

The Metabolites of the Cardioprotective Drug Dexrazoxane Do Not Protect Myocytes from Doxorubicin-Induced Cytotoxicity

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Received February 23, 2003; accepted May 20, 2003

This article is available online at <http://molpharm.aspetjournals.org>

ABSTRACT

The clinically approved cardioprotective agent dexrazoxane (ICRF-187) and two of its hydrolyzed metabolites (a one-ring open form of dexrazoxane and ADR-925) were examined for their ability to protect neonatal rat cardiac myocytes from doxorubicin-induced damage. Dexrazoxane may protect against doxorubicin-induced damage to myocytes through its strongly metal-chelating hydrolysis product ADR-925, which could act by displacing iron bound to doxorubicin or chelating free or loosely bound iron, thus preventing site-specific iron-based oxygen radical damage. The results of this study showed that whereas dexrazoxane was able to protect myocytes from doxorubicin-induced lactate dehydrogenase release, neither of the metabolites displayed any protective ability. Dexrazoxane also reduced apoptosis in doxorubicin-treated myocytes. The

ability of dexrazoxane and its three metabolites to displace iron from a fluorescence-quenched trapped intracellular iron-calcein complex was also determined to see whether the metabolites were taken up by myocytes. Although ADR-925 was taken up in the absence of calcium in the medium, in the presence of calcium, its uptake was greatly slowed, presumably because it formed a complex with calcium. Both of the one-ring open metabolites were taken up by myocytes and displaced iron from its complex with calcein. These results suggest either that the anionic metabolites do not have the same access to iron pools in critical cellular compartments, that their uptake is slowed in the presence of calcium, or, less likely, that dexrazoxane protects by some other mechanism.

Dexrazoxane (ICRF-187; Zinecard; Fig. 1) is clinically used to reduce doxorubicin-induced cardiotoxicity (Hasinoff, 1998; Hasinoff et al., 1998). There is now considerable evidence to indicate that this toxicity may be due to iron-dependent oxygen free radical formation (Meyers, 1998) on the relatively unprotected cardiac muscle. Dexrazoxane likely acts by diffusing into the cell and hydrolyzing (Hasinoff, 1994a,b, 1998; Hasinoff et al., 1998) to its ring-opened metal ion-binding form, ADR-925 (Fig. 1), which has a structure similar to that of EDTA. ADR-925 may then either remove iron from the iron-doxorubicin complex (Buss and Hasinoff, 1993) or bind free or loosely bound iron, thus preventing oxygen radical formation. ADR-925 binds Fe^{2+} and Fe^{3+} with formation constants of 10^{10} and $10^{18.2} \text{ M}^{-1}$, respectively, compared with formation constants of $10^{14.3}$ and $10^{25.1} \text{ M}^{-1}$ for EDTA (Huang et al., 1982; Diop et al., 2000). Thus, dexrazoxane, which is easily permeable to cells (Dawson, 1975), can be considered a neutral prodrug analog of EDTA.

We previously showed in isolated hepatocytes (Hasinoff et al., 1994), in a rat model (Schroeder and Hasinoff, 2002), and in humans (Schroeder et al., 2003) that dexrazoxane was rapidly metabolized to the one-ring open compounds **B** and **C**, and ADR-925 (Fig. 1). This metabolism may be due, in part, to the ability of dihydropyrimidinase, which is present in the liver and kidney, but not the heart, to enzymatically hydrolyze dexrazoxane to **B** and **C**, but not to ADR-925 (Hasinoff et al., 1998). We also recently showed that dihydroorotase, which is present in all three of these organs, enzymatically hydrolyzes **B** and **C** to ADR-925 but does not act on dexrazoxane (Schroeder et al., 2002). We also previously used a neonatal cardiac myocyte model to compare the ability of dexrazoxane and deferiprone to protect against doxorubicin-induced cytotoxicity (Barnabé et al., 2002). Given the rapid appearance of these metabolites in plasma, we decided to examine whether these metabolites could prevent doxorubicin-induced damage to isolated neonatal rat myocytes and, thus, whether they might be useful as drugs. To accompany these studies, we also investigated the uptake of dexrazoxane and its metabolites in myocytes by following the fluorescence dequenching of an intracellular iron-calcein

This work was supported by the Canadian Institutes of Health Research, the Canada Research Chairs Program, and a Canada Research Chair in Drug Development for B.H.

ABBREVIATIONS: DF- α , Dulbecco's modified Eagle's medium/Ham's F-12 medium with 50 mM HEPES (pH 7.4) (α is v/v % serum); HBS, HEPES/NaCl buffer (20/150 mM, pH 7.4); k_{obs} , pseudofirst-order rate constant; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling; LDH, lactate dehydrogenase; $t_{1/2}$, half-time; $t_{1/2\beta}$, beta phase pharmacokinetic elimination half-time; v , initial velocity; λ_{ex} , λ_{em} , fluorescence excitation and emission wavelengths, respectively.

complex (Barnabé et al., 2002; Esposito et al., 2002; Hasinoff, 2002; Kakhlon and Cabantchik, 2002).

Materials and Methods

Materials. DF-15 medium [with 7.5% (v/v) fetal bovine serum and 7.5% (v/v) horse serum], fetal bovine and horse serum, penicillin, streptomycin, and fungizone were obtained from Invitrogen (Burlington, ON, Canada). Trypsin, collagenase, and DNase were from Worthington Biochemicals (Freehold, NJ). The black plastic 96-well plates with clear bottoms used in the fluorescence plate reader studies were obtained from Corning Glassworks (Corning, NY). Unless specified, other reagents were obtained from Sigma-Aldrich (Oakville, ON, Canada). Stock calcein (Molecular Probes, Eugene, OR) solutions were prepared in water and stored dark in the cold over the chelating resin Chelex (0.2 g/ml), which was found to greatly increase its stability. HBS buffers were likewise stored over Chelex to reduce levels of adventitious iron. Metabolites **B** and **C** were prepared from NaOH-hydrolyzed dexrazoxane and separated by reverse-phase C_{18} and then ion-exchange column chromatography as described (Schroeder et al., 2002). As determined by high-performance liquid chromatography, **B** and **C** contained less than 1.0 and 1.6 mol% ADR-925, respectively. The ApoAlert DNA fragmentation assay TUNEL kit was from BD Biosciences Clontech (Palo Alto, CA). Nonlinear least-squares curve fitting was done with SigmaPlot (SPSS Science, Chicago, IL). The errors shown were least-squares calculated S.E. values. Where significance is indicated, an unpaired *t* test was used with $p < 0.05$ considered as significant.

