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Modulation of the in vitro cardiotoxicity of doxorubicin by flavonoids

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Abstract Cancer therapy with the anthracycline doxorubicin (Dox) is limited by cardiomyopathy, which develops in animals and patients after cumulative dosing. Generation of free radicals by Dox may be involved in this cardiotoxicity. Dox binds strongly to metal ions, especially iron(III). This Dox-metal complex stimulates the generation of free radicals through self-reduction of the complex. We investigated the possibility of inhibiting Dox-induced cardiotoxicity by scavenging of free radicals and/or chelating metal ions. The effects of Dox, both alone and in combination with iron-chelating agents, were studied on inotropy of the isolated mouse left atrium, lipid peroxidation (LPO) in cardiac microsomal membranes, ferricytochrome c (cyt.c^{3+}) reduction, and oxygen consumption. The flavonoids 7-monohydroxyethylrutoside (mono-HER) and 7,3′,4′-trihydroxyethylrutoside (tri-HER) and the ethylenediaminetetraacetic acid (EDTA) analogue ICRF-198 and its precursor ICRF-187 were used as iron-chelating agents. The latter were used for comparison since ICRF-187 has been reported to inhibit the cardiotoxic effects of Dox both in vitro and in vivo. Only the flavonoids could inhibit the negative inotropic effect of Dox (35 μ*M*) on the mouse left atrium; in the presence of tri-HER (500 μ*M*) the beating force decreased by 18% instead of 50%, whereas mono-HER completely prevented the Dox-induced negative inotropic effect. ICRF-198 and both flavonoids (500 μ*M*) completely inhibited Dox (35 μ*M*)-induced LPO, whereas ICRF-187 provided 65% inhibition. The observation that both cyt.c^{3+} reduction and oxygen consumption induced by the Dox-iron(III) complex (50/16.6 μ*M* Dox_3Fe^{3+}) could be inhibited by superoxide dismutase proved the involvement of superoxide anions ($O_2^{\cdot -}$). The iron-chelating agents (50 μ*M*) inhibited cyt.c^{3+} reduction by 91% (mono-HER), 43% (tri-HER), and 100% (ICRF-198). Only mono-HER and ICRF-198 (50 μ*M*) were capable of inhibiting the oxygen consumption by 70% and 43%, respectively. It is concluded that flavonoids offer a good perspective for further studies on the prevention of Dox-induced cardiomyopathy.

Key words Flavonoids · Doxorubicin-iron complex · ICRF-187

Introduction

The anthracycline doxorubicin (Dox) is a highly effective anticancer drug. It can intercalate cell DNA and thus interfere with the processes of replication and DNA synthesis. Its clinical use is limited by cardiomyopathy, which can develop in patients when cumulative doses exceed 500 mg/m^2 [33]. Dox can also cause acute cardiotoxicity by exerting a direct negative inotropic effect on isolated mouse atria that does not involve the ß-adrenoceptor [5, 22]. We found the mouse left atrium to be a sensitive and useful in vitro model to estimate the acute cardiotoxic effect of anthracyclines [5].

The mechanism behind the cardiotoxicity associated with Dox has remained elusive, but several hypotheses have been put forward, including strand scission in the DNA molecule [29]; generation of free radicals, which is stimulated when Dox is chelated to metal ions [10, 11, 37, 38]; and subsequent lipid peroxidation [14, 24, 34]. This occurs through formation of the Dox semiquinone free radical [1] and via reactive oxygen species through redox cycling [9, 12]. Whether

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formation of Dox-metal complexes plays a role in the cardiotoxicity observed in vivo is a matter of debate. The concentration of free iron(III) and free copper(II) is very low in vivo, but the affinity constants are so high (10^{28}–10^{33} M^{-3} [3, 25] and 10^{12}–10^{16} M^{-2} [26], respectively) that the presence of the Dox_3Fe^{3+} and Dox_2Cu^{2+} complexes cannot be merely hypothetical. In vitro, transfer of iron from ferritin [7] and microsomes [8] to Dox has been demonstrated, indicating that bound iron may nonetheless be available for complex formation. Dox-metal complexes can also give rise to reactive oxygen species [14, 17, 38].

