from the preceding considerations that the differences in the attenuation of doxorubicin toxicity do not correlate with the solubilities of the various bisdiketopiperazines.

Cellular uptake of bisdiketopiperazines

Cellular uptake is another property that could influence the biological activity of the ICRF compounds. Only very limited information is available on the rate of penetration of bisdiketopiperazines into cells. Dawson [7] compared the uptake of ICRF-154, ICRF-159 and ICRF-192 into cultured BHK-21S cells and showed that differences in cytotoxic activity against these cells were independent of cellular uptake, as the reactive compounds (ICRF-154 and ICRF-159) and the inactive compound (ICRF-192) entered the cells at the same rate by simple diffusion. The uptake of ICRF-187 into isolated adult rat cardiac myocytes is extremely rapid, as maximal levels are detected within 60 s of exposure to the drug [8]. The intracellular accumulation of ICRF-187 was not altered by changes in temperature or ATP status, again suggesting that simple diffusion is the driving force for this process [8]. Thus, it does not seem likely that differences in cellular uptake account for the variations in the cardioprotective activity exerted by the bisdiketopiperazines. In addition, we attribute the lack of protective activity of ICRF-198 to poor penetration of this highly polar derivative across cell membranes, although the uptake of this compound into cells has not been specifically evaluated.

Formation of open-ring, iron-chelating derivatives

The data in the present study demonstrate only relatively minor differences in the rates of ring-opening of bisdiketopiperazines to form the metal-chelating derivatives. It should be emphasized that in the present study these rates were determined on the basis of experiments in which the reaction occurred nonenzymatically at an alkaline pH (at which the reaction proceeds more rapidly than at a neutral pH). Other studies have shown that this ring opening also can be catalyzed by an enzyme present in liver [12]; however, such an enzyme has not yet been identified in heart. In isolated adult

myocytes, the ring opening of ICRF-187 occurs within 60 s [8]. Under the conditions of the present study, the rates of formation of the open-ring derivatives of ICRF-187, ICRF-154 and ADR-559, the most cardioprotective compounds, were 2.11 ± 0.1, 1.60 ± 0.02 and 1.81 ± 0.01 min^{-1}, respectively, which did not differ significantly from a rate of 1.93 ± 0.01 min^{-1} for ICRF-197, which was less protective, and 2.01 ± 0.01 min^{-1} for ICRF-192, which was not protective. These data indicate that differences in the cardioprotective activity of these compounds are not related to the rates of formation of their open ring iron–chelating derivatives.

Affinity of open-ring derivatives for iron (III)

The ability of the open-ring derivative of the various bisdiketopiperazines to remove iron from the iron–doxorubicin complex did not vary greatly. The $t_{1/2}$ values for this reaction were 6.6, 6.5 and 8.3 min for the derivatives of ICRF-187, ICRF-154 and ADR-559, respectively. These rates were only slightly different from that of 8.8 min for ICRF-192, which was not cardioprotective. The rate of iron removal for ICRF-198 (the racemic mixture of the open-ring derivatives of ICRF-186 and ICRF-187) was similar to that reported previously for ADR-925 (the open-ring derivative of ICRF-187) [9]. Thus, these results indicate that the differences in cardioprotective activity among the various bisdiketopiperazines are not a function of their relative abilities to displace iron from the doxorubicin–iron complex.

Cytotoxicity and inhibition of DNA topoisomerase IIby bisdiketopiperazines

In studies of structure–activity relationships, Creighton et al. [5] found large differences in cytotoxic activity among a series of structural analogues of ICRF-159. Both ICRF-154 and ICRF-159 exerted similar degrees of inhibitory activity toward the proliferation of BHK-21S cells, while ICRF-193 and ICRF-202 were at least 50 times more potent [5]. In contrast, ICRF-192 was essentially inactive. ICRF-154, ICRF-159 and ICRF-193 have been shown to be inhibitors of DNA topoisomerase II [25, 30].