Myocyte Isolation and Culture. Ventricular myocytes were isolated from 1- to 3-day-old Sprague-Dawley rats as described (Barnabé et al., 2002; Hasinoff, 2002). Briefly, minced ventricles were serially digested with collagenase and trypsin in Dulbecco's

phosphate-buffered saline /1% (w/v) glucose at 37°C in the presence of DNase and preplated in large Petri dishes to remove fibroblasts. The preparation, which was typically greater than 90% viable by trypan blue exclusion, yielded an almost confluent layer of uniformly beating heart myocytes by day 2. For the LDH release experiments, the myocyte-rich supernatant was plated on day 0 in 24-well plastic culture dishes (5×10^5 myocytes/well, 750 μ l/well) in DF-15 to yield an almost confluent layer of beating heart myocytes by day 2. On days 2 and 3, the medium was replaced with 750 μ l of fresh DF-10 containing 10% (v/v) fetal bovine serum. To lower the background LDH levels, on day 4, 24 h before the drug treatments, the medium was changed to DF-2 and again on day 5 just before the addition of drugs. The animal protocol was approved by the University of Manitoba Animal Care Committee.

LDH Determination and Drug Treatments. LDH released into the myocyte growth medium was determined as previously described (Barnabé et al., 2002; Hasinoff, 2002). In all experiments at the end of the 3-h doxorubicin treatment, the myocytes were washed (two 20-min incubations followed by replacement with fresh medium) with fresh DF-2 medium containing 0, 20, or 100 μ M dexrazoxane, **B**, or ADR-925, respectively, and maintained in this medium for a further 72 h. Starting on day 6 after plating, samples (80 μ l) of the myocyte supernatant were collected every 24 h for 3 days after treatment with doxorubicin and/or dexrazoxane, **B**, or ADR-925. The samples were frozen at -80°C and analyzed within 1 week. After the last supernatant sample was taken, the myocytes were lysed with 250 μ l of 1% (v/v) Triton X-100/2 mM EDTA/1 mM dithiothreitol/0.1 M phosphate buffer (pH 7.8) for 20 min at room temperature. The total cellular LDH activity, from which the percentage of LDH was calculated, was determined from the activity of the lysate plus the activity of three 80- μ l samples previously taken. The LDH activity was determined in quadruplicate, in a kinetic

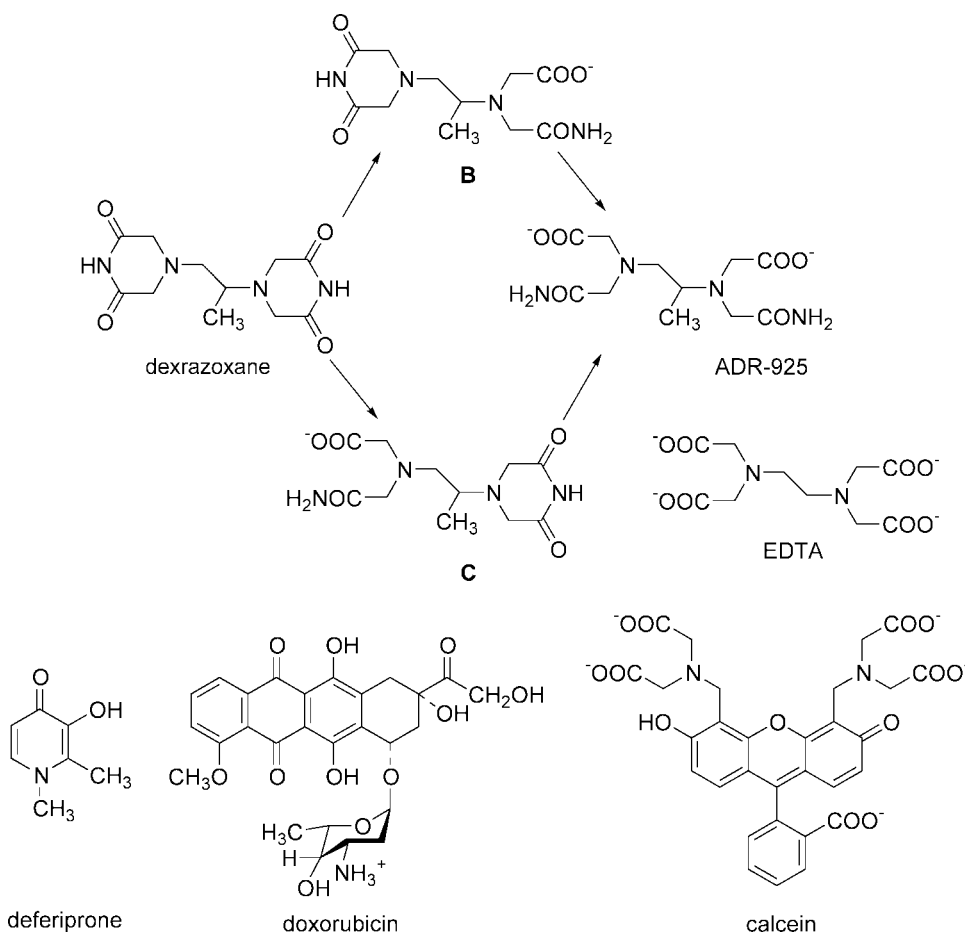


Fig. 1. Structure of dexrazoxane, its one-ring open hydrolysis intermediates **B** and **C**, and its metal-chelating hydrolysis product ADR-925. Also shown are doxorubicin and the iron chelators, EDTA, deferiprone, and the fluorescent iron sensor calcein.

assay in a 96-well plate in a Molecular Devices Corp. (Sunnyvale, CA) plate reader. The initial velocity of the LDH-catalyzed reaction of NAD^+ with lactate to produce NADH and pyruvate was determined by measuring the rate of increase in absorbance at 340 nm at 25°C. The assay buffer contained 2.4 mM NAD^+ and 290 mM sodium lactate in 28 mM Tris buffer (pH 8.8).

