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dl-N,N'-Dicarboxamidomethyl-*N,N'*-dicarboxymethyl-1,2-diaminopropane (ICRF-198) and *d*-1,2-Bis(3,5-dioxopiperazine-1-yl)propane (ICRF-187) Inhibition of Fe^{3+} Reduction, Lipid Peroxidation, and CaATPase Inactivation in Heart Microsomes Exposed to Adriamycin[1]

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ABSTRACT

Iron-catalyzed free radical reactions, such as the peroxidation of membrane lipids or the inactivation of critical enzymes, have been implicated in the cardiotoxicity of Adriamycin. Fe^{3+} reduction is an important step in both processes. The reduction of Fe^{3+}, Fe^{3+}ADP, or Fe^{3+}-ferritin by rabbit heart microsomes, Adriamycin, and NADPH was 10% inhibited by ICRF-187 (ADR-529) in N_2 and 77% inhibited by ICRF-198, the hydrolysis product of ICRF-159 (the racemic form of ICRF-187). Lipid peroxidation and CaATPase inactivation catalyzed by Fe^{3+}, Fe^{3+}ADP, or Fe^{3+}-ferritin were substantially inhibited by ICRF-198 but only partially inhibited by ICRF-187. The cardioprotective action of ICRF-187 during Adriamycin treatment may be a result of its hydrolysis to the *d* isomer of ICRF-198 which inhibits reduction of Fe^{3+}, thus limiting the role of iron in tissue damaging free radical reactions.

INTRODUCTION

Attempts to ameliorate the cardiotoxicity of Adriamycin have involved either development of analogues that may be less cardiotoxic (1, 2) or coadministration of compounds that have been shown to decrease Adriamycin induced damage *in vitro* (3–5). A recent development in the last category is the compound ICRF-187[3] (ADR-529) which effectively diminishes Adriamycin cardiotoxicity without compromising antitumor activity (6, 7). ICRF-187 is the more soluble *d* form of ICRF-159 (razoxane), a bis-cyclic imide. ICRF-187 is membrane permeable and can enter cells, where it is hydrolyzed to ICRF-198 (8), which is structurally similar to EDTA. The rationale for developing these types of compounds is their potential to act as intracellular chelators. On this basis it is reasonable to propose that ICRF-187 protects against the cardiotoxicity of Adriamycin by a mechanism involving metal binding by the *d* isomer of ICRF-198.

There is substantial evidence that free radical reactions are involved in the cardiotoxicity of Adriamycin (9–11). Reduction of Adriamycin by microsomal and mitochondrial enzyme systems yields an Adriamycin semiquinone and subsequent free radical species that initiate membrane lipid peroxidation (11–14), inactivation of critical thiol containing enzymes such as those involved with calcium transport (15–17), and the production of highly reactive hydroxyl radicals ($OH^{\cdot}$) (10, 18, 19). These reactions have all been shown to require iron in catalytic amounts. In some cases reactions involve iron-Adriamycin complexes (20, 21), but in most the iron concentration required is very low, and complexation is not required. Free iron, iron bound to low molecular weight chelators such as ADP, or iron released from ferritin are all good catalysts of Adriamycin induced lipid peroxidation and CaATPase inactivation in cardiac microsomes (14, 17). Both $OH^{\cdot}$ production and lipid peroxidation require the reduction of Fe^{3+} or Fe^{3+}-chelates (22, 23), and recently we have shown that Adriamycin enhances microsomal reduction of Fe^{3+}, Fe^{3+}ADP, and Fe^{3+}-ferritin (24).

Protection against cardiotoxicity afforded by ICRF-198 may be related to its ability to complex iron in such a way that the iron is no longer able to take part in free radical reactions. In this study we have investigated the effects of ICRF-187 and ICRF-198 on rabbit heart microsomes treated with Adriamycin, NADPH, and low molecular weight iron complexes or ferritin. We have determined whether they inhibit reduction of iron complexes and their subsequent effect on CaATPase inactivation and lipid peroxidation.

MATERIALS AND METHODS

All biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO) except Adriamycin (Farmitalia Carlo Erba, Milan, Italy) and ICRF-198 and ICRF-187 (ADR-529) (a gift from Dr. Verhoef, Adria Laboratories, Columbus, OH). Ferritin (Sigma) contained 1.04 nmol/μg protein and was free of loosely bound iron since it gave no immediate formation of Fe^{2+}-ferrozine when added to ferrozine and ascorbate. Microsomes were prepared from the hearts of New Zealand White rabbits (25) and protein content was determined by the method of Lowry *et al.* (26). All procedures were carried out in acid washed glassware and levels of adventitious iron were decreased by treating solutions with Chelex resin (Bio-Rad Laboratories, Richmond, CA). Reactions were carried out in 10 mM phosphate buffer, pH 7.4, except calcium uptake experiments which were carried out in 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, pH 7.4. Where the effect of ICRF-198 or -187 with an existing iron complex was investigated, the iron complex was preformed in buffer.