Hasinoff et al. [14] examined the ability of ICRF-154, ICRF-187, ICRF-192 and ICRF-198 to inhibit the growth of Chinese hamster ovary (CHO) cells and the activity of DNA topoisomerase II. These investigators found significant differences in the growth-inhibiting ability among various bisdiketopiperazines. These findings were almost identical to those reported earlier for BHK-21S cells by Creighton et al. [5] in that ICRF-193 was the most potent, ICRF-154, ICRF-159 and ICRF-187 were intermediate in potency, and ICRF-192 and ADR-925 were essentially inactive. The degree of cytotoxicity toward the CHO cells showed a high degree of correlation with the extent to which these agents inhibited the activity of DNA topoisomerase II [14].

Increased cytotoxicity could be a factor in the high incidence of deaths (> 50%) which occurred in the groups of animals pretreated with ICRF-192, ICRF-198 and ICRF-239. However, the results are interpreted as indicating a lack of protective activity, since almost all the deaths in these groups occurred within a similar time frame to that of animals dying in the group that received doxorubicin alone.

At present it has not been established to what extent DNA topoisomerase II participates in the pathogenesis of anthracycline cardiotoxicity. DNA topoisomerase II is targeted by both doxorubicin and by several bisdiketopiperazines [6, 14]. The mechanism of the cytoxicity of doxorubicin is thought to involve the stabilization by the drug of the "cleavable complex" formed by DNA and the enzyme [6]. This stabilized complex acts as a cellular poison [6]. Among the best cardioprotectors, ICRF-154 and ICRF-187 also are reasonably good inhibitors of DNA topoisomerase II (IC_{50} of 7.3 and 3 μM, respectively), whereas ICRF-192 and ADR-925 are weak inhibitors of the enzyme (IC_{50} of 91 and > 100 μM, respectively) and lack cardioprotective activity [14]. Compared to that by doxorubicin, the inhibition of DNA topoisomerase II by bisdiketopiperazines occurs at a different point in the series of reactions involving the enzyme and is mediated by the formation of a closed protein clamp [28]. The assay used to examine inhibition of topoisomerase II was performed in nuclear extracts. Consequently, penetration of the bisdiketopiperazines into the cells is not a factor affecting the drugs' interactions with the enzyme [28]. Nevertheless, the role of such an inhibition in mediating the protective effects of ICRF-187 on the cardiotoxicity of doxorubicin remains unclear, because ADR-925 (which, as discussed previously, is the iron-chelating derivative of ICRF-187 and is formed very rapidly after cellular uptake of the parent compound) does not inhibit DNA topoisomerase II [14]. The intrinsic properties responsible for the differences in cardioprotective activity among the bisdiketopiperazines and the possible alterations induced by these agents in the actual formation of reactive oxygen intermediates remain to be determined.

References

1. Billingham ME (1991) Role of endomyocardial biopsy in diagnosis and treatment of heart disease. In: Silver MD (ed) Cardiovascular pathology, 2nd edn. Churchill Livingstone, New York, p 1465
2. Burke JF Jr, Laucius JF, Brodovsky HS, Soriano RZ (1977) Doxorubicin hydrochloride-associated renal failure. Arch Intern Med 137: 385
3. Buss JL, Hasinoff BB (1993) The one-ring open hydrolysis product intermediates of the cardioprotective agent ICRF-187 (dexrazoxane) displace iron from iron-anthracycline complexes. Agents Actions 40: 86
4. Creighton AM, Hellmann K, Whitecross S (1969) Antitumour activity in a series of bisdiketopiperazines. Nature 222: 384
5. Creighton AM, Jeffery WA, Long J (1979) Bisdioxopiperazines. In: Simkins MH (ed) Medicinal chemistry I. Proceedings