Calcein Loading of Myocytes and Displacement of Iron from the Fluorescence-Quenched Intracellular Calcein-Iron Complex by Dexrazoxane, B, C, and ADR-925. Calcein was loaded into attached myocytes 6 to 10 days after plating in 96-well plates (125,000 myocytes/well, 200 μl of medium/well), essentially as described (Cabantchik et al., 1996; Zanninelli et al., 1997; Barnabé et al., 2002; Hasinoff, 2002). Briefly, myocytes were incubated with 125 nM calcein-AM (Molecular Probes), the cell-permeant acetoxymethyl ester of calcein, for 5 min at 37°C in serum-free DF-0 medium, followed by three changes of medium at room temperature to remove extracellular ester. The kinetic fluorescence measurements were conducted on a BMG Labtechnologies Inc. (Durham, NC) Fluostar Galaxy fluorescence plate reader (λ_{ex} of 485 nm, λ_{em} of 520 nm, 30°C) equipped with excitation and emission probes directed to the bottom of the plate. To reduce background fluorescence from DF-0 medium, the medium was changed to HBS buffer (100 μl) a couple of minutes before the addition of the drug. After initial baseline fluorescence intensity data were collected, dexrazoxane or its metabolites were added to the attached myocytes by pipetting stock drug solution into the well and gently mixing the solution with a pipette. The increase in fluorescence as the drug displaced iron from the fluorescence-quenched trapped intracellular calcein-iron complex was recorded relative to untreated controls as a function of time. The initial velocities (v) for the fluorescence change occurring upon the addition of chelators were calculated by linear least-squares fits of the fluorescence-time data over the first 20 min for dexrazoxane and 50 min for the metabolites. The neutral iron chelator deferiprone (Fig. 1), which rapidly enters cells and displaces iron from its complex with calcein, was used as a positive control (Zanninelli et al., 1997; Barnabé et al., 2002).

Solution Kinetics of the Reaction of ADR-925 and B with Fe^{2+} -Calcein. The displacement of Fe^{2+} from its complex with calcein by ADR-925 and B was followed in the fluorescence plate reader at 30°C in HBS buffer containing 1 mM ascorbic acid and 0.1 mg/ml catalase. Because the Fe^{2+} -calcein complex is rapidly oxidized to Fe^{3+} -calcein under aerobic conditions (Breuer et al., 1995), ascorbic acid was used to maintain the iron in its ferrous state. The catalase was used to decompose H_2O_2 formed from oxidation of Fe^{2+} -calcein and prevent the oxidation of calcein to a nonfluorescent product (Petrat et al., 2002). The Fe^{2+} -calcein complex (1 μM) was formed by adding FeSO_4 to calcein in the well of the 96-well plate, mixing, and waiting 10 s for the reaction to complete. The chelator was then added, and the fluorescence was followed with time. The initial velocities were calculated from the first 0.7 to 1.5 min of the reaction.

TUNEL Assay of Doxorubicin- and Dexrazoxane-Treated Myocytes, Epifluorescence Microscopy, and Image Analysis. To determine whether doxorubicin treatment of myocytes induced apoptosis, a TUNEL assay was carried out along with a nuclear propidium iodide counterstain to determine the percentage of apoptotic cells as per the manufacturer's directions. The terminal deoxynucleotidyl transferase catalyzes incorporation of fluorescein-dUTP at the free 3'-hydroxy ends of fragmented DNA. During late-stage apoptosis, cellular endonucleases cleave DNA between nucleosomes. The wet-mounted fixed RNase and anti-fade-treated cells were imaged on a Zeiss Axioscop 2 MOT epifluorescence microscope with fluorescein and propidium iodide filter sets, respectively. The percentage of TUNEL-positive cells in 20 randomly chosen fields (approximately 125 cells/field) per treatment was determined from counting the number of TUNEL-positive cells in the green plane relative to the total number of cells in the red plane.

Results

Doxorubicin-Induced LDH Release from Cardiac Myocytes Preincubated with Dexrazoxane, B, and ADR-925. As shown in Fig. 2, a to c, a 3-h treatment with 1.5 μM doxorubicin, which was followed by washing doxorubicin off and maintaining the myocytes in doxorubicin-free medium, resulted in a significant increase in the cumulative amount of the LDH released, compared with untreated myocytes at all three times up to 72 h. The release of the cytosolic enzyme LDH from myocytes is commonly used as a measure of doxorubicin and other drug-induced damage (Hershko et al., 1993; Barnabé et al., 2002). The data of Fig. 2a also show that preincubation of the myocytes with either 20 or 100 μM dexrazoxane for 3 h before doxorubicin treatment resulted in a significant reduction in doxorubicin-induced LDH release similar to what we observed before (Barnabé et al., 2002). The data of Fig. 2, b and c, show that preincubation of the myocytes with either 20 or 100 μM B or ADR-925 for 3 h before doxorubicin treatment did not result in any significant decrease in doxorubicin-induced LDH release. In similar experiments carried out using 0.6 μM doxorubicin, preincubation with either 20 or 100 μM B also did not reduce doxorubicin-induced LDH release (data not shown).

Kinetics of Displacement of Intracellular Iron from Its Complex with Calcein by ADR-925, Metabolites B and C, and Dexrazoxane in Myocytes. As shown in Fig. 3a, the addition of various concentrations of ADR-925 to attached calcein-loaded myocytes in HBS resulted in increases in fluorescence intensity consistent with the removal of iron from the trapped intracellular iron-calcein complex. Upon the addition of 50 to 1000 μM ADR-925, the initial rate of the fluorescence change increased with an increase in the ADR-925 concentration as seen previously (Hasinoff, 2002). A plot of the initial velocities (v) as a function of ADR-925 concentration (Fig. 3b) suggested that ADR-925 displacement of iron from its complex with calcein was a saturable process. A nonlinear least-squares fit of the initial velocity data v to the Michaelis-Menten equation, $v = V_{\text{max}} \cdot [\text{ADR-925}] / (1 + K_m \cdot [\text{ADR-925}])$ gave a K_m of $103 \pm 26 \mu\text{M}$.

Because ADR-925 binds Ca^{2+} and Mg^{2+} with formation constants of $10^{6.9}$ and $10^{5.1} \text{ M}^{-1}$ (Huang et al., 1982), respectively, in vivo, in plasma the ADR-925 is likely complexed to Ca^{2+} and to Mg^{2+} , to a lesser extent. Thus, to determine the effect of Ca^{2+} and Mg^{2+} in the medium on the ADR-925-induced fluorescence dequenching of the intracellular iron-calcein complex in attached myocytes, the experiments were repeated in the presence of Ca^{2+} and Mg^{2+} at concentrations typically found in plasma (Sheppard and Kontoghiorghes, 1993). As can be seen from Fig. 4a, the initial rate of calcein fluorescence dequenching by 500 μM ADR-925 was greatly decreased (6.6-fold) by the addition of 2.5 mM Ca^{2+} to the HBS medium, but not by the addition of 1.0 mM Mg^{2+} . A control experiment (Fig. 4b) was carried out with deferiprone, which has a low affinity for Ca^{2+} and Mg^{2+} (Sheppard and Kontoghiorghes, 1993). As previously seen, deferiprone in HBS caused a rapid fluorescence dequenching of calcein (Barnabé et al., 2002). However, in contrast to the ADR-925 results, in the presence of a mixture of Ca^{2+} and Mg^{2+} , the deferiprone-induced fluorescence dequenching was only slightly affected. Similar results with both ADR-925 and deferiprone were seen with calcein-loaded myocytes in

