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Reduction of chronic doxorubicin cardiotoxicity in beagle dogs by bis-morpholinomethyl derivative of razoxane (ICRF-159)

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Summary. Addition of morpholinomethyl substituents to razoxane (ICRF-159) produced a compound (bis-4-morpholinomethyl-3,5-dioxopiperazinyl-1,2-propane (MM-159) considerably more water-soluble than razoxane. The increased solubility allowed MM-159 to be examined for protective activity against chronic doxorubicin (DXR) cardiotoxicity. Adult beagle dogs of either sex were given, i. v. at 3-week intervals, either DXR (1.75 mg/kg) alone or DXR 15 min after MM-159 (25 mg/kg). Control animals received MM-159 (25 mg/kg) or saline without DXR. The experiment was terminated 3 weeks after the ninth injection (total DXR dose, 15.75 mg/kg). Of the eight animals given DXR alone, five died after receiving seven to eight injections (12.25–14 mg/kg DXR) and the remaining three were killed after eight injections because they were in poor condition. Marked ascites was noted in four of these eight dogs. When frequency and extent of myocardial lesions (vacuolation and myofibrillar loss) were assessed on a scale from 0 to 4+, severe lesions (3+) were present in all eight dogs given DXR alone, but no abnormalities (lesion score 0) were found in the hearts of three of eight dogs given MM-159 and DXR and the five remaining animals in this group had minimal (1+; four dogs) or mild (2+; one dog) alterations. DXR reduced the erythrocyte count, hemoglobin, and hematocrit when administered alone, but not in combination with MM-159. Such protection against DXR hematologic effects was not noted previously [14] when dogs were pretreated with ICRF-187, the *d*-isomer of razoxane, despite the fact that pretreatment with ICRF-187 was as effective as MM-159 in reducing chronic DXR cardiotoxicity. It remains to be determined whether there are other differences in biological activity between MM-159 and ICRF-187.

Introduction

Certain bis(dioxopiperazines) have been shown to elicit antitumor activity against a variety of in vitro tumor systems [5, 21]. One of these compounds, razoxane [ICRF-159; *dl* 1,2-bis(3,5-dioxopiperazinyl)propane] was subsequently found also to possess clinical antitumor activity [7, 13, 15, 16]. In experimental studies, pretreatment with razoxane significantly reduced high-dose daunorubicin toxicity [10]. However, a major problem in the use of razoxane in all studies was its low solubility in water. The use of cosolvents, complexation, chemically derived prodrugs or crystalline modification to overcome the low solubility were unsuccessful [20]. However, a significant increase in solubility was noted when razoxane was resolved into the individual *d*- and *l*- enantiomers [20]. ICRF-187, the more water-soluble *d*-isomer of razoxane, was examined in a number of animal models of anthracycline toxicity and was found [11] to reduce the acute toxicity induced by high doses of daunorubicin, in a manner similar to that seen with razoxane. Pretreatment with ICRF-187 also reduced chronic anthracycline-induced cardiotoxicity in rabbits, miniature pigs, and dogs [8, 9, 12–14]. A number of analogues of razoxane have been synthesized [15]. One of these, bimolane, a morpholinomethyl derivative of the bis-(dioxopiperazine) compound ICRF-154 has been reported to show enhancement of both the experimental and the clinical biological activity of the parent compound [19]. The addition of N-morpholinomethyl groups to the razoxane molecule has resulted in a compound which is much more soluble than razoxane. The present study was initiated to determine whether pretreatment with MM-159, the bis-morpholinomethyl derivative of razoxane, would protect against chronic doxorubicin (DXR) cardiotoxicity in beagle dogs.

Materials and methods

Synthesis of the bis-morpholinomethyl derivative of razoxane. Razoxane (3.5 g, 13. 0 mmol) was dissolved in dimethylsulfoxide (35 ml) by stirring at 55 °C and the solution was filtered. To the filtrate was added morpholine (3.5 g, 40 mmol) and 37% formaldehyde solution (3.5 ml, 40 mmol). The mixture was stirred at 55 °C for 1 h and then for 20 h at 20 °C. The crystalline solid that separated was stirred with a mixture of ether (50 ml) and ethanol (10 ml), filtered, and washed with ether. The 4.9 g (81.7%) yield of pure bis-4-morpholinomethyl-3,5-dioxopiperazinyl-1,2-propane (MM-159) (Fig. 1) had a melting point of 169°–170 °C.