NADPH Oxidation and Cytochrome *c* Reduction. The activity of the microsomes was monitored by assaying NADPH oxidation by microsomal NADPH cytochrome P-450 reductase. Microsomes (0.5 mg protein/ml) were incubated with NADPH (100 μM) and the decrease in A_{340} was monitored (ϵ_{340} = 6220). The effect of ICRF-198 or ICRF-187 on Adriamycin-enhanced reduction of cytochrome *c* was measured by adding the compounds to an incubation of microsomes, NADPH, cytochrome *c* (30 μM), and Adriamycin (30 μM) in phosphate buffer and monitoring ΔA_{550} ($\Delta\epsilon_{550}$ reduced-oxidized = 21,100).

Microsomal Reduction of Fe^{3+} Chelates. $FeCl_3$ (5 μM) and either ICRF-198 or ICRF-187 (20 μM) were premixed in buffer and then incubated with microsomes (0.5 mg/ml), NADPH (100 μM), ferrozine (30 μM), and Adriamycin (30 μM). Reduction was followed by monitoring the increase in A_{562} of the Fe^{2+}-ferrozine complex in either air or N_2 (ϵ_{562} = 27,900), as previously (24). Solutions were equilibrated with O_2-free N_2 by flushing for 5 min in a sealed tonometer and the reaction was started by injecting NADPH through a rubber septum.

Lipid Peroxidation. The effect of ICRF-198 or ICRF-187 on iron-dependent lipid peroxidation was examined by incubating microsomes (0.5 mg/ml), NADPH (100 μM), Adriamycin (30 μM), and $FeCl_3$ (5 μM) premixed with either ICRF-198 (20 μM) or ICRF-187 (20 μM).

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[3] The abbreviations used are: ICRF-187, *d*-1,2-bis(3,5-dioxopiperazine-1-yl)propane; ICRF-198, *dl-N,N'*-dicarboxamidomethyl-*N,N'*-dicarboxymethyl-1,2-diaminopropane.

Reactions were carried out in stoppered 10-ml tubes continuously rotated at 22°C. The pO_2 was adjusted by bubbling with O_2-free N_2 and adding the appropriate volume of air by gas tight syringe after removal of the requisite volume of N_2. After 30 min lipid peroxidation was measured as the formation of thiobarbituric acid reactive products at 532 nm (27).

CaATPase Activity. Microsomes (0.5 mg/ml) were incubated in 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, pH 7.4, containing 125 mM KCl, 2 mM K_2HPO_4, and 4 mM $MgCl_2$ at 22°C with NADPH (100 μM), Adriamycin (30 μM), H_2O_2 (100 μM), and $FeCl_3$ (5 μM) that had been premixed with either ICRF-198 (20 μM) or ICRF-187 (20 μM). After 30 min $CaCl_2$ (20 μM), arsenazo(III) (30 μM), and ATP (100 μM) were added and calcium uptake was followed by monitoring the loss of the calcium-arsenazo(III) complex at 654 nm (ϵ_{654} = 21,400).

RESULTS

Iron Reduction. As observed previously (24) incubation of Fe^{3+} with microsomes, Adriamycin, and NADPH in N_2 resulted in the formation of Fe^{2+} as detected by the accumulation of a colored complex with ferrozine (Fig. 1). There was no detectable Fe^{3+} reduction in the absence of NADPH or microsomes. Premixing of Fe^{3+} with ICRF-198 substantially inhibited Fe^{3+} reduction and the extent of inhibition was relatively unaltered by increasing the ICRF-198 concentration from 10 to 50 μM. ICRF-187 also inhibited Fe^{3+} reduction, but only by about 10% at concentrations from 10 to 50 μM.