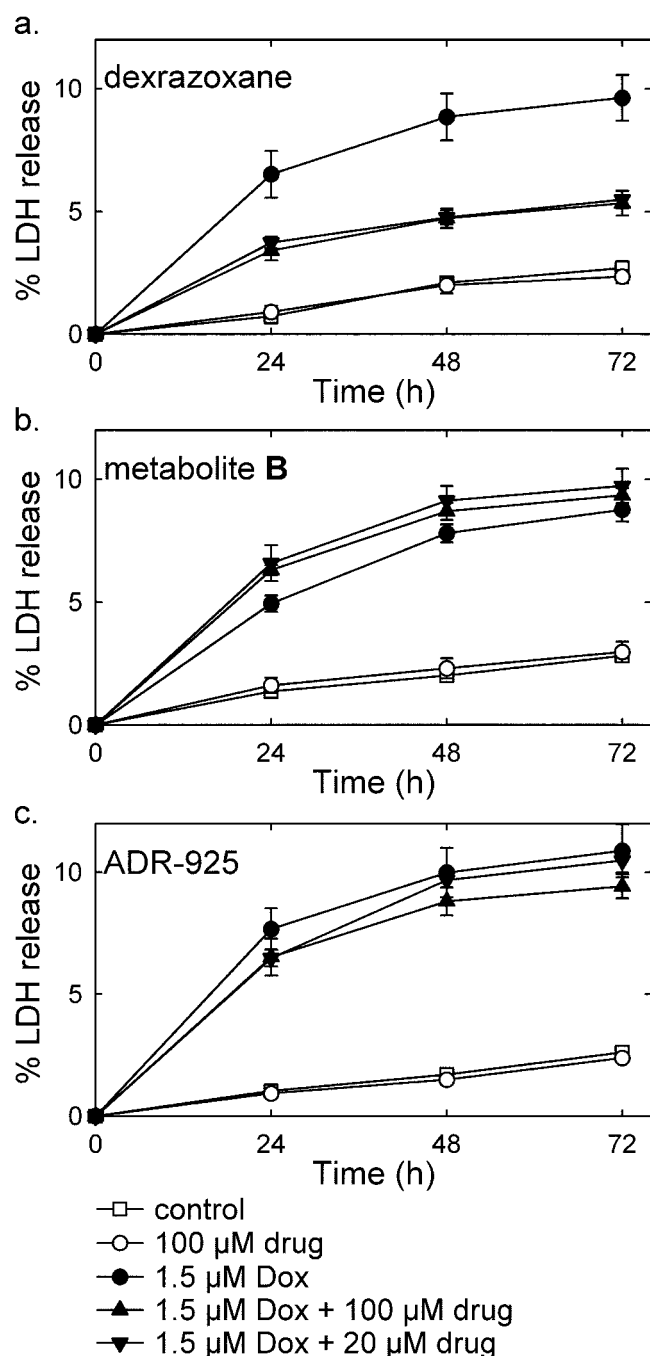


Fig. 2. Effect of dexrazoxane and the dexrazoxane metabolites **B** and ADR-925 on the release of LDH from doxorubicin-treated cardiac myocytes. **a**, plot of cumulative percentage of LDH release from cardiac myocytes that were untreated (\square) or treated with 1.5 μM doxorubicin (\bullet), 100 μM dexrazoxane and 1.5 μM doxorubicin (\blacktriangle), 20 μM dexrazoxane and 1.5 μM doxorubicin (\blacktriangledown), or 100 μM dexrazoxane (\circ). The data were from a typical single experiment in which the percentage of LDH release was measured in four separate wells. Where error bars are not seen, they were smaller than the size of the symbol. **b**, plot of cumulative percentage of LDH release from cardiac myocytes that were untreated (\square) or treated with 1.5 μM doxorubicin (\bullet), 100 μM **B** and 1.5 μM doxorubicin (\blacktriangle), 20 μM **B** and 1.5 μM doxorubicin (\blacktriangledown), or 100 μM **B** (\circ). **c**, plot of cumulative percentage of LDH release from cardiac myocytes that were untreated (\square) or treated with 1.5 μM doxorubicin (\bullet), 100 μM ADR-925 and 1.5 μM doxorubicin (\blacktriangle), 20 μM ADR-925 and 1.5 μM doxorubicin (\blacktriangledown), or 100 μM ADR-925 (\circ). The percentage of LDH release was significantly increased ($p < 0.001$) by incubation with doxorubicin in all experiments compared with untreated controls. Dexrazoxane significantly reduced (p values of 0.006–0.02) doxorubicin-induced LDH release after pretreatment with

Hanks' buffer containing 1.25 mM Ca^{2+} , 0.81 mM Mg^{2+} , 20 mM HEPES (pH 7.4). Probenecid is well known to inhibit the uptake of organic anions into cells (Tsuji et al., 1990). To determine whether uptake of dianionic ADR-925 (Fig. 1) was inhibited by probenecid, ADR-925 fluorescence dequenching of calcein-loaded myocytes was determined in the absence and presence of 1 mM probenecid. The prior addition of probenecid to HBS had no effect on ADR-925-induced fluorescence dequenching of myocytes.

Because significant levels of the intermediate metabolites **B** and **C** are found in plasma after the administration of dexrazoxane to both rats (Schroeder and Hasinoff, 2002) and humans (Schroeder et al., 2003), the ability of **B** and **C** to displace iron from the trapped intracellular iron-calcein complex were also examined. As seen from Fig. 5, **b** and **c**, both **B** and **C** were able to dequench calcein fluorescence in myocytes in a concentration-dependent manner, although at rates that were, respectively, 5.1- and 3.9-fold lower than ADR-925 at a 500 μM drug concentration. The rate at which dexrazoxane dequenched calcein fluorescence (Fig. 5a) was much lower than either **B**, **C**, or ADR-925 (maximally 7.4-fold less) and, as previously seen, did not measurably vary with the dexrazoxane concentration (Hasinoff, 2002).