It has been shown in vitro that damage through reactive oxygen species to biological targets requires iron [11, 14, 19, 25, 39]. Ways to inhibit the iron-induced effects include (a) scavenging of reactive oxygen species ($O_2^{\cdot -}$, H_2O_2) by superoxide dismutase (SOD) and catalase and (b) chelating of iron by metal-ion-complexing agents. Thus, either the formation of the Dox_3Fe^{3+} complex is prevented or it is dissociated. It has been shown that ICRF-187 [(+)-1,2-bis(3,5-dioxopiperazin-l-yl) propane], a lipophilic ethylenediaminetetraacetic acid (EDTA) analogue (Fig. 1), inhibits iron-induced lipid peroxidation (LPO) [27]. Both in animal models [13, 21] and in a clinical study [30], ICRF-187 offered protection against Dox-induced cardiotoxicity. ICRF-187 is the (S)-enantiomer of the racemic mixture known as ICRF-159. It can pass cell membranes and hydrolyze intracellularly, rendering the ring-opened form ICRF-198, which chelates metal ions [16] and is thought to be the active species.

Flavonoids, which are ubiquitous in photosynthesizing cells and therefore occur in various plants, also possess iron-chelating capacity. For centuries, preparations that contain flavonoids have been used by physicians for the treatment of human diseases [20]. Due to their metal-ion-chelating effect and their antioxidant activity [20, 31], flavonoids offer a good prospect for inhibiting the effects of Dox in vitro and in vivo. Cardioprotective properties have been shown in vivo by using a synthetically modified flavonoid mixture [32]. Two compounds present in this mixture were chosen for further study of this protection in vitro by measuring their influence of effects induced by Dox and Dox_3Fe^{3+}: 7, 3′4′-trihydroxyethylrutoside (tri-HER) and 7-monohydroxyethylrutoside (mono-HER; Fig. 1). Their antioxidant activities are equal, but mono-HER is a much better iron chelator than tri-HER [2].

Our aim was to investigate the potential of these flavonoids in protecting against the cardiotoxic effects of Dox in vitro. To that end, we studied the effect of Dox and the Dox_3Fe^{3+} complex, both alone and in combination with the iron-chelating agents, on isolated mouse left-atrium inotropy, LPO in cardiac microsomal membranes, ferricytochrome c (cyt.c^{3+}) reduction, and oxygen consumption.

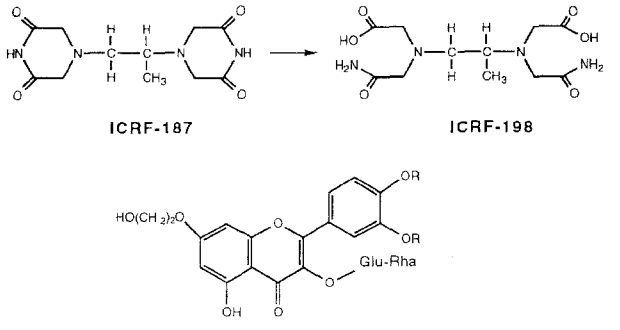

Fig. 1 Structural formulas of the iron-chelating (modulators) used in this study

Materials and methods

Animals

Male BALB/c mice aged 6–8 weeks (weight, 18–22 g) were obtained from C.P.B Harlan Olec (Zeist, The Netherlands).

Chemicals

Doxorubicin-HCl was kindly supplied by Farmitalia Carlo Erba (Milan, Italy). TRIS-base, SOD (bovine blood), catalase (bovine liver), and cyt.c^{3+} (grade III) were obtained from Sigma (St. Louis, USA). Tri-HER and mono-HER were gifts from Zyma (Nyon, Switzerland) and ICRF-187 (Cardioxane) and ICRF 198, from EuroCetus (Amsterdam, The Netherlands). All other chemicals were analytically pure. Ultrapure water was prepared using a Nanopure apparatus (Barnstead, Boston, USA).

For the isolated left-atrium inotropy and LPO experiments, Dox was dissolved in 0.9% NaCl. The stock solution Dox_3Fe^{3+} (2/0.66 μM) was prepared by adding 1.32 μM $FeCl_3 \cdot 6H_2O$ dissolved in 1.5 μM HCl [to prevent Fe(III) hydroxide polymer formation] to an equal volume of 4 μM Dox dissolved in water. Polypropylene tubes were used throughout for dissolving Dox (to minimize Dox adsorption). ICRF-187 was dissolved in water, in which only minimal hydrolysis occurred, and prepared fresh each experimental day. The flavonoids were dissolved in dimethylsulfoxide (DMSO) and further diluted with ultrapure water. TRIS/KCl (50/150 μM) buffer (pH 7.4) was passed over a Chelex-100 (Bio-Rad, Veenendaal, The Netherlands) column. Ascorbic acid was neutralized (pH 7.4) with NaOH. All experiments were carried out at 37°C.