Animal experiments. Beagle dogs (1–1.5 years old; 6.2–11.4 kg) of either sex were assigned to four groups, with eight animals each in group 1 and group 2, five animals in group 3, and three animals in group 4. Groups 1 and 2 received DXR 1.75 mg/kg i. v. once every 3 weeks. Group 2 was pretreated 15 min before DXR administration with MM-159 25 mg/kg i. v. Group 3 was given MM-

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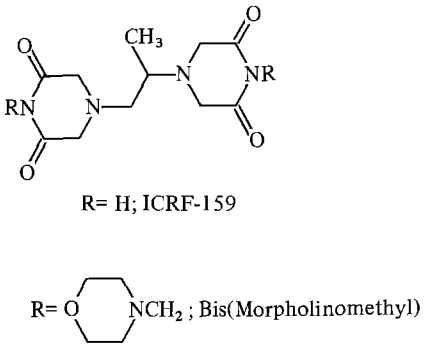

Fig. 1. Structure of MM-159

159 25 mg/kg i. v., and group 4 was given saline without DXR. MM-159 20 mg/ml and DXR 10 mg/ml were dissolved in physiologic saline just before use. A total of seven or eight i. v. injections of DXR (12.25–14 mg/kg total dose) were given to animals not receiving MM-159. Nine DXR injections (15.75 mg/kg total dose) were given to animals pretreated with MM-159.

The animals were returned to their cages after each injection, observed daily, and weighed weekly. Blood samples for biochemical and hematological analysis were collected from the jugular vein prior to initiation of drug treatment and at 3-week intervals thereafter. The final sample was obtained just before the animals were killed 3 weeks after the seventh or eighth injection (DXR only) or the ninth treatment (MM-159 and DXR, MM-159, or saline). A complete blood count and serum determinations of glucose, urea nitrogen, creatinine, total protein, albumin, globulin, total bilirubin, direct bilirubin, cholesterol, sodium, potassium, phosphorus, calcium, chloride, magnesium, alkaline phosphatase, *gamma*-glutamyl transpeptidase (GGTP), lactic dehydrogenase (LDH), serum glutamate-pyruvic transaminase (SGPT), serum glutamate-oxalate transaminase (SGOT), and creatine phosphokinase (CK) were performed on each sample by Vet-Path Laboratories, Teterboro, NJ. A two-tailed, paired-sample *t*-test statistical analysis was used to analyze treatment-related differences in biochemical and hematological results.

Three weeks after the eighth or ninth injection the dogs were killed with an overdose of pentobarbital sodium, and the entire heart and samples of liver, kidney, small intestine, diaphragm, and lung were excised and fixed in 10% neutral formalin. Blocks of heart tissue were embedded in glycol methacrylate plastic resin. One-micron-thick sections from the left ventricular free wall, septum, and left ventricular papillary muscles were prepared and stained with hematoxylin-eosin and toluidine blue. All other tissues were embedded in paraffin and stained with hematoxylin-eosin. The frequency and severity of DXR-induced cardiac lesions were assessed by light-microscopic examination of three left ventricular sections. The changes were graded on a scale of 0 to 4+ on the basis of the numbers of muscle cells showing myofibrillar loss and cytoplasmic vacuoliztion. The scale was defined as follows: 0 = no damage; 1+ = involvement of only an occasional cell; 4+ = severe involvement of 50% or more cells in the visual field; 2+ and 3+ = intermediate degrees of involvement. The reported cardiomyopathy score for each animal represents the mean value rounded to the nearest whole number for all three sections examined. Sections were evaluated without prior knowledge of the treatment given to the dogs. Differences in severity of the cardiomyopathy scores between the groups were analyzed by the chi-square test.

Results

Clinical chemistry and hematologic determinations

The serum concentrations of glucose, creatinine, urea nitrogen, total and direct bilirubin, total protein, albumin,

Table 1. Effects of doxorubicin (DXR), bis(morpholinomethyl)-ICRF-159 (MM-159), and MM-159 plus DXR on mean body weight and hematologic values (± SD) before and 3 weeks after chronic dosing in beagle dogs

Treatment	DXR[a]	MM-159 + DXR	MM-159	Saline
Number of Dogs	8	8	5	3
Body wt (kg)				
Before	9.40 ± 1.3	9.40 ± 1.4	9.90 ± 0.7	10.00 ± 0.6
After	8.60 ± 1.8	8.80 ± 1.6	10.70 ± 1.0	10.90 ± 0.8
WBC ($\times 10^3$)				
Before	8.70 ± 2.6	9.80 ± 3.4	10.40 ± 1.5	9.00 ± 1.7
After	10.60 ± 2.9	9.90 ± 2.5	11.00 ± 0.5	11.00 ± 0.9
RBC ($\times 10^6$)				
Before	7.02 ± 0.3	6.85 ± 0.46	7.01 ± 0.3	7.27 ± 0.46
After	6.16 ± 0.3*	6.86 ± 0.58	6.80 ± 0.6	7.25 ± 0.27
Hemoglobin (g/dl)				
Before	17.50 ± 1.1	17.90 ± 1.5	17.90 ± 0.9	18.10 ± 1.2
After	14.60 ± 0.7*	16.20 ± 1.5	17.20 ± 0.9	17.70 ± 0.6
Hematocrit				
Before	50.20 ± 2.6	49.70 ± 4.0	50.30 ± 2.9	51.30 ± 2.9
After	43.10 ± 2.3*	47.90 ± 4.3	49.20 ± 3.0	50.80 ± 1.5