Solution Kinetics of the Displacement of Fe^{2+} from Its Complex with Calcein by ADR-925 and Metabolite B. The kinetics of the displacement of Fe^{2+} from its complex with calcein by ADR-925 and **B** were studied in solution under reducing conditions, as would exist in the cell, to see whether these reactions might be rate-limiting and, thus, whether they could affect the observed rate of fluorescence dequenching observed in myocytes. As shown in Fig. 6a, upon the addition of ADR-925 to Fe^{2+} -calcein, a rapid ($t_{1/2}$ of 2.5 min at 100 μM chelator) increase in fluorescence resulted, due to the displacement of Fe^{2+} from its complex with calcein. Under conditions where ADR-925 was present in large excess, the fluorescence increased exponentially with time (Fig. 6a). To obtain k_{obs} , the observed pseudofirst-order rate constant for the reaction, the observed fluorescence changes were nonlinear least-squares fitted to a three-parameter exponential equation:

$$F = \Delta F(1 - e^{-k_{\text{obs}}t}) + F_0 \quad (1)$$

in which F is the fluorescence at time t , ΔF is the total change in fluorescence, and F_0 is the fluorescence at time 0. The plots of k_{obs} as a function of the ADR-925 concentrations are shown in Fig. 6b and increase with an increase in chelator concentration. However, the reaction of **B** with Fe^{2+} -calcein was much slower ($t_{1/2}$ of 10 min at 100 μM **B**) and could not be accurately fit to eq. 1 because it displayed both a fast initial

either 20 or 100 μM dexrazoxane. Pretreatment of myocytes with either 20 or 100 μM metabolite **B** did not significantly reduce doxorubicin-induced LDH release (p values of 0.087–0.65) except for 100 μM **B** on day 1 (p of 0.012) in which an increase was seen. Pretreatment of myocytes with either 20 or 100 μM metabolite ADR-925 did not significantly reduce doxorubicin-induced LDH release (p values of 0.24–0.78). In each of the three experiments described above, the myocytes were pretreated with the concentration of drug indicated for 3 h, doxorubicin was added for a further 3 h, and the myocytes were washed three times with medium containing the indicated concentration of dexrazoxane or dexrazoxane metabolite and kept in this medium for the next 3 days. Whereas the data shown for **B** were from a single experiment, the results were typical of those obtained in experiments utilizing two other separate myocyte isolations.

phase and a slower second phase. To obtain a k_{obs} for the first initial reaction, k_{obs} was calculated from $v/\Delta F$, which assumed that the fast initial reaction was a first-order process. The values of k_{obs} initially increased and then leveled off at about 100 μM **B** (Fig. 6b). In the presence of 2.5 mM Ca^{2+} in the medium, the k_{obs} for the reaction of ADR-925 and **B** with Fe^{2+} -calcein decreased from 1.4- to 3.4-fold, between 100 and 1000 μM ADR-925. However, the presence of Ca^{2+} in the medium increased the rate at which **B** displaced Fe^{2+} from its complex with calcein. The k_{obs} increased from 3.0- to 4.5-fold, between 50 and 1000 μM **B**. A k_{obs} was also determined at 200 μM EDTA in the absence of any added Ca^{2+} . The k_{obs} for EDTA under these conditions was 0.53 min^{-1} compared with 0.43 min^{-1} for ADR-925.

TUNEL Assay of Doxorubicin- and Dexrazoxane-Treated Myocytes. To determine whether doxorubicin induced apoptosis in myocytes and whether dexrazoxane was able to prevent doxorubicin-induced apoptosis, a TUNEL assay was carried out on myocytes. As shown in Fig. 7, myocytes that were treated with doxorubicin for 3 h, washed, and TUNEL-assayed 72 h later showed a significant ($p < 0.001$) increase in the proportion of TUNEL-positive myocytes compared with untreated controls. Myocytes that were pretreated with 100 μM dexrazoxane for 3 h, then treated with doxorubicin for a further 3 h, and washed with medium containing dexrazoxane showed a significant ($p < 0.001$) reduction in the proportion of TUNEL-positive myocytes compared with doxorubicin-treated myocytes. Thus, dexra-

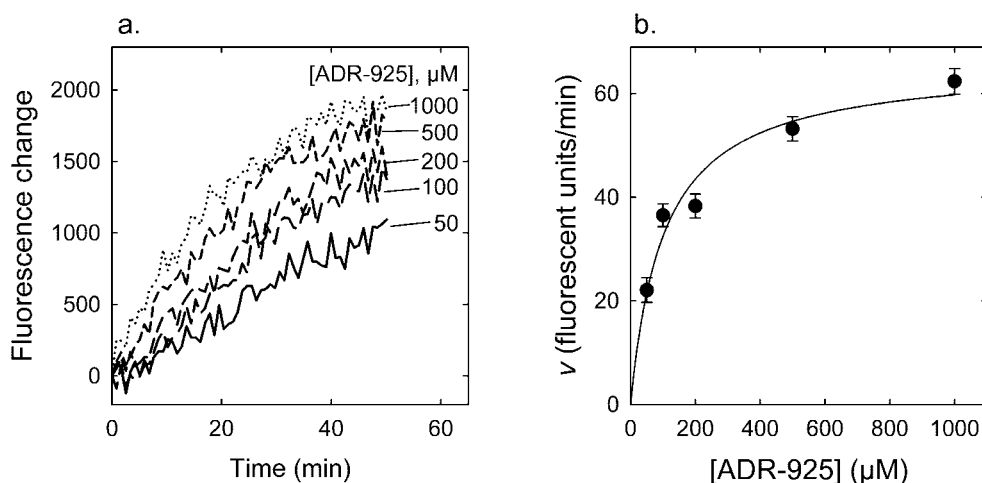


Fig. 3. Effect of ADR-925 on the intracellular calcein fluorescence upon displacement of intracellular iron from its complex with calcein in attached cardiac myocytes at 30°C. a, change in fluorescence with time upon the addition of various concentrations of ADR-925. b, initial velocity for the fluorescence change as a function of the ADR-925 concentration. The smooth curved line is a nonlinear least-squares calculated fit to the Michaelis-Menten equation, which gave a K_m of 103 ± 26 μM . The growth medium was replaced with HBS buffer just before the addition of ADR-925. Although the results shown were from one experiment, they were typical of experiments utilizing six different myocyte isolations.