Isolated left-atrium inotropy

This method has been outlined extensively elsewhere [5]. Only a concise description is given herein. Animals were killed by decapitation and the entire heart was quickly removed. To arrest contraction, the heart was put in calcium-free Dulbecco's buffer (pH 7.4) containing (μM):NaCl (136.9), KCl (2.68), KH_2PO_4 (1.47), and Na_2HPO_4 (8.0). Left atria were separated from the heart and placed in a 20-μl organ bath containing Krebs solution of the following

composition (μ*M*): NaCl (117.5), KCL (5.6), $MgSO_4$ (1.18), $CaCl_2$ (2.5), NaH_2PO_4 (1.28), $NaHCO_3$ (25), and glucose (11.1; pH 7.4) gassed with 5% CO_2 in O_2. A preload of 0.4 g and a stimulation frequency of 4 Hz (Grass S88 stimulator) were applied to the atria. The atria were preincubated for 30 min with modulator (mono-HER, tri-HER, or ICRF-187) or 2 h (ICRF-187) before the addition of Dox at the IC_{50} (35 μ*M*, causing a negative inotropic effect of 50% after 1 h). The effect of Dox was tested by measuring the negative inotropic effect during 1 h of incubation with Dox.

Preparation of heart microsomes

Mice were killed by decapitation. The hearts were removed, rinsed in 50 μ*M* potassium phosphate buffer (pH 7.4; 10%, w/v), and homogenized. The homogenates were centrifuged (10,000 *g*, 20 min) and the supernatants, decanted and again centrifuged under the same conditions. In the next step the supernatants were centrifuged at 105,000 *g* for 60 min. The remaining pellets were resuspended in phosphate buffer (16%, w/v), repelleted (105,000 *g*, 60 min), re-homogenized in phosphate buffer under the same conditions, and stored at − 80°C until use.

Lipid peroxidation

The heart microsomal fractions were thawed on ice and centrifuged (135,000 *g*, 45 min). The pellets obtained were suspended in TRIS/KCl buffer (16%, w/v). Incubation medium (1.0 ml) consisting of a reduced nicotinamide adenine dinucleotide phosphate (NADPH)-generating system ($NADP^+$, 1.9 μ*M*; glucose-6-phosphate, 20 μ*M*; glucose-6-phosphate dehydrogenase, 1.1 U/ml; and $MgCl_2 \cdot H_2O$, 4.3 μ*M* in TRIS/KCl buffer) was mixed with Dox (70 μ*M*) and modulator (twice the final concentration). After preincubation of the mixture for 5 min at 37°C the reaction was started by the addition of 1.0 ml of the microsomal fraction (≈0.1 mg protein/ml, 37°C). At $t = 0$, 20, 40, 60, and 120 min, 0.3-ml aliquots were taken from the incubate and mixed with 2.0 ml of thiobarbituric acid (TBA) reagent [15]. After 15 min at 80°C the TBA mixture was centrifuged and the absorption at 535 nm was measured. Values were corrected for $t = 0$ min absorption (due to Dox and iron chelator).

$Cyt.c^{3+}$ reduction

$Cyt.c^{3+}$ was dissolved in TRIS/KCl buffer. At $t = 0$ min, Dox_3Fe^{3+} (50/16.6 μ*M*) and/or modulator or SOD were added to a microcuvette containing 20 μ*M* $cyt.c^{3+}$. The reduction was followed spectrophotometrically by measuring ferrocytochrome c ($cyt.c^{2+}$) at 550 nm for 30 min. A molar extinction coefficient for ($cyt.c^{2+}$) of 29.5 $\mu M^{-1} cm^{-1}$ was used [17].

O_2 consumption

A 1.0-ml air-tight chamber at 37°C was filled with air-saturated Tris/KCl buffer, which under these conditions contained 199 μ*M* O_2 [4]. During the entire experiment the solution was stirred at a constant rate. The decrease in oxygen tension induced by the removal of O_2 was measured using a Clark electrode and a YSI 5300 biological oxygen monitor (Tamson, Zoetermeer, The Netherlands). The oxygen consumption caused by 50/16.6 μ*M* Dox_3Fe^{3+} (25 μl) alone (no other addition) and in combination with 50 μ*M* modulator (25 μl), SOD (25 ml), and catalase (25 μl) was measured. In all cases the O_2 consumption induced by Dox_3Fe^{3+} was catalyzed by using 400 μ*M* ascorbic acid (10 μl).

Statistical analysis

When appropriate, statistical significance was evaluated using Student's *t*-test.

