Reduction of Chronic Doxorubicin Cardiotoxicity in Dogs by Pretreatment with (±)-1,2-Bis(3,5-dioxopiperazinyl-1-yl)propane (ICRF-187)

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INTRODUCTION

A major limitation to the effective use of daunorubicin or doxorubicin is dose-related cardiomyopathy (5, 19, 20). Considerable efforts are under way to find a means of reducing the risk of cardiomyopathy while at the same time maintaining the therapeutic efficacy of these agents. Recent attempts to solve the problem have involved the use of modified drug scheduling (14). Recently, pretreatment with ICRF-187 was found to reduce cardiac toxicity in rabbits treated chronically at 3-week intervals with daunorubicin (16). Since doxorubicin is at present much more widely used clinically than is daunorubicin, the present studies were undertaken to determine whether pretreatment with ICRF-187 would also protect against chronic doxorubicin cardiotoxicity. The beagle dogs were selected as the animal model to study because these animals are largely free of the respiratory infections and renal toxicity that are frequently encountered in chronic studies with rabbits (29, 34). In addition, we have found that the cardiac lesions are more consistent in the dog than in the rabbit.

MATERIALS AND METHODS

Fifteen beagle dogs (1 to 1.5 years old) of either sex, weighing between 8.2 and 11.5 kg, were divided into 2 groups of 6 animals and 1 group of 3 animals. The dogs in Groups 1 (4 male, 2 female) and 2 (2 male, 4 female) received weekly i.v. injections of doxorubicin (1.0 mg/kg body weight) for a total of 15 weeks. In Group 2, the dogs were pretreated 30 min before doxorubicin administration with ICRF-187 (12.5 mg/kg body weight i.p.). Group 3, composed of 3 dogs (2 female, 1 male), received ICRF-187 (12.5 mg/kg i.p.), followed in 30 min by an i.v. injection of 0.9% NaCl solution. ICRF-187 and lyophilized doxorubicin were dissolved in 0.9% NaCl solution just before use and injected in doses of 10 and 5 mg/ml, respectively. Animals were returned to their pens, observed daily, and weighed weekly. Blood samples for biochemical and hematological analysis were obtained from the jugular vein just before giving the drug and at 3-week intervals thereafter. The last sample was obtained just before the animals were sacrificed, 1 week after the 15th drug injection. A complete blood count and serum determinations of urea nitrogen, creatinine, glucose, total protein, albumin, globulin, totalbilirubin, direct bilirubin, total lipids, triglycerides, uric acid, cholesterol, sodium, potassium, phosphorus, calcium, chloride, serum glutamate-pyruvate transaminase LDH, alkaline phosphatase, SGOT, and CPK of each sample were performed by Met-Path Laboratories, Rockville, Md. A 2-tailed, paired-sample t test statistical analysis was used to determine treatment-related differences in biochemical and hematological results.

One week after the 15th injection (total dose of doxorubicin, 15.0 mg/kg), the animals were killed, and the entire heart and samples of liver, kidney, small intestine, skeletal muscle, and diaphragm were excised from each animal and fixed in 10% neutral formalin. Four blocks of tissue from each heart, including sections from the left ventricular free wall, anterior and posterior papillary muscles, and ventricular septum, were embedded in paraffin and in glycol methacrylate plastic resin. Sections of plastic-embedded tissues were stained with hematoxylin-eosin or with toluidine blue. All other tissues were embedded in paraffin and stained with hematoxylin-eosin.

The frequency and severity of doxorubicin-induced cardiac lesions were assessed by light microscopic examination of sections stained with toluidine blue. These changes were graded on a scale of 0 to 4+ on the basis of the number of muscle cells showing myofibrillar loss and cytoplasmatic vacuolization: 0 = no damage; 1+ = involvement of only an occasional cell; 4+ = severe involvement of 50% or more
cells, and 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 4 sections examined. Sections were evaluated without prior knowledge of the pretreatment given to the animals. A $\chi^2$ test was utilized to determine the significance of differences in the severity of cardiomyopathy scores between groups.