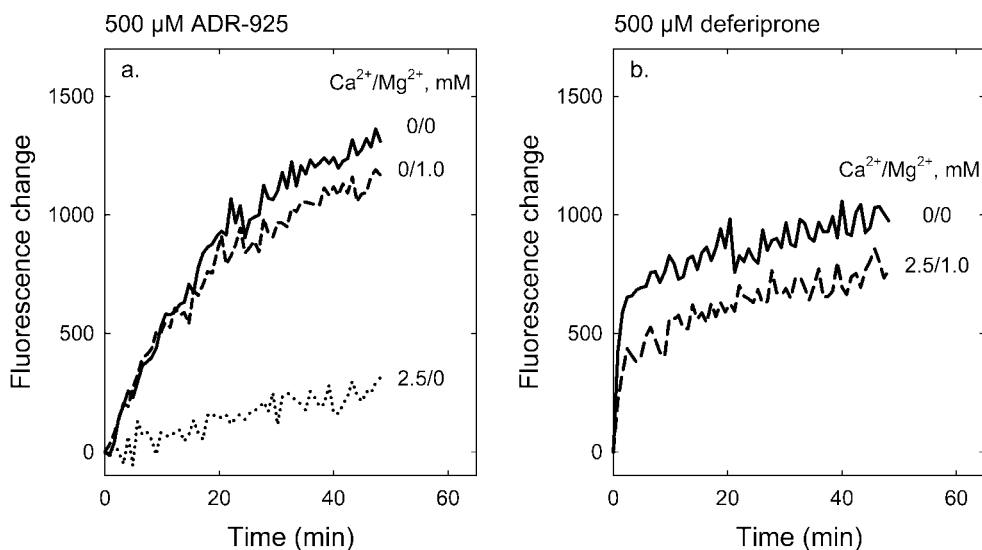


Fig. 4. Effect of Ca^{2+} or Mg^{2+} in the medium on displacement of intracellular iron from its complex with calcein by ADR-925 or deferiprone in attached cardiac myocytes. a, increase in intracellular calcein fluorescence upon displacement of intracellular iron from its complex with calcein in attached cardiac myocytes occurring upon the addition of 500 μM ADR-925 in the absence (solid line) or presence of either 2.5 mM Ca^{2+} (dotted line) or 1.0 mM Mg^{2+} (broken line) in the HBS buffer at 30°C. b, increase in intracellular calcein fluorescence upon displacement of intracellular iron from its complex with calcein in attached cardiac myocytes occurring upon the addition of 500 μM deferiprone in the absence (solid line), or presence of a mixture of 2.5 mM Ca^{2+} and 1.0 mM Mg^{2+} (broken line) in the HBS buffer at 30°C. The growth medium was replaced with HBS buffer containing either Ca^{2+} or Mg^{2+} , or neither, just before the addition of the ADR-925. Although the results shown were from one experiment, they were typical of experiments utilizing two myocyte isolations.

zoxane acted as an antiapoptotic agent. Dexrazoxane was not able to reduce the proportion of TUNEL-positive myocytes to control values ($p < 0.001$). This result is consistent with the LDH results of Fig. 2a in which LDH levels of dexrazoxane-treated myocytes did not reach levels of control myocytes. Dexrazoxane-treated myocytes also showed a significant ($p < 0.001$) increase in the proportion of TUNEL-positive myocytes compared with untreated controls. This result might be

due to the fact that dexrazoxane is also a strong inhibitor of DNA topoisomerase II (Hasinoff et al., 1995) that can induce apoptosis in K562 cells (Hasinoff et al., 2001). The high percentage of TUNEL-positive control apoptotic myocytes is likely a result of the collagenase/trypsin tissue dissociation treatment used to obtain the primary cell culture.

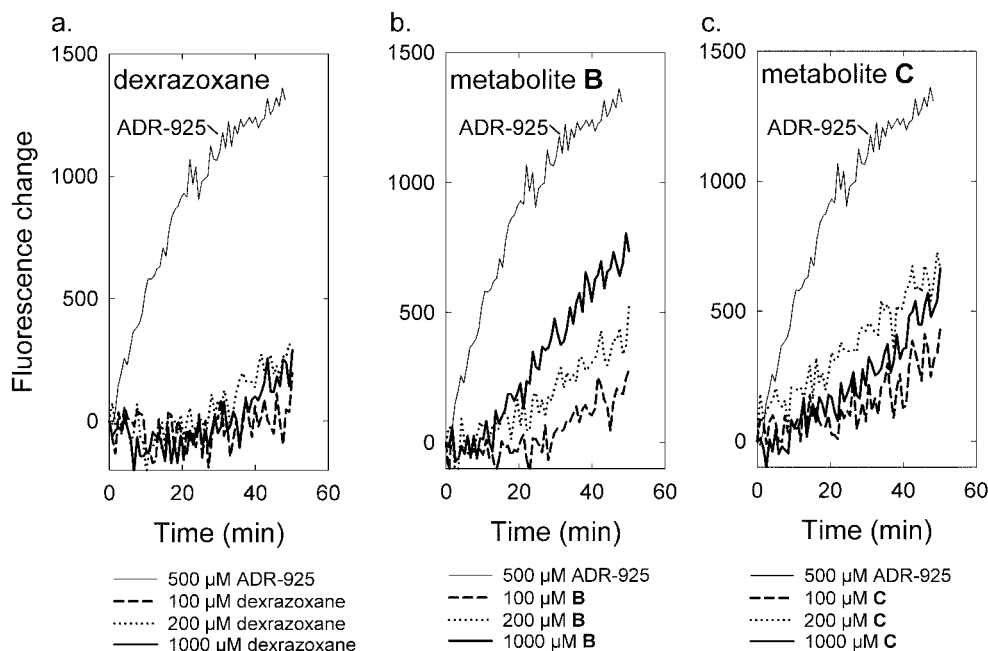


Fig. 5. Effect of dexrazoxane and metabolites **B** and **C** on displacement of intracellular iron from its complex with calcein in attached cardiac myocytes at 30°C in HBS buffer. For comparison, data obtained at 500 μM ADR-925 are also plotted on each graph. a, change in intracellular calcein fluorescence upon addition of various concentrations of dexrazoxane to attached calcein-loaded cardiac myocytes. b, change in intracellular calcein fluorescence upon addition of various concentrations of metabolite **B** to attached calcein-loaded cardiac myocytes. c, change in intracellular calcein fluorescence upon addition of various concentrations of metabolite **C** to attached calcein-loaded cardiac myocytes. The growth medium was replaced with HBS buffer just before the addition of drug. Although the results shown were from one experiment, they were typical of experiments utilizing three different myocyte isolations. Data obtained for dexrazoxane, **B**, and **C** concentrations at 50 and 500 μM were not plotted for clarity.