of the 6th International Symposium on Medicinal Chemistry. Research Studies Press, Forrest Grove, p 281
6. D'Arpa P, Liu LF (1989) Topoisomerase-targeting antitumor drugs. Biochim Biophys Acta 989: 163
7. Dawson KM (1975) Studies on the stability and cellular distribution of dioxopiperazines in cultured BHK-21S cells. Biochem Pharmacol 24: 2249
8. Doroshow JH (1995) Role of reactive-oxygen metabolism in cardiac toxicity of anthracycline antibiotics. In: Priebe W (ed) Anthracycline antibiotics: new analogues, methods of delivery, and mechanisms of action. American Chemical Society, Washington, p 259
9. Hasinoff BB (1989) The interaction of the cardioprotective agent ICRF-187 (+)-1,2-bis(3,5-dioxopiperazinyl-1-yl)propane); its hydrolysis product (ICRF-198); and other chelating agents with the Fe(III) and Cu(II) complexes of adriamycin. Agents Actions 26: 378
10. Hasinoff BB (1990) The iron(III) and copper(II) complexes of adriamycin promote the hydrolysis of the cardioprotective agent ICRF-187 ((+)-1,2-bis(3,5-dioxopiperazinyl-1-yl)propane). Agents Actions 29: 374
11. Hasinoff BB (1990) The hydrolysis activation of the doxorubicin cardioprotective agent ICRF-187 [+)-1,2-bis(3,5-dioxopiperazinyl-1-yl)propane). Drug Metab Dispos 18: 344
12. Hasinoff BB (1993) Enzymatic ring-opening reactions of the chiral cardioprotective agent (+) (S)-ICRF-187 and its (−) (R)-enantiomer ICRF-186 by dihydropyrimidine amidohydrolase. Drug Metab Dispos 21: 883
13. Hasinoff BB (1994) An HPLC and spectrophotometric study of the hydrolysis of ICRF-187 (dexrazoxane, (+)-1,2-bis(3,5-dioxopiperazinyl-1-yl)propane) and its one-ring opened intermediates. Int J Pharm 107: 67
14. Hasinoff BB, Kuschak TI, Yalowich JC, Creighton AM (1995) A QSAR study comparing the cytotoxicity and DNA topoisomerase II inhibitory effects of bisdioxopiperazine analogs of ICRF-187 (dexrazoxane). Biochem Pharmacol 50: 953
15. Herman EH, Ferrans VJ (1981) Reduction of chronic doxorubicin cardiotoxicity in dogs by pretreatment with (+/−)-1,2-bis(3,5-dioxopiperazinyl-1-yl)propane (ICRF-187). Cancer Res 41: 3436
16. Herman EH, Mhatre RM, Chadwick DP (1974) Modification of some of the toxic effects of daunomycin (NSC-82, 151) by pretreatment with the antineoplastic agent ICRF 159 (NSC-129, 943). Toxicol Appl Pharmacol 27: 517
17. Herman E, Ardalan B, Bier C, Waravdekar V, Krop S (1979) Reduction of daunorubicin lethality and myocardial cellular alterations by pretreatment with ICRF-187 in Syrian golden hamsters. Cancer Treat Rep 63: 89
18. Herman EH, EL-Hage AN, Creighton AM, Witiak DT, Ferrans VJ (1985) Comparison of the protective effect of ICRF-187 and structurally related analogues against acute daunorubicin toxicity in Syrian golden hamsters. Res Commun Chem Pathol Pharmacol 48: 39
19. Herman EH, EL-Hage AN, Ferrans VJ, Ardalan B (1985) Comparison of the severity of the chronic cardiotoxicity produced by doxorubicin in normotensive and hypertensive rats. Toxicol Appl Pharmacol 78: 202
20. Herman EH, Ferrans VJ, Bhat HB, Witiak DT (1987) Reduction of chronic doxorubicin cardiotoxicity in beagle dogs by bis-morpholinomethyl derivative of razoxane (ICRF-159). Cancer Chemother Pharmacol 19: 277