RESULTS

General Toxicity and Weight Change. None of the dogs receiving doxorubicin, the combination of doxorubicin and ICRF-187, or ICRF-187 alone died during the 15-week experimental period. Except for alopecia, the 1-mg/kg dose of doxorubicin had little overt effect on the dogs. By the fifth week, alopecia was noted around the limbs. As the dosing continued, the alopecia spread to the head, trunk, and tail. Similar effects were noted in the dogs given the combination of ICRF-187 and doxorubicin. Animals receiving ICRF-187 alone did not show this effect.

A temporary reduction in food consumption was noted during the first 24 hr after doxorubicin administration, regardless of pretreatment with ICRF-187. During the course of the studies, the dogs receiving only ICRF-187 increased in body weight from 10.5 ± 0.6 (S.E.) to 10.8 ± 0.4 kg; animals receiving doxorubicin alone lost an average of 1.2 kg (from 9.7 ± 0.8 to 8.5 ± 0.6 kg), and those receiving ICRF-187 and doxorubicin lost an average of 0.4 kg (from 9.7 ± 0.4 to 9.3 ± 0.5 kg). The difference in amount of weight change was statistically significant ($p < 0.05$) only in the group receiving doxorubicin alone.

Gross Anatomic Changes. There were few gross anatomic changes suggestive of cardiomyopathy in any of the animals. Edema and cardiac enlargement were not apparent. One doxorubicin-treated dog showed mild right ventricular dilation and pulmonary congestion.

Myocardial Alterations. The cardiac lesions observed in the present experiments were comparable to those observed previously in doxorubicin-treated humans (3, 5), dogs (12), rabbits (18, 27), and rats (23, 28). The lesions showed 2 characteristics: cytoplasmic vacuolization and myofibrillar loss. Both of these changes involved progressively larger numbers of cells as the lesions increased in severity. The vacuolization 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. Both types of change often coexisted in the same cells. Animals given doxorubicin alone showed the most severe cardiac alterations; typical examples are shown in Fig. 1. Five of 6 animals from this group had a lesion score of 4+ (Table 1). In contrast, lesions were absent in 4 of the 6 animals given doxorubicin in combination with ICRF-187; a lesion score of 1+ was observed in the other 2 animals in this group. The difference in cardiomyopathy severity scores in the group given doxorubicin alone and the group given doxorubicin together with ICRF-187 was highly significant ($p < 0.001$) (Table 1). An example of the most severe lesions in the animals receiving doxorubicin and ICRF-187 is shown in Fig. 2. No cardiac lesions were present in animals receiving ICRF-187 alone (Fig. 3).

Pathology of Noncardiac Tissues. At the dosage schedules used, there were no histological lesions in kidney, lung, small intestine, diaphragm, or skeletal muscle that were attributable to doxorubicin or to ICRF-187. A few scattered inflammatory cells were noted in the livers of 3 animals given doxorubicin and of 1 animal given ICRF-187.

Clinical Chemistry and Hematological Determinations. At the end of the experiment, the serum concentrations of glucose, creatinine, urea nitrogen, bilirubin, total lipids, total protein, triglycerides, uric acid, cholesterol, sodium, potassium, SGOT, LDH, CPK, and alkaline phosphatase in all groups were essentially unchanged from the control values obtained at the beginning of the experiment. The average serum iron concentrations were significantly reduced from base-line control levels in the groups given doxorubicin alone (198 ± 24 to 124 ± 12 µg/100 ml; $p < 0.02$) and doxorubicin with ICRF-187 (231 ± 14 to 113 ± 10 µg/100 ml; $p < 0.005$). The degree of reduction was comparable in both groups. The serum iron concentration was unchanged in those animals receiving ICRF-187 alone (239 ± 14 to 240 ± 33 µg/100 ml).