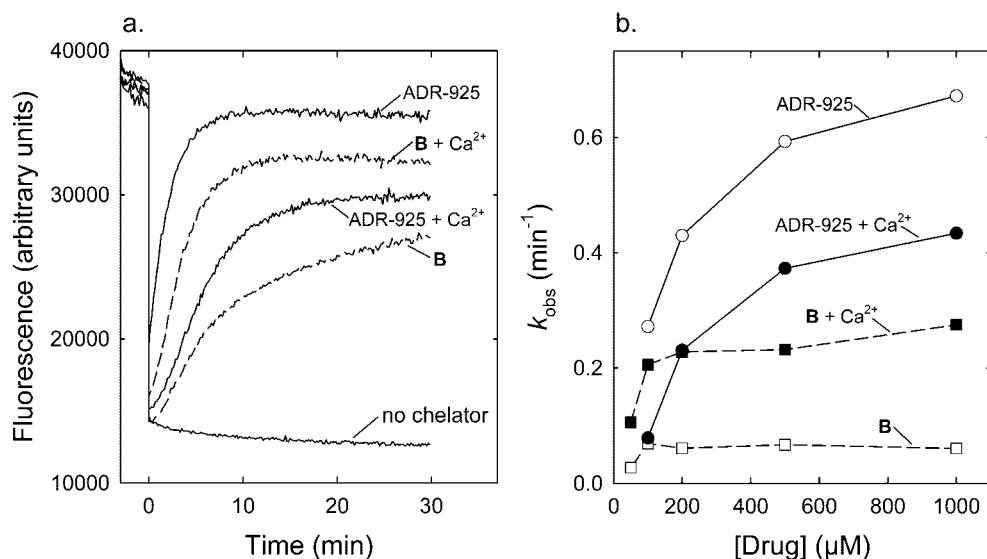


Fig. 6. Effect of ADR-925 and **B** on the calcein fluorescence upon displacement of Fe^{2+} from its complex with calcein in the presence and absence of added Ca^{2+} . a, change in fluorescence with time upon the addition of 100 μM ADR-925 (solid line) or **B** (broken line) in the presence or absence of 2.5 mM Ca^{2+} . b, plot of the first-order rate constant k_{obs} as a function of the concentration of ADR-925 (solid line) in the absence (\circ) or presence (\bullet) of 2.5 mM Ca^{2+} , or **B** (broken line) in the absence (\square) or presence (\blacksquare) of 2.5 mM Ca^{2+} . The reaction was followed in HBS buffer at 30°C containing 1 mM ascorbic acid and 0.1 mg/ml catalase. At time 0, the chelator was added to the preformed Fe^{2+} -calcein complex. The Fe^{2+} -calcein complex was formed by adding Fe^{2+} to calcein (each 1 μM final concentration). The fluorescence shown before time 0 was that of calcein before the addition of Fe^{2+} . Upon the addition of Fe^{2+} to calcein, there was a rapid drop in fluorescence corresponding to binding of Fe^{2+} to calcein (bottom trace).

Discussion

The concentrations of dexrazoxane and its metabolites that were used in this study were well within pharmacological levels achieved when it is used as a doxorubicin cardioprotective agent (Hochster et al., 1992; Schroeder et al., 2003). The peak plasma concentrations of dexrazoxane, ADR-925, **B**, and **C** in humans dosed at 1500 mg/m² are 211, 30, 19, and 9 μ M, respectively (Schroeder et al., 2003). The concentrations of doxorubicin used in these studies were even smaller (1.5 compared with 12 μ M) than the plasma concentrations seen clinically at the end of a 60 mg/m² doxorubicin infusion period ($t_{1/2\beta}$ of 1.8 h) (Hochster et al., 1992). Thus, the doxorubicin concentrations and the time over which the myocytes were exposed to doxorubicin were also in a pharmacologically relevant range.

The rapid rate of displacement of Fe²⁺ from its complex by calcein by ADR-925 in solution (Fig. 6b) in either the presence or absence of Ca²⁺ suggests that this reaction was not likely to be rate-limiting when ADR-925 was added to calcein-loaded myocytes. However, the slower rate at which **B** displaced Fe²⁺ (Fig. 6b) suggests that this reaction partly

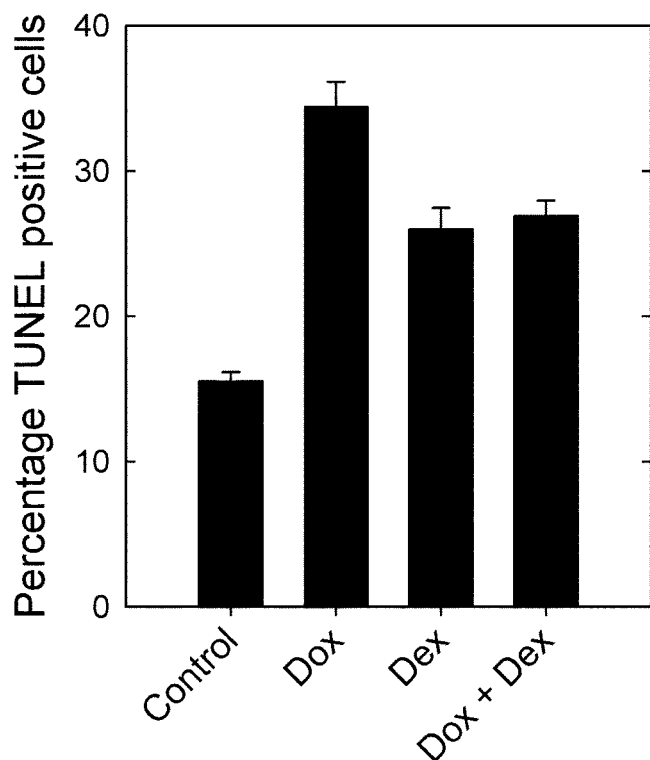


Fig. 7. Induction of apoptosis in doxorubicin-treated (Dox) myocytes and its prevention by pretreatment with dexrazoxane (Dex). The percentage of apoptosis was determined by epifluorescence microscopy using a TUNEL assay. Where indicated, myocytes were pretreated with 100 μ M dexrazoxane for 3 h, then treated with doxorubicin for a further 3 h, and then washed with medium containing dexrazoxane only. A TUNEL assay was then conducted 72 h later. Myocytes that were treated with doxorubicin for 3 h and then washed with doxorubicin-free medium showed a significant ($p < 0.001$) increase in the proportion of TUNEL-positive myocytes compared with untreated controls. Myocytes pretreated with dexrazoxane and then doxorubicin showed a significant ($p < 0.001$) reduction in the proportion of TUNEL-positive myocytes compared with doxorubicin-treated myocytes. Dexrazoxane was, however, not able to reduce the proportion of TUNEL-positive myocytes to control values ($p < 0.001$).