Results

Modulation of the effect of Dox on isolated left-atrium inotropy by iron-chelating agents

Dox exerts a concentration-dependent negative inotropic effect on isolated mouse atrium [5, 22]. The IC_{50} (the concentration at which the contractile force is reduced by 50% after 1 h) for Dox was determined to be 35 μ*M* [5]. At this concentration, we investigated the inhibitory action of the modulators on the Dox-induced negative inotropic effect after a 30-min period of preincubation. For mono-HER and tri-HER, concentrations ranging from 50 to 500 μ*M* were evaluated, the results of which are shown in Fig. 2. Mono-HER was effective at all concentrations, providing full inhibition of the Dox-induced effect at 500 μ*M*, whereas tri-HER showed an appreciable inhibition only at the highest concentration. At this level, ICRF-187 was not effective. When atria were preincubated with ICRF-187 for 2 h instead of 30 min, a small, although nonsignificant, protective effect was observed for this compound, amounting to 66% ± 12% of the control value as compared with 52% ± 8% following incubation with Dox alone.

Modulation of Dox-induced LPO

Dox induced LPO in mouse heart microsomes. Figure 3 shows that all iron-chelating agents (500 μ*M*)

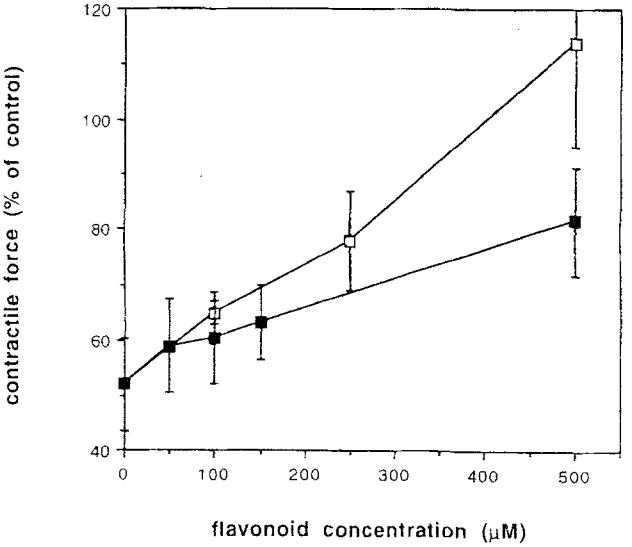

Fig. 2 Concentration-effect curves generated for mono-HER (□) and tri-HER (■) against the Dox (35 μ*M*)-induced decrease in the contraction of paced mouse left atrium (mean values ± SD, $n = 4$)

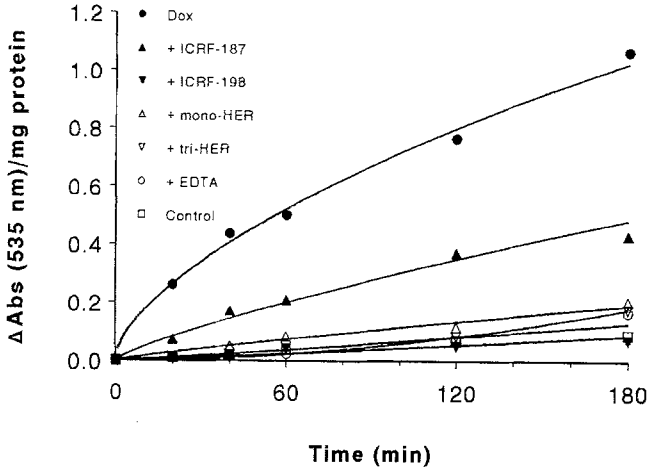

Fig. 3 Time course of lipid peroxidation induced by 35 μ*M* Dox alone and in combination with 500 μ*M* of modulating agent. Incubations were carried out in TRIS-KCl buffer at pH 7.4 and 37°C (mean values for 3–4 experiments; error bars have been omitted for clarity)