Compared to control values, the RBC, WBC, and hemoglobin concentrations were essentially unchanged from base-line control levels in the groups given doxorubicin alone (198 ± 24 to 124 ± 12 µg/100 ml; $p < 0.02$) and doxorubicin with ICRF-187 (231 ± 14 to 113 ± 10 µg/100 ml; $p < 0.005$). The degree of reduction was comparable in both groups. The serum iron concentration was unchanged in those animals receiving ICRF-187 alone (239 ± 14 to 240 ± 33 µg/100 ml).

### Table 1

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>No. of dogs attaining following cardiomyopathy score</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICRF-187 control</td>
<td>0 1 2 3 4 $\leq 1$^b</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0 0 1 0 5 0/6^c</td>
</tr>
<tr>
<td>ICRF-187-doxoru-</td>
<td>4 2 0 0 0 6/6^d</td>
</tr>
<tr>
<td>bicin</td>
<td></td>
</tr>
</tbody>
</table>

Where ratios are given, the numerator denotes the number of animals with a cardiomyopathy score of 1 or less, and the denominator denotes the number of animals examined.

**Table 2**

<table>
<thead>
<tr>
<th>WBC (×10^3)</th>
<th>RBC (×10^3)</th>
<th>Hemoglobin (g/100 ml)</th>
<th>Hematocrit (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 16 wk</td>
<td>Control 16 wk</td>
<td>Control 16 wk</td>
<td>Control 16 wk</td>
</tr>
<tr>
<td>ICRF-187</td>
<td>11.8 ± 1.1^a</td>
<td>9.6 ± 1.0</td>
<td>6.63 ± 0.4</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>12.2 ± 0.9^b</td>
<td>7.05 ± 0.9^c</td>
<td>6.89 ± 0.16</td>
</tr>
<tr>
<td>ICRF-187-doxoru-</td>
<td>10.6 ± 1.1^b</td>
<td>7.6 ± 0.8^c</td>
<td>6.88 ± 0.29</td>
</tr>
</tbody>
</table>

Notes:
- Mean ± S.E. (n = 3).
- Mean ± S.E. (n = 6).
- Significantly different from control ($p < 0.001$).
- Significantly different from control ($p < 0.02$).
- Significantly different from control ($p < 0.01$).
concentration were significantly reduced in the groups treated with doxorubicin (p < 0.001) and with the combination of ICRF-187 and doxorubicin (p < 0.02) (Table 2). The hematocrit also was reduced in both groups, but only in the doxorubicin group was this decrease significant (p < 0.01). The values obtained in the animals treated with ICRF-187 alone remained essentially unchanged during the course of the experiments.

DISCUSSION

Three methods of reducing anthracycline cardiotoxicity are currently under investigation. Dose schedule modification has been attempted with varying degrees of success. The incidence of cardiac failure was reduced when smaller doses of doxorubicin were given at weekly intervals or over prolonged infusion periods (8, 21, 32, 34). These schedules result in lower peak levels of doxorubicin in plasma compared to the every-3-week schedule (7). The lower levels of doxorubicin to which the heart is exposed appear to be a key feature in reducing the incidence of congestive heart failure. The clinical effectiveness of small weekly doses of doxorubicin is still being evaluated. Detailed anatomic evaluation of the cardiac lesions developing in humans after exposure to more frequent, lower doses of doxorubicin has not been reported.

Small weekly doses of doxorubicin produced extensive myocardial alterations in rabbits (34). Similar observations were made in the present experiments, in which 5 of 6 dogs given 15 weekly doses of doxorubicin (1 mg/kg; 300 mg/sq m, total dose) had evidence of severe cardiomyopathy. The main features of this cardiomyopathy, intracellular vacuolization and myofibrillar loss, were similar to those caused by doxorubicin in humans and in a variety of animal species (3, 5, 12, 18, 23, 26, 27) when doxorubicin was given less frequently but at higher doses (12). These lesions were also similar to those found in beagle dogs. In previous studies of doxorubicin-treated dogs, the most severe damage was reported in the left ventricle and ventricular septum (30). In the present study, these areas were found to have consistently high cardiomyopathy scores. The lesions were scattered diffusely throughout the various layers of the myocardium and were not localized in the vicinity of the coronary vessels as has been reported previously in the rabbit and rat (18, 26, 36). In spite of the severity of these lesions, all animals in the present study survived the entire experimental period; however, in another study the majority of dogs given the same weekly dose (1 mg/kg) died from cardiotoxicity when the duration of the experiment was extended to 20 weeks (30). Clearly, the cardiotoxic effects of doxorubicin can occur in spite of different treatment schedules.