In the absence of Adriamycin the rate of reduction of Fe^{3+} was 2–3-fold less than with Adriamycin (Table 1; Ref. 24). In the presence of Adriamycin, Fe^{3+}, Fe^{3+}ADP, and Fe^{3+}-desferrioxamine were all reduced. Under the same conditions Fe^{3+}-EDTA is reduced at a rate of 0.51 μM/min (24). ICRF-198 inhibited microsomal reduction of Fe^{3+} by 80% in the absence and 77% in the presence of Adriamycin. It also inhibited Adriamycin dependent reduction of Fe^{3+}ADP to a rate similar to that obtained with no ADP present. In contrast, ICRF-198 did not inhibit Fe^{3+}-desferrioxamine reduction. ICRF-187 inhibited reduction of the Fe^{3+} complexes, but to a lesser extent than ICRF-198 (Table 1). Fe^{3+} reduction of microsomes, NADPH, and Adriamycin is progressively inhibited by O_2 (24). In air the rate of reduction was 0.19 μM/min which was decreased to 0.04 μM/min by ICRF-198 but was scarcely affected by ICRF-187 (0.17 μM/min).

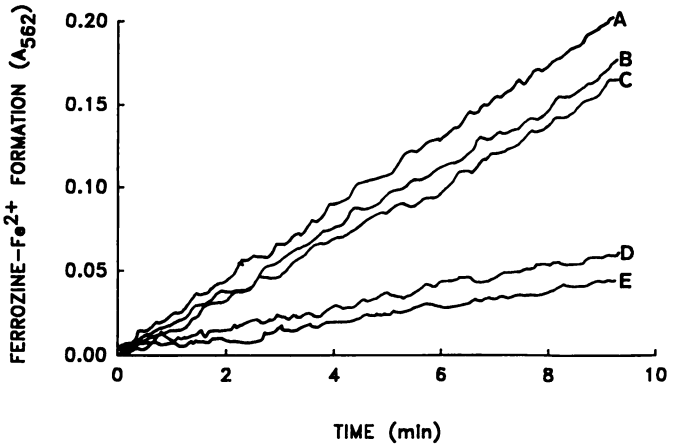

Fig. 1. Effect of ICRF-198 and ICRF-187 on the microsomal reduction of Fe^{3+} in N_2. Incubations contained heart microsomes (0.5 mg/ml), NADPH, Adriamycin, ferrozine, and 10 μM $FeCl_3$ with (*A*) no addition, (*B*) 10 μM ICRF-187, (*C*) 20 or 50 μM ICRF-187, (*D*) 10 μM ICRF-198, and (*E*) 20 or 50 μM ICRF-198. Each *curve* is representative of three experiments.

Table 1 *Effect of ICRF-198 and ICRF-187 on Adriamycin-promoted microsomal reduction of Fe^{3+} in N_2*

Incubations contained rabbit heart microsomes (0.5 mg/ml), NADPH, Adriamycin (Adr), $FeCl_3$ (5 μM), and additions as shown below. Reactions were performed in N_2. Fe^{3+} reduction was measured with ferrozine (30 μM). Each value is the mean of three measurements which differed by no more than 10%. ND, not done; desf, desferrioxamine.

Addition	Rate of Fe^{3+} reduction (μM/min)		
	None	+ ICRF-198	+ ICRF-187
Fe^{3+}	0.34	0.07	0.21
Fe^{3+} + Adr	0.82	0.19	0.74
Fe^{3+} + ADP (500 μM) + Adr	1.13	0.23	0.75
Fe^{3+} + desf (50 μM) + Adr	0.80	0.83	ND

ICRF-198 and ICRF-187 had no effect on the microsomal reductase enzymes since neither altered the rate of NADPH-cytochrome P-450 reductase catalyzed oxidation of NADPH (1.8 ± 0.2 μM/min) or the reduction of cytochrome *c* (3.0 ± 0.3 μM/min) in the presence of Adriamycin. Increasing the ferrozine concentration 10-fold had no effect on the rate of accumulation of the Fe^{2+}-ferrozine complex.

Lipid Peroxidation. Microsomes incubated with NADPH, Adriamycin, and Fe^{3+} or Fe^{3+}ADP undergo lipid peroxidation that is maximal at a pO_2 of 4–8 mm Hg and is inhibited by EDTA or desferrioxamine (Table 2; Ref. 14). ICRF-198 was as good as EDTA or desferrioxamine at inhibiting Fe^{3+} dependent microsomal peroxidation promoted by Adriamycin at low pO_2 (Table 2). Lipid peroxidation promoted by Fe^{3+}-ADP was also inhibited by ICRF-198. ICRF-187 inhibited lipid peroxidation, but not to the same extent as ICRF-198.

CaATPase Inactivation. NADPH dependent reduction of Adriamycin by microsomes results in iron dependent thiol oxidation and associated loss of microsomal CaATPase activity (Table 2; Ref. 17). Inactivation can be suppressed partially by EDTA and almost completely by desferrioxamine (Table 2; Ref. 17). ICRF-198 almost completely protected CaATPase from Adriamycin dependent inactivation catalyzed by Fe^{3+} (Table 2). ICRF-187 was less effective. Neither of the ICRF compounds had any effect on the uptake of calcium in the absence of iron.