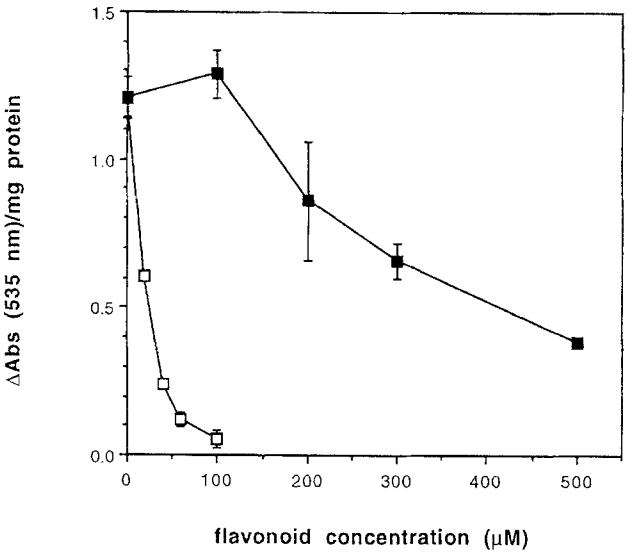

Fig. 4 Concentration-effect curves generated for mono-HER (□) and tri-HER (■) against Dox (35 μ*M*)-induced LPO after 2 h of incubation (mean values ± SD, $n = 3$)

inhibited Dox (35 μ*M*)-induced LPO. The flavonoids mono-HER and tri-HER were effective inhibitors, preventing the formation of TBA-reactive substances almost completely in concentration-dependent fashion (Fig. 4) . ICRF-187 was capable of reducing Dox-induced LPO by 65% after 2 h. Its ring-opened derivative ICRF-198 as well as EDTA provided complete inhibition.

Cyt.c^{3+} reduction induced by Dox_3Fe^{3+}

Superoxide anions ($O_2^{\cdot -}$) are capable of reducing cyt.c^{3+} to cyt.c^{2+}. Without the addition of reducing agents, superoxide anions were apparently generated via self-reduction of the Dox_3Fe^{3+} complex [14, 17, 38]. The increase in absorption at 550 nm (characteristic for cyt.c^{2+}) was measured for 30 min. Neither Dox nor Fe(III) alone could reduce cyt.c^{3+} nonenzymatically. Dox_3Fe^{3+}, however, caused a cyt.c^{3+} reduction of 0.244 ± 0.040 μ*M*/min. The involvement of superoxide anions in this reaction was confirmed by measuring the effect of SOD on (Dox_3Fe^{3+}-induced) cyt.c^{3+} reduction. SOD inhibited the cyt.c^{3+} reduction induced by Dox_3Fe^{3+} in a concentration-dependent fashion, with 91% inhibition being observed at 3,700 U/ml. Since Dox and Fe(III) alone did not cause cyt.c^{3+} reduction, an alternative possibility of inhibiting the cyt.c^{3+} reduction induced by Dox_3Fe^{3+} could be dissociation of the complex. Therefore, we investigated this aspect by the use of iron chelators, which possibly possess a higher affinity than Dox for iron, such as mono-HER, tri-HER, and ICRF-198.

Figure 5 shows the inhibition of the cyt.c^{3+} reduction caused by 50/16.6 μ*M* Dox_3Fe^{3+} in the presence of mono-HER and tri-HER at several concentrations.

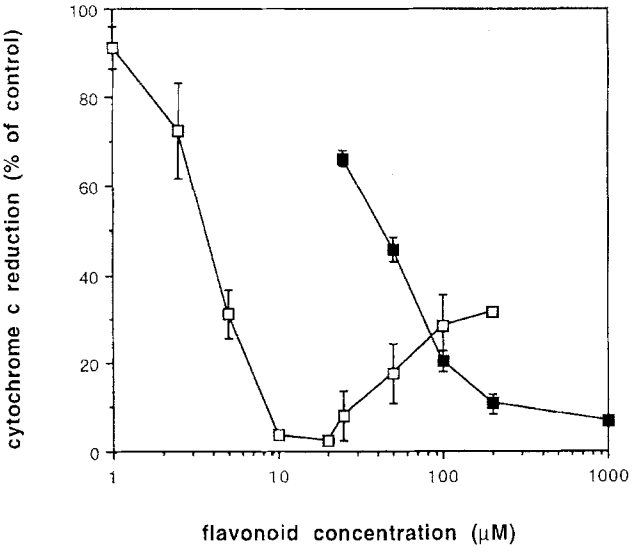

Fig. 5 Concentration-effect curves generated for mono-HER (□) and tri-HER (■) against cyt.c^{3+} reduction induced by the doxorubicin-iron complex (50/16.6 μ*M*; mean values ± SD, $n = 3$)

Whereas the latter compound caused a gradual decrease in cyt.c^{3+} reduction, mono-HER behaved differently, a sharp decrease down to full inhibition of the Dox_3Fe^{3+}-induced cyt.c^{3+} reduction being followed by a gradual increase. For purposes of comparison, all other test compounds were tested only at 50 μ*M*, equal to the Dox concentration; the results are shown in Table 1. At this concentration, mono-HER, ICRF-198, and EDTA were the most potent inhibitors of Dox_3Fe^{3+}-induced cyt.c^{3+} reduction, followed by tri-HER and ICRF-187.