A second approach to overcoming current limitations on the use of doxorubicin has been to develop less cardiotoxic anthracycline analogs. No analog yet tested with antitumor activity in experimental systems has been totally devoid of cardiac toxicity in the rat or rabbit models (11). Analogs of doxorubicin which have been used clinically include rubidazone, carminomycin, AD 32, aclacinomycin A, quelamycin, 4-epiadiramycin, and detorubicin (6, 28). These compounds have been examined to various extents, but none has demonstrated a clear advantage over doxorubicin with regard to both antineoplastic activity and cardiac toxicity (6, 28).

It has been suggested that the cardiotoxicity of doxorubicin is mediated through the production of superoxides or by activation of the anthracyclines to a free radical state (1). This finding led to the observation that lipid peroxidation and acute myocardial damage induced by anthracyclines in mice, rats, and rabbits could be prevented by pretreatment with the free radical scavenger vitamin E (22, 23, 33). In chronic studies, comparable protection by vitamin E has not been observed (4, 15, 19).

In the present study, pretreatment with ICRF-187 caused a significant reduction in doxorubicin cardiotoxicity. One important effect was that the hearts of 4 of the 6 dogs pretreated with ICRF-187 remained essentially normal despite the 15 weekly injections of doxorubicin. This finding is all the more striking since the same dose of doxorubicin caused severe lesions in 5 of the 6 dogs which were not pretreated with ICRF-187. A second aspect of this protection was noted in the frequency and extent of the alterations in the remaining 2 animals pretreated with ICRF-187. Vacuolization and myofibrillar loss in these 2 animals occurred only to a minor extent; in fact, these changes were significantly less extensive than the mildest lesions seen in any of the animals not pretreated with ICRF-187.

A reduction in doxorubicin-induced myocardial cellular injury would be expected to be accompanied by other signs of protection, such as an increase in the tolerated cumulative dose and a decrease in the incidence of cardiac-related deaths. All animals survived under the conditions of the present experiment, and it is possible that these other effects would have become apparent with a longer experimental period.

The most obvious general toxic effect caused by doxorubicin in the dog was alopecia. Pretreatment with ICRF-187 did not prevent this toxicity. In rats and rabbits, doxorubicin has been reported to cause toxicity in organs such as the kidney (23, 31, 36). With the exception of bone marrow suppression, doxorubicin elicited no alterations in any of the extracardiac tissues examined. This observation is supported by the fact that the majority of the results of blood chemical determinations remained essentially unchanged throughout the duration of the experiment. Three plasma enzymes (CPK, LDH, and SGOT) which have been used as indicators of cardiac damage were not altered in the animals showing severe cardiomyopathy. These findings are similar to observations made in other animals receiving doxorubicin (18, 26) and suggest that serum levels of the 3 enzymes are not reliable indicators of slowly progressive cardiac damage such as that elicited by the anthracyclines.

As mentioned above, potentially lethal extracardiac toxicity, such as the bone marrow suppression and the gastrointestinal damage found in some other studies (12), was not a problem at the dose and duration of treatment used in the present experiments. In the hamster, acute single high doses of daunorubicin significantly lowered the WBC, and this effect was greater in those animals pretreated with ICRF-187 (14). The additive effect on bone marrow suppression seen when high doses are used apparently does not occur when the 2 agents are given chronically at lower doses. Significant depression of WBC, RBC, and hemoglobin occurred in the dogs with chronic doxorubicin administration. A similar effect was noted in animals given the combination of ICRF-187 and doxorubicin; however, in these animals, the magnitude of these changes was no greater than that observed in dogs receiving doxorub-
icin alone. The average serum iron concentrations were reduced to a similar degree in the groups treated with doxorubicin alone and with doxorubicin and ICRF-187. The mechanism of this reduction is not clear.