All values are mean ± SD

[a] Final hematologic values in DXR groups were obtained from blood collected prior to killing or prior to the last DXR injection before the animal died

* Significantly different from control ($p < 0.05$)

globulin, cholesterol, sodium, potassium, magnesium, calcium, chloride, phosphorus, SGOT, SGPT, LDH, CK, and alkaline phosphatase in all groups were not significantly altered over the course of the study. Likewise, no significant changes were found in the red and white blood cell counts, hemoglobulin concentration, and hematocrit during the experimental period in the groups given MM-159 and DXR, MM-159 alone, or saline (Table 1). Compared with control values, the red blood cell count, the hemoglobin concentration, and the hematocrit were significantly reduced in the group given DXR alone (Table 1). However, these reduced levels were still within the normal ranges of red blood cell, hemoglobin, and hematocrit values found by Vet-Path Laboratories in untreated beagle dogs.

General toxicity and weight change

Five of the eight dogs given DXR alone died before the end of the study, two after seven injections (2 and 3 weeks after DXR) and three after eight injections (1 day (one) and 5 days (two) after DXR). Ascitic fluid was found in four of these dogs. The remaining three dogs in this group were killed 3 weeks after the eighth dose. None of the animals given the combination of MM-159 and DXR died before the end of the study (3 weeks after the ninth dose). Except for alopecia, the 1.75 mg/kg dose of DXR had little overt effect on the dogs. By the fourth dose, alopecia had spread to the head, trunk, and tail. Similar effects were noted in the dogs given the combination of MM-159 and DXR. Animals receiving MM-159 alone did not show this effect. A rapid bolus injection of MM-159 caused half of the dogs to immediately lie down for less than 5 min, after which the animals appeared entirely normal. This phenomenon was not noted when the injection was made slowly over a period of 1 min. A temporary reduction in food consumption was noted within 24 h in dogs given DXR with or without MM-159. At the end of the study, DXR-treated animals had lost an average of 0.8 kg body weight compared to 0.6 kg in the dogs given both MM-159 and DXR (Table 1). Animals receiving MM-159 or saline without DXR gained an average of 0.8 and 0.9 kg body weight respectively (Table 1).

Table 2. Effect of pretreatment with the N-morpholinomethyl derivative of ICRF-159 (MM-159) on the incidence and severity of doxorubicin-induced chronic cardiomyopathy in dogs

Treatment group	Incidence		Cardiomyopathy score				
	Death	Lesions	0	1	2	3	Ratio[a]
Saline control	0/3	0/3	3	0	0	0	3/3
MM-159	0/5	0/5	3	0	0	0	5/5
Doxorubicin	5/8	8/8	0	0	0	8	0/8*
MM-159/ doxorubicin	0/8	5/8	3	4	1	0	8/8

[a] Where ratios are given, the numerator denotes the number of animals with a cardiomyopathy score equal to or less than 2 and the denominator denotes the number of animals examined

* Severity scores of doxorubicin group significantly greater than that of MM-159/doxorubicin group by chi-square analysis ($p < 0.01$)

Myocardial alterations

The cardiac lesions observed in the present study were similar to those observed previously in DXR-treated humans [1, 2], dogs [8, 14], rabbits [12], and pigs [9]. The lesions showed two characteristics, cytoplasmic vacuolation and myofibrillar loss (Fig. 2). Both of these changes involved larger numbers of cells as lesions increased in severity. The vacuolation involved the formation of multiple, clear, membrane-limited vacuoles that filled the cytoplasm of the affected cells and often caused them to appear larger than normal. The myofibrillar loss resulted in a pale but nonvacuolated appearance of the cytoplasm. These two types of change often coexisted in the same cells. Animals given DXR alone showed the most severe cardiac alterations. All eight animals from this group had a lesion score of 3+ (Table 2). In contrast, lesions were absent in three of eight dogs given the combination of MM-