Table 1 Cyt.c^{3+} reduction and oxygen consumption (mean ± SD) induced by the doxorubicin-iron complex in the presence or absence of inhibitors at 50 μ*M*[a]

Addition	Cyt.c^{3+} reduction (μ*M*/min)	Oxygen consumption (μ*M*/min)
None	0	0.3 ± 0.1
Mono-HER	0	0.3
Tri-HER	0	0.3
$FeCl_3$ (16.6 μ*M*)	0	0.8 ± 0.2
Dox (50 μ*M*)	0	1.4 ± 0.3
$FeCl_3$ + Dox	–	2.1
Dox-Fe (50/16.6 μ*M*)	0.244 ± 0.040	4.8 ± 0.8
+ mono-HER	0.022 ± 0.002	1.4 ± 0.6
+ tri-HER	0.115 ± 0.014	4.7 ± 0.8
+ ICRF-187	0.207 ± 0.007	6.6 ± 2.7
+ ICRF-198	0	2.7 ± 0.5

[a]Buffer: TRIS-KCl, 50/150 μ*M*, pH 7.4, 37°C. Cyt.c^{3+} reduction was measured in the presence of 0.25 mg cyt.c/ml. The determination of O_2 consumption was performed using a 1-ml measuring cell equipped with a Clark electrode in the presence of 400 μ*M* ascorbic acid

Modulation of the oxygen consumption induced by Dox_3Fe^{3+}

The production of $O_2^{\cdot -}$ by Dox_3Fe^{3+} is reflected by a decrease in O_2-tension, although not all of the O_2 is transformed into $O_2^{\cdot -}$. The expected decrease in O_2 tension caused by Dox_3Fe^{3+} (under the same conditions as the cyt.c^{3+} reduction) was too low to be measured (below the detection limit of 0.1 μ*M* O_2/min). The addition ascorbic acid (0.4 μ*M*) catalyzed the $O_2^{\cdot -}$-production through Dox_3Fe^{3+}. Therefore, all oxygen consumption measurements were carried out in the presence of ascorbic acid. As can be seen in Table 1, there was a clear-cut difference in the oxygen consumption caused by 50 μ*M* Dox and 16.6 μ*M* Fe(III), on the one hand, and 50/16.6 μ*M* Dox_3Fe^{3+}, on the other.

Since SOD catalyzes the transformation of $O_2^{\cdot -}$ into $O_2^{\cdot -}$ and H_2O_2, the addition of SOD to the measuring cell should result in a less pronounced decrease in oxygen tension because of the regeneration of O_2. Indeed, adding 725 U SOD/ml to the solution decreased Dox_3Fe^{3+}-induced O_2 consumption by 31%. The subsequent addition of a 2,750-U/ml concentration of catalase, which can regenerate O_2 from H_2O_2, decreased the O_2 consumption by another 45%.

Iron chelators would be expected to inhibit the O_2 consumption of Dox_3Fe^{3+}. Table 1 shows that ICRF-198 and mono-HER (50 μ*M*) were capable of inhibiting the O_2 consumption by 70%, or down to the value measured for 50 μ*M* Dox alone. Neither tri-HER (50 μ*M*) nor ICRF-187 (50 μ*M*) affected Dox_3Fe^{3+}-induced O_2 consumption.

Discussion

In this study we compared three metal-chelating agents and a (lipophilic) precursor with respect to their potential to inhibit processes tentatively involved in Dox-induced cardiotoxicity. Dox directly affects the functionality of heart muscle that does not involve the ß-adrenoceptor [5, 22]. The flavonoids mono- and tri-HER clearly showed better inhibition of the effect of Dox on atrial inotropy than did ICRF-187. Some reasons for ICRF-187 not being effective may be that (a) the compound was not taken up in the atrial cells or (b) it was not (or not yet) hydrolyzed to ICRF-198, the active ligand. In vivo, ICRF-187 has afforded protection against the cardiac side effects of Dox [13, 21, 30]. However, the hydrolysis of ICRF-187 to ICRF-198 is very slow as indicated by a half-life of 7.8 h in 66 μ*M* phosphate buffer (pH 7.4, 37°C) [16] and a biological half-life of 12 h [38]. This observation may explain the slight, albeit not significant, increase in protection occurring in our model following a longer period of preincubation. Another factor may be the amount of ICRF-187 that is needed for significant protection. Measuring the concentration of Dox in the atrial tissue after incubation revealed this to be 20- to 30-fold higher than the incubation concentration [6]. Thus, it may be hypothesized that the intracellular ICRF-198: Dox ratio was too low to affect Dox-induced atrial toxicity. Third, although less obvious because of the relatively low affinity constant [23], an effect of ICRF-198 on the Ca^{2+} homeostasis, directly influencing the contractile properties, may be considered. Another factor may be that the flavonoids decrease the membrane permeability [20], thus influencing the uptake of Dox.