21. Herman EH, El-Hage A, Ferrans VJ (1988) Protective effect of ICRF-187 on doxorubicin-induced cardiac and renal toxicity in spontaneously hypertensive (SHR) and normotensive (WKY) rats. Toxicol Appl Pharmacol 92: 42
22. Herman EH, Ferrans VJ, Sanchez JA (1992) Methods of Reducing the Cardiotoxicity of Anthracyclines. In: Muggia FM, Green MD, Speyer JL (eds) Cancer treatment and the heart. Johns Hopkins University Press, Baltimore, p 114
23. Huang Z-X, May PM, Quinlan KM, Williams DR, Creighton AM (1982) Metal binding by pharmaceuticals. Part 2. Interactions of Ca(II), Cu(II), Fe(II), Mg(II), Mn(II) and Zn(II) with the intracellular hydrolysis products of the antitumour agent ICRF 159 and its inactive homologue ICRF 192. Agents Actions 12: 536
24. Huang Z-X, Creighton AM, Williams DR (1985) Metal binding properties of two homologues of razoxane, a bisdioxopiperazine antitumor drug. Inorg Chem Acta 107: L29
25. Ishida R, Miki T, Narita T, Yui R, Sato M, Utsumi KR, Tanabe K, Andoh T (1991) Inhibition of intracellular topoisomerase II by antitumor bis(2,6-dioxopiperazine) derivatives: mode of cell growth inhibition distinct from that of cleavable complex-forming type inhibitors. Cancer Res 51: 4909
26. Myers CE, Gianni L, Simone CB, Klecker R, Greene R (1982) Oxidative destruction of erythrocyte ghost membranes catalyzed by the doxorubicin-iron complex. Biochemistry 21: 1707
27. Repta AJ, Baltezor MJ, Bansal PC (1976) Utilization of an enantiomer as a solution to a pharmaceutical problem: application to solubilization of 1,2-di(4-piperazine-2,6-dione) propane. J Pharm Sci 65: 238
28. Roca J, Ishida R, Berger JM, Andoh T, Wang JC (1994) Antitumor bisdioxopiperazines inhibit yeast DNA topoisomerase II by trapping the enzyme in the form of a closed protein clamp. Proc Natl Acad Sci USA 91: 1781
29. Speyer JL, Green MD, Zeleniuch-Jacquotte A, Wernz JC, Rey M, Sanger J, Kramer E, Ferrans V, Hochster H, Meyers M (1992) ICRF-187 permits longer treatment with doxorubicin in women with breast cancer [published erratum appears in J Clin Oncol 1992 May; 10(5):867]. J Clin Oncol 10: 117
30. Tanabe K, Ikegami Y, Ishida R, Andoh T (1991) Inhibition of topoisomerase II by antitumor agents bis(2,6-dioxopiperazine) derivatives. Cancer Res 51: 4903
31. Wexler LH, Andrich MP, Venzow D, Berg SL, Weaver-McClure L, Chen CC, Dilsizan V, Auila N, Jaronski P, Balis FM, Poplack DG, Horowitz ME (1996) Randomized trial of the cardioprotective agent ICRF-187 in pediatric sarcoma patients treated with doxorubicin. J Clin Oncol 14: 362
32. Zhang J, Herman EH, Ferrans VJ (1993) Dendritic cells in the hearts of spontaneously hypertensive rats treated with doxorubicin with or without ICRF-187. Am J Pathol 142: 1916
33. Zhang J, Herman EH, Ferrans VJ (1994) Effects of ICRF-186 [(L)1,2-bis(3,5-dioxopiperazinyl-1-yl)propane] on the toxicity of doxorubicin in spontaneously hypertensive rats. Toxicology 92: 179
34. Zhang J, Clark JR Jr, Herman EH, Ferrans VJ (1996) Doxorubicin-induced apoptosis in spontaneously hypertensive rats: differential effects in heart, kidney and small intestine, and inhibition by ICRF-187. J Mol Cell Cardiol 28: 1931