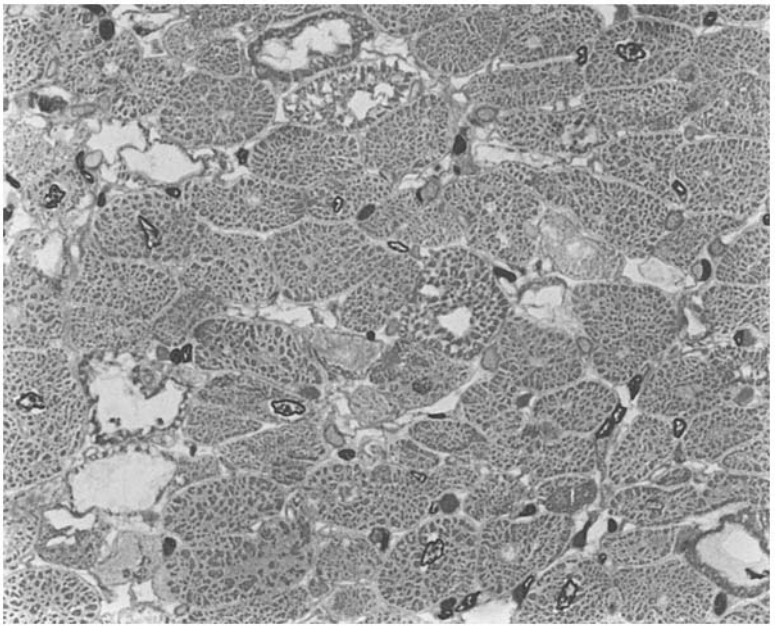

Fig. 2. Photomicrograph of histologic section of heart of beagle dog given 12.25 mg/kg total cumulative dose of doxorubicin. Myocytes show vacuolation and myofibrillar loss. Hematoxylin-eosin stain, X 250

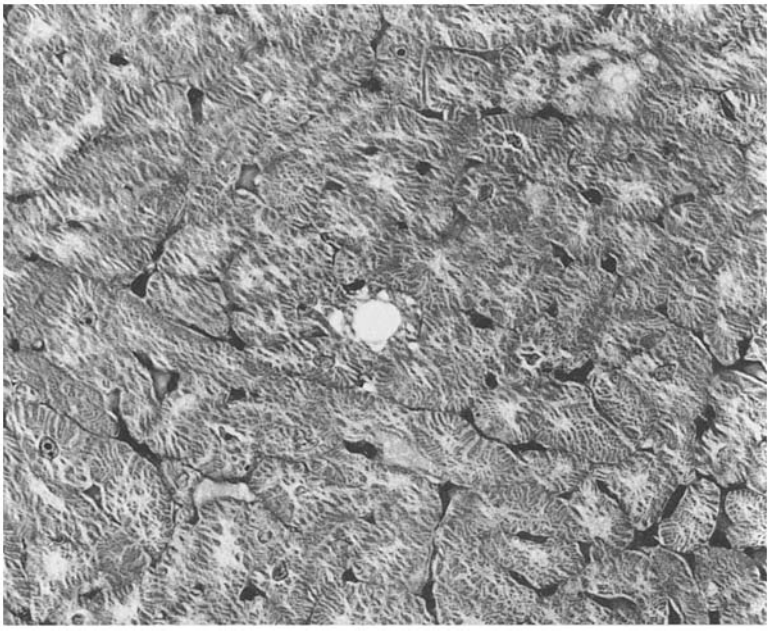

Fig. 3. Photomicrograph of histologic section of heart of beagle dog given a combination of MM-159 and doxorubicin (total cumulative dose 15.75 mg/kg). Only one myocyte in the field shows cytoplasmic vacuolation. Hematoxylin-eosin stain, X 250

159 and DXR (Fig. 3); of the five other animals in this group, four had a lesion score of 1+ (Fig. 3) and one had a score of 2+. The difference in severity of cardiomyopathy scores between the group given DXR alone and the group given DXR together with MM-159 was highly significant ($p<0.01$) (Table 2). No cardiac lesions were present in animals receiving MM-159 or saline alone.

Pathology of noncardiac tissues

At the dosage schedules used in the present study no morphologic alterations attributable to DXR or MM-159 were found in the samples of liver, kidney, lung, small intestine, or diaphragm.

Discussion

Certain modifications have been made in the basic structure of bis-diketopiperazine compounds in order to explore the variety of biological actions possessed by these agents. Recently, bimolane, the morpholinomethyl derivative of ICRF-154 [1,2-bis(morpholinomethyl-3,5-dioxopiperazinyl)ethane], was found to have greater activity against various human and experimental malignancies than ICRF-154, the parent compound [19]. Bimolane is insoluble in water and, therefore, cannot be readily administered parenterally.