Also in the LPO experiments, the flavonoids showed a concentration-dependent inhibition of the Dox-induced effect (Fig. 4). At 500 μ*M* (the concentration used in the inotropy experiments), both flavonoids, ICRF-198, and EDTA completely inhibited Dox (35 μ*M*)-induced LPO, whereas ICRF-187 provided only 65% inhibition (Fig. 3). Although Ryan et al. [27] have suggested that liver microsomes do not hydrolyze ICRF-187, an additional experiment revealed that mouse liver microsomes showed the same degree of protection by ICRF-187 as did mouse heart microsomes (data not shown). Thus, probably ICRF-187 was indeed partly hydrolyzed in microsomes. Mono-HER and tri-HER would be capable of inhibiting the LPO both by their metal-ion-chelating activity and by their antioxidant property. It is not clear, however, to what extent both activities might have contributed to the inhibition of the LPO induced by Dox. EDTA and ICRF-198 also inhibited Dox-induced LPO, although their only activity is metal-ion chelation. Thus, Dox-induced LPO depends on the presence of iron. In an additional experiment the LPO induced by the doxorubicin-iron complex was measured. Its effect

exceeded that of free Dox by 40% after 2 h of incubation (results not shown).

To evaluate further the possible consequences of iron chelation by anthracyclines, a simple chemical model was chosen using the artificially produced doxorubicin-iron complex in the presence or absence of the compounds tested for their protective ability. It was shown that Dox_3Fe^{3+} (50/16.6 μM) was capable of reducing cyt.c^{3+} nonenzymatically, whereas Dox alone (50 μM) could not reduce cyt.c^{3+}. SOD, which transforms $O_2^{\cdot -}$ into O_2 and H_2O_2, inhibited Dox_3Fe^{3+} (50/16.6 μM)-induced cyt.c^{3+} reduction. Thus, the reduction of cyt.c^{3+} is mediated by superoxide anions that are generated via a so-called self-reduction of Dox_3Fe^{3+}. In this process an intramolecular electron transfer from one of the Dox ligands to the Fe^{3+} takes place, rendering a Dox-semiquinone radical, followed by an electron transfer to an oxygen molecule [17]. There are two explanations for the observation that inhibition of the cyt.c^{3+} reduction by SOD occurred only at rather high enzyme concentrations (3700 U/ml to achieve 91% inhibition): (1) the suggestion that the semiquinone radical itself can reduce cyt.c^{3+} directly by transferring the electron to the cytochrome heme iron and (2) the competition of both SOD and cyt.c^{3+} for $O_2^{\cdot -}$. Winterbourn, in studying cyt.c^{3+} reduction by the alloxane radical [35] and semiquinone radicals [36], elegantly explained the high concentrations of SOD needed by assuming an indirect inhibitory effect of SOD on cyt.c^{3+} reduction: by trapping superoxide anion radicals, SOD forces the equilibrium of reaction 1 (see below) to the right, thereby inhibiting reaction 2:

$$R^{\cdot} + O_2 \rightarrow R + O_2^{\cdot -} \quad (1)$$

$$R^{\cdot} + \text{cyt}\,c^{3+} \rightarrow R + \text{cyt}\,c^{2+}. \quad (2)$$

The faster reaction 2 proceeds, the more SOD will be needed to obtain an inhibition of cyt.c^{3+} reduction.

The flavonoid mono-HER and ICRF-198 were the most effective inhibitors of Dox_3Fe^{3+} (50/16.6 μM)-induced cyt.c^{3+} reduction. Tri-HER, which has a lower iron-chelating activity than mono-HER [2], inhibited cyt.c^{3+} reduction by 59% at 50 μM (Table 1). These results indicate that the iron-chelating activity of the compounds plays an important role in inhibiting the cyt.c^{3+} reduction. The role of the antioxidant property is unknown. ICRF-187 inhibited cyt.c^{3+} reduction by a mere 15%, which is probably attributable to its hydrolysis during the incubation period. Sobol et al. [28] suggested a mode of action of ICRF-187 itself, namely, the inhibition of Dox_3Fe^{3+}-induced radical formation by steric hindrance through the formation of a ternary complex (Dox/Fe/ICRF-187). This could also be an explanation for the results we obtained with ICRF-187.