Ferritin Dependence. Adriamycin incubated with microsomes and NADPH releases iron from ferritin (Table 3; Refs. 28 and 29), and this iron is able to catalyze peroxidation of lipids and the inactivation of CaATPase in heart microsomes (14, 17). The rate of ferritin iron release is decreased with increasing pO_2 to a rate in air that is 20% of the rate in N_2 (24). As shown in Table 3 desferrioxamine inhibited both processes, indicating that released iron is required. ICRF-198 did not alter the rate of iron release from ferritin (Table 3). However, it completely

Table 2 *Effect of ICRF-198 and ICRF-187 on Adriamycin-promoted microsomal lipid peroxidation and CaATPase inhibition, catalyzed by $FeCl_3$*

Solutions contained heart microsomes (0.5 mg/ml), $FeCl_3$ (5 μM) except where indicated, Adriamycin, NADPH, and chelator as indicated below. Lipid peroxidation was performed at pO_2 = 8 mm Hg and was measured by the formation of thiobarbituric reactive species (A_{532}) read against a microsome, Adriamycin, no NADPH blank. CaATPase activity was measured by monitoring the uptake of calcium in air. The basal rate of calcium uptake prior to incubation with Adriamycin, NADPH, and iron was 91 ± 2 nM calcium/min. Each value is the mean of two sets of duplicates that differed by no more than 10%. ND, not done.

Chelator	Lipid peroxidation (A_{532})	Rate of calcium uptake (nM/min)
None (no added Fe^{3+})	0.07	90
None	0.34	20
EDTA (100 μM)	0.05	68
Desferrioxamine (50 μM)	0.01	79
ICRF-198 (50 μM)	0.02	77
ADP (1 mM)	0.44	ND
ADP (1 mM) + ICRF-198 (50 μM)	0.02	ND
ICRF-187 (50 μM)	0.19	48

Table 3 *Effect of ICRF-198 and ICRF-187 on Adriamycin promoted microsomal lipid peroxidation and CaATPase inactivation, catalyzed by ferritin*

Solutions contained microsomes (0.5 mg/ml), NADPH, Adriamycin, ferritin (100 μg/ml), and chelators as indicated above. Lipid peroxidation was performed at pO_2 = 8 mm Hg and was measured by the formation of thiobarbituric acid reactive species (A_{532}) against a microsome, Adriamycin, no NADPH blank. CaATPase activity was measured by monitoring the uptake of calcium in air. The basal rate of calcium uptake before incubation with Adriamycin, iron, and NADPH was 91 ± 2 nM calcium/min. Each value is the mean of two sets of duplicates that differed by no more than 10%. ND, not done.

Chelator	Rate of iron reduction (μM/min)	Lipid peroxidation (A_{532})	Rate of calcium uptake (nM/min)
None (no added ferritin)	0	0.07	78
None	0.57	0.26	31
Desferrioxamine (50 μM)	ND	0.01	67
ICRF-198 (50 μM)	0.56	0.02	70
ICRF-187 (50 μM)	0.56	0.16	45

inhibited lipid peroxidation and prevented most of the CaATPase inactivation catalyzed by iron from ferritin (Table 3). ICRF-187 was much less effective at inhibiting both ferritin dependent processes.

DISCUSSION

Adriamycin participates in a variety of iron dependent free radical reactions that are potentially cytotoxic. These include lipid peroxidation, the production of strong oxidants, *e.g.*, $OH^{\cdot}$, and the inactivation of thiol dependent enzymes, *e.g.*, CaATPase. Reduction of Fe^{3+} is an important step in all these processes (22, 23). Recently we demonstrated that microsomes and NADPH, in the presence of Adriamycin, can reduce a variety of Fe^{3+} chelates (24). In this study we have demonstrated that the reduction of Fe^{3+} by heart microsomes is considerably diminished by ICRF-198. This lack of reducibility is reflected in the inability of Fe^{3+}, in the presence of ICRF-198, to catalyze lipid peroxidation and CaATPase inactivation. ICRF-187, which does not inhibit microsomal reduction of Fe^{3+} by microsomes, Adriamycin, and NADPH to the same extent as ICRF-198, is also less effective at inhibiting lipid peroxidation or protecting CaATPase from inactivation.