The addition of morpholinomethyl substituents to razoxane (ICRF-159) produced a compound, MM-159, which is considerably more water-soluble than razoxane, the original compound. At present there is little information regarding the biological activity of this compound. The increased solubility allowed MM-159 to be examined by the i. v. route of administration for protective activity against chronic DXR cardiotoxicity.

In the present study DXR (1.75 mg/kg) was administered to beagle dogs at 3-week intervals over a 21- to 24-week period (12.25–14 mg/kg total dose). The hearts of all eight animals receiving these doses of DXR had severe myocyte damage. The most prominent features of the cardiac lesions, intracellular vacuolation and myofibrillar loss, had been observed previously in humans [1, 2] and in a variety of animal species [8, 9, 12, 17, 24].

Several observations indicated that pretreatment with MM-159 caused a marked reduction in DXR cardiotoxicity. A major finding was that the hearts of three of eight dogs given MM-159 and DXR remained essentially normal despite administration of a cumulative dose of 15.75 mg/kg DXR. A second indication of protection was noted in the incidence and severity of myocyte alterations in the remaining five animals in this group. Vacuolation and myofibrillar loss were minimal in four hearts (lesion score 1+) and mild in one heart (lesion score 2+) among the hearts from dogs treated with the combination of MM-159 and DXR. These lesions were significantly less extensive than the severe lesions seen in each of the eight animals receiving DXR (12.25–14 mg/kg) alone.

Damage to noncardiac tissues such as the kidney has been reported to occur in rats and rabbits treated chronically with DXR [17, 24]. Alterations in renal function are extremely rare when DXR is used therapeutically in humans [3]. Renal toxicity did not develop in the present study, nor has it been observed in other investigations in which DXR was administered chronically to dogs [8, 14]. With the exception of bone marrow suppression, DXR caused no alterations in any of the other extracardiac tissues examined. Also, the majority of the blood chemistry determinations remained essentially unchanged throughout the duration of the study. Thus, it would seem that a reduction in DXR-induced myocardial cellular injury would be expected to be accompanied by other signs of protection, such as an increase in the tolerated cumulative DXR dose and a decrease in the incidence of cardiac-related deaths. These factors were not directly addressed in the present study. However, it should be noted that five of eight dogs treated with DXR alone died and the remaining three were killed after the seventh or eighth injection (12.25 to 14 mg/kg cumulative dose) because of their poor condition. In contrast, all animals given the combination of MM-159 and DXR were alive after nine injections (15.75 mg/kg cumulative tolerated dose) of DXR.

MM-159 is the second bisdiketopiperazine compound found to ameliorate DXR cardiotoxicity. The degree of reduction in chronic cardiotoxicity as a result of pretreatment with MM-159 was similar to that previously reported in a variety of animals given ICRF-187 prior to DXR [8, 9, 12, 14]. In this instance, protective activity was elicited by i. v. administration instead of the i. p. route utilized in the previous ICRF-187 studies. The main structural differences between MM-159 and ICRF-187 are that MM-159 is a bis-morpholinomethyl derivative and is a racemic mixture. Substances with morpholinomethyl groups are thought to be unstable and to undergo hydrolysis to the parent compound [4]. Nevertheless, some morpholinomethyl derivatives have found to be more potent than their parent imides [22, 23]. If the hydrolysis were complete, the 25 mg/kg pretreatment dose utilized in the present study would be equivalent to approximately 14 mg/kg of the razoxane parent compound. For comparison, in studies utilizing a dog model, pretreatment doses of ICRF-187 have ranged from 12.5 mg/kg when 1 mg/kg DXR was given on a weekly schedule [8], to 25 mg/kg when the DXR dosing was identical to that used in the present study [14]. At present there is little information regarding the pharmacokinetic properties of MM-159, and thus the extent to which it remains intact or is converted to the parent compound or other compounds is unknown.

There is some evidence to indicate that the cardiotoxic effects of DXR are mediated by the formation of an iron-doxorubicin complex which is capable of generating highly reactive oxygen-containing free radicals [18]. The similarity in the degree of cardioprotective action exerted by ICRF-187 and MM-159 indicates that this activity could occur without the morpholinomethyl-N groups. Razoxane can passively diffuse through the cell membrane and by hydrolysis be converted to a diacid diamide [6]. The structural similarity of the hydrolyzed product to EDTA suggests that both MM-159 and ICRF-187 could ultimately function as chelating agents. Chelation of iron by these two agents could decrease the in vivo formation of reactive oxygen radicals and thereby protect against chronic DXR cardiotoxicity.

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