In the presence of ascorbic acid, free Dox can be reduced to its semiquinone free radical. Under aerobic conditions this semiquinone free radical is capable of forming superoxide anions through redox cycling. The transformation of oxygen to superoxide anions can be determined by measuring the decrease in O_2 tension. The O_2 consumption caused by 50 μM Dox (in the presence of 400 μM ascorbic acid) was 1.4 μM O_2/min. The O_2 consumption caused by Dox_3Fe^{3+} (50/16.6 μM) was approximately 4-fold higher, whereas control experiments using a combination of free Dox and Fe^{3+} showed a merely additive effect (Table 1). Thus, the complex is not destroyed by ascorbic acid.

SOD catalyzes the reaction:

$$2\,H^+ + 2\,O_2^{\cdot -} \xrightarrow{SOD} O_2 + H_2O_2. \quad (3)$$

SOD (725 U/ml) decreased Dox_3Fe^{3+}-induced O_2 consumption by 31%. For calculations, the blank value (400 μM ascorbic acid) of 0.3 $\pm$ 0.1 μM/min was taken into account. Catalase (*CAT*) catalyzes the reaction:

$$2\,H_2O_2 \xrightarrow{CAT} 2\,H_2O + O_2. \quad (4)$$

When catalase (2,750 U/ml) was added following SOD, the O_2 consumption decreased by another 31%. The net SOD and catalase reaction is:

$$4\,O_2^{\cdot -} \longrightarrow 3\,O_2 + 2\,H_2O. \quad (5)$$

Thus, theoretically, after the addition of SOD and catalase a 75% decrease in the initial O_2 consumption would be expected. In practice, however, a 62% reduction in O_2 consumption was found. This might indicate that not all of the consumed O_2 was transformed into $O_2^{\cdot -}$, in which case SOD would not be capable of preventing the decrease in O_2 tension by the full 50%. Moreover, H_2O_2 may also have reacted via the Fenton reaction to form $\cdot OH$ and OH^-. In that case, not all of the H_2O_2 formed via reaction 1 would be available for reaction 2. Zweier et al. [39] found that SOD (120 U/ml) and catalase (400 U/ml) decreased the Dox_2Fe^{3+} (0.2/0.1 μM)-induced O_2 consumption of 7.5 μM/min by 37.5%. Two reasons for the discrepancy with our data are apparent: (1) the Dox/Fe ratio (2: 1) used by Zweier et al. [39] is not optimal and (2) the concentration of the complex is too high in relation to the concentration of (especially) SOD. Thus, the latter authors did not obtain the theoretical decrease of 75%. In our setup, mono-HER (50 μM) could inhibit the O_2 consumption of Dox_3Fe^{3+} (50/16.6 μM) by 70%, i.e., to a level as low as the O_2 consumption caused by free Dox. The flavonoid tri-HER (50 μM) and ICRF-187 (50 μM) had no effect on the O_2 consumption of Dox_3Fe^{3+}. The values measured after the addition of ICRF-187 showed a large variation, but no significant effect was found. Like Sobol et al. [28], Hasinoff [18] has also hypothesized that ICRF-187 binds to Dox_3Fe^{3+}, forming the complex Fe^{3+}-Dox_2-(ICRF-187). This complex would be formed directly

and would inhibit the reduction of the Dox_3Fe^{3+} complex. Hasinoff, however, claims that this accelerates the hydrolysis of ICRF-187, resulting in dissociation of the complex (yielding Fe^{3+}-ICRF and Dox). If this process indeed occurs, it probably takes more than a few minutes to take effect. In our experiments, no inhibition by ICRF-187 of Dox_3Fe^{3+}-induced oxygen consumption was observed.

In vivo, ICRF-187 has proved to be an inhibitor of Dox-induced cardiotoxicity [13, 21, 30, 32]. In this study we showed the flavonoids mono- and tri-HER to be more active than ICRF-187 in inhibiting the in vitro cardiotoxicity of Dox and about as active as ICRF-198 in inhibiting radical formation caused by Dox and/or Dox_3Fe^{3+}. In conclusion, these compounds offer a good perspective for lowering the cardiotoxicity of Dox in vivo both by inhibiting the generation of free radicals and by their scavenging properties. Results obtained in in vivo studies with a flavonoid mixture are encouraging [32]; investigations into the protective capacity of mono-HER alone are under way.

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