The mechanism by which ICRF-187 decreases the cardiotoxicity of Adriamycin is unknown. However, recent interest has focused on the ability of its *in vivo* hydrolysis product, ICRF-198, to chelate iron (20, 30). ICRF-198 binds a variety of polyvalent metal ions, including Fe^{2+}, with a high affinity (31). The effects of ICRF-198 that we observed can be explained in terms of iron binding. Reduction of Fe^{3+} by the microsomal system and subsequent lipid peroxidation and thiol oxidation require the enzymatic reduction of Adriamycin to its semiquinone. The effects of ICRF-198 and -187 were not on this step, since neither altered the rate of NADPH oxidation or the rate of radical generation, measured with cytochrome *c*. An iron binding mechanism would explain why ICRF-198 was maximally effective at concentrations only just above that of iron and why it did not inhibit reduction of Fe^{3+}-desferrioxamine. The latter suggests that ICRF-198 is not as strong a chelator of Fe^{3+} as desferrioxamine. ICRF-198 did inhibit Fe^{3+} reduction and lipid peroxidation in the presence of a 20-fold excess of ADP, implying that it can compete effectively with ADP as an iron ligand. It also follows from this result that ICRF-198 should be able to bind iron intracellularly in the presence of chelators with affinities comparable to ADP. ICRF-187 was considerably less effective than ICRF-198 at inhibiting Fe^{3+} reduction and subsequent lipid peroxidation and CaATPase inactivation. ICRF-187 does not bind iron (30). However, it does undergo slow hydrolysis to the *d* form of ICRF-198, and this may explain why it caused some inhibition.

The majority of nonheme iron is stored intracellularly in ferritin and can be released by reducing species such as microsomally reduced Adriamycin (28, 29). Ferritin, therefore, could provide iron for Adriamycin-dependent damage to heart cells. ICRF-198 did not inhibit iron release from ferritin, presumably because it cannot complex the iron within the ferritin core. Ferritin dependent peroxidation of lipids and inactivation of CaATPase require released iron which is recycled catalytically (14, 17). By binding this iron, ICRF-198 would suppress this recycling; this explains why ICRF-198 inhibited both processes.

Lipid peroxidation of platelet membranes, catalyzed by preformed Fe^{3+}-Adriamycin complexes, has been shown to be inhibited by ICRF-198 (20). In this case the mechanism of inhibition was explained in terms of ICRF-198 removing iron from the Fe^{3+}-Adriamycin complex. A relatively high affinity constant (log k = 9.99) for ICRF-198 and Fe^{2+} has been measured (31) and Hasinoff (30) recently reported that ICRF-198 rapidly removes Fe^{3+} from Fe^{3+}-Adriamycin complexes. It is unlikely that an Fe^{3+}-Adriamycin complex is involved in microsomal lipid peroxidation and CaATPase inactivation reactions reported here and elsewhere (13, 14, 17, 32) since the iron concentration is low. We propose that the significance of chelation of Fe^{3+} by ICRF-198 is that it prevents reduction to Fe^{2+}. In addition to inhibiting Fe^{3+} reduction mediated by the Adriamycin semiquinone, ICRF-198 inhibited direct reduction of Fe^{3+} by the microsomes, and Adriamycin dependent reduction in air, where $O_2^{\cdot-}$ is presumed to be the reductant (24). Therefore, the effects of ICRF-198 are not confined to reactions involving Adriamycin.

Fe^{3+}-EDTA is a good catalyst of $OH^{\cdot}$ production (33) and although EDTA inhibits Fe^{3+} catalyzed lipid peroxidation, it is only partially protective against CaATPase inactivation in microsomes (12, 17). Thus EDTA would not necessarily protect against radical mediated injury, and in contrast to ICRF-198, EDTA forms an Fe^{3+} complex that is readily reduced by Adriamycin radicals and the microsomal system (24). Iron complexes must undergo reduction in order to promote free radical reactions (22–24). Thus development of effective intracellular chelators must consider not only iron binding characteristics but also low biological reducibility.

It is still unresolved whether free radicals are involved in the antitumor activity of Adriamycin. If they are then it is possible that ICRF-198, by binding and inhibiting redox cycling of iron, could decrease its efficacy. However, clinical trials of ICRF-187 suggest that this may not be a problem (6). Heart microsomal and mitochondrial reductases are involved in the cellular reduction of Adriamycin and Fe^{3+} (11, 12, 17, 24). Subsequent production of free radical species that initiate inactivation of CaATPase and the peroxidation of lipids have been implicated in the cardiotoxicity of Adriamycin. Our demonstration that ICRF-198 binds Fe^{3+} so that it is unable to take part in free radical reactions is support for this mechanism and may also explain why ICRF-187 is able to diminish Adriamycin cardiotoxicity *in vivo*.

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