The mechanism by which ICRF-187 alters the chronic cardiotoxicity caused by doxorubicin has not been clarified. The racemic compound ICRF-159 exerts antineoplastic effects by normalizing the vasculature of developing tumors (22). Both experimental and clinical evidence indicates that ICRF-159 enhances the effect of ionizing radiation (2). The reduction in doxorubicin cardiotoxicity would seem to be independent of these 2 actions. An additional mechanism of action is suggested by the structure of ICRF-187. Since this compound is a nonpolar derivative of EDTA, it could enter myocardial cells and exert protective effects by functioning as a chelating agent and altering critical concentrations of divalent cations. The hearts of rabbits treated with doxorubicin have elevated levels of calcium (27), but it is uncertain whether this increase is a cause or a result of the toxicity of doxorubicin. At present, there is little information regarding pharmacokinetic properties of ICRF-187, and thus the extent to which either of the compounds alters the intracellular concentration of calcium in myocardium has not been determined. Likewise, it is not known whether ICRF-187 alters intracellular concentrations of other cations such as copper and iron. There is evidence that copper can react directly with the unhydrolyzed ICRF-159 molecule (10). The same sort of reaction between ICRF-187 and iron could have important implications, particularly if the cardiotoxic action of doxorubicin is mediated through production of highly reactive oxygen-containing radicals. Iron and doxorubicin have recently been shown to form a complex that catalyzes the in vitro formation of such radicals (13). An ICRF-187-induced decrease in iron concentration could conceivably lead to a reduction in the formation of hydroxyl radicals in vivo. These questions are currently under investigation.

The reduction in doxorubicin toxicity by ICRF-187 is not uniform for all tissues. Studies in hamsters showed that pretreatment with ICRF-187 did not ameliorate the bone marrow depression but did reduce the mortality produced by daunorubicin (14). In the present experiments, doxorubicin-induced bone marrow depression and alopecia were not influenced by pretreatment with ICRF-187. Furthermore, the data available indicate that pretreatment with ICRF-187 does not interfere with the antitumor activity of daunorubicin or doxorubicin (35). These factors, together with the potential for significant ICRF-187 cardioprotection, would seem to provide the basis for more effective use of doxorubicin. In addition, elucidation of the nature of the protective effect of ICRF-187 could provide a critical insight into the pathogenesis of daunorubicin or doxorubicin cardiotoxicity.

ACKNOWLEDGMENTS

The authors acknowledge the valuable technical assistance provided by Donald Gates.

REFERENCES

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Fig. 1. Histological section of heart of dog treated with 1-mg/kg dose of doxorubicin weekly for 15 weeks and sacrificed 1 week after last dose. Widespread vacuolization and loss of myofibrils are evident. This area corresponds to a cardiomyopathy score of 3+. One-μm-thick plastic section. Toluidine blue, x 300.

Fig. 2. Histological section of heart of dog pretreated with 12.5-mg/kg dose of ICRF-187 before each of 15 weekly injections of doxorubicin (1 mg/kg) and sacrificed 1 week after last dose. Only minimal changes are present in the field, in the form of one single vacuolated cell. This area corresponds to a cardiomyopathy score of 1+. One-μm-thick plastic section. Toluidine blue, x 300.

Fig. 3. Normal histology is shown in this section of heart from dog sacrificed 1 week after last of 15 weekly injections of ICRF-187 (12.5 mg/kg). This area corresponds to a cardiomyopathy score of 0. One-μm-thick plastic section. Toluidine blue, x 300.
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*Cancer Res* 1981;41:3436-3440.