limited the kinetics of fluorescence dequenching in calcein-loaded myocytes in the absence of added Ca²⁺ (Fig. 5b). We previously showed that **B** and **C** are also chelators that are capable of displacing Fe³⁺ from its complex with doxorubicin (Buss and Hasinoff, 1993). The similarity in the k_{obs} values for the ADR-925 and EDTA displacement reactions in solution suggests that there was a common rate-determining step that was most likely the breaking of an Fe²⁺-calcein bond. The Ca²⁺-induced decrease in rate of displacement of Fe²⁺ from its complex with calcein in solution is likely due to the requirement that the Ca²⁺-ADR-925 complex dissociate before it can complex Fe²⁺ released from calcein. We previously showed that Ca²⁺ binds to **B** (Buss and Hasinoff, 1997), although likely much less strongly than to ADR-925. The reason for the Ca²⁺-induced increase in rate of reaction of **B** with Fe²⁺-calcein is not known but may be due to a faster dissociation of the weaker Ca²⁺-**B** complex.

Our recent pharmacokinetic studies showed that dexrazoxane was rapidly metabolized in rats (Schroeder and Hasinoff, 2002) and in humans (Schroeder et al., 2003) to the one-ring open compounds **B** and **C** and ADR-925 (Fig. 1). Given the rapid metabolism of dexrazoxane in vivo, we tested the hypothesis that circulating **B** or ADR-925 in plasma might be the active form of dexrazoxane that protects myocytes from doxorubicin-induced LDH release. However, as shown in Fig. 2, neither **B** nor ADR-925 protected myocytes against doxorubicin. The inability of ADR-925 to protect myocytes from doxorubicin-induced damage is in accord with the inability of ICRF-198 (the racemic form of ADR-925) to protect hamsters from acute toxic effects of the doxorubicin analog daunorubicin (Herman et al., 1985). The reason that both **B** and ADR-925 do not protect may be due to their being anionic species that enter cells more slowly than neutral dexrazoxane does (Dawson, 1975).

To determine whether the lack of uptake of these anionic metabolites prevented them from protecting myocytes, **B**-, **C**-, and ADR-925-induced fluorescence dequenching of the iron-calcein complex as they were taken up in myocytes was determined. However, the results of Fig. 3 show that there was a saturable uptake of ADR-925 into myocytes. The fact that ADR-925 uptake was saturable suggests that dianionic ADR-925 may have been taken up by an anion transport system that was, however, not inhibited by probenecid. However, in the presence of Ca²⁺ concentrations similar to those in plasma, the rate of entry of ADR-925 was greatly reduced. This probably occurred because ADR-925 can form a complex with Ca²⁺ (K of $10^{6.9}$ M⁻¹; Huang et al., 1982). Although the presence of 2.5 mM Ca²⁺ did decrease the rate of the reaction of ADR-925 with Fe²⁺-calcein in solution, this decrease was not wholly consistent with the large decrease in rate of fluorescence dequenching of calcein in myocytes that was caused by the addition of Ca²⁺ to the medium (Fig. 4a). However, the reduced rate of uptake of the Ca²⁺-ADR-925 complex may, in part, be responsible for the inability of ADR-925 to protect myocytes from doxorubicin.

The results of Fig. 5 show that **B** and **C** were taken up by myocytes more rapidly than ADR-925. However, the uptake of **B** did not result in its protecting myocytes from doxorubicin (Fig. 2). Together, these results suggested that these anionic metabolites did not have the same access that dexrazoxane had to iron pools in critical cellular compartments. Doxorubicin has been shown by fluorescence microscopy to

localize in the mitochondria of myocytes (Swift and Sarvazyan, 2000) with doxorubicin-induced dichlorofluorescein oxidation occurring close to the mitochondria (Sarvazyan, 1996). Cardiac mitochondria are also a prominent site of injury by doxorubicin (Gianni et al., 1983; Sokolove and Shinaberry, 1988). We have shown that dexrazoxane reduces doxorubicin-induced oxidation of intracellular dichlorofluorescein and prevents mitochondrial damage (Hasinoff et al., 2003). Thus, dexrazoxane may be protecting mitochondria because it is neutral and permeable to mitochondria, whereas the anionic metabolites **B** and ADR-925 are not. The intracellular Ca^{2+} concentration is so low that the ADR-925 would not be present as its neutral Ca^{2+} -ADR-925 complex. The calcein in myocytes is located mainly in the cytoplasm and, thus, our experiments do not give us any information on the displacement of iron from the mitochondria. The results of Fig. 7 showing that dexrazoxane reduced doxorubicin-induced apoptosis are consistent with its ability to prevent daunorubicin-induced apoptosis of myocytes (Sawyer et al., 1999).

An alternative, although less likely possibility, is that dexrazoxane did not act by preventing iron-based oxidative damage, but through its ability to catalytically inhibit DNA topoisomerase II (K_i 13 μM) (Hasinoff et al., 1995). It is, however, unclear how preventing doxorubicin from targeting topoisomerase II could protect myocytes. Dexrazoxane is clearly able to prevent oxygen free radical damage inasmuch as it has been shown that dexrazoxane can protect myocytes from hypoxia-reoxygenation and other drug-induced free radical damage (Hasinoff, 2002; Hasinoff et al., 1998).

The fact that dexrazoxane only very slowly removed iron from its complex with calcein in myocytes (Fig. 5a) is explained by the fact that at the pH and temperature of this study, it is only slowly hydrolyzed to its one-ring open intermediates **B** and **C** (Fig. 2) ($t_{1/2}$ of 14 and 29 h, respectively) and then to ADR-925 ($t_{1/2}$ 23 h) (Hasinoff, 1994b). We previously showed that ferrous ion strongly promoted the ring opening of **B** and **C** to ADR-925 (Buss and Hasinoff, 1995). Given that the intracellular environment of the cell is highly reducing, free or loosely bound intracellular iron would be expected to be largely present in the ferrous state. Thus, by reacting with ferrous ion, the strongly chelating ADR-925 could be formed intracellularly more quickly. From the kinetics of the formation of **B** and **C** (Hasinoff, 1994b), and assuming rapid ferrous ion-promoted ring opening, it can be estimated that a 3-h preincubation with 100 μM dexrazoxane could yield an intracellular ADR-925 concentration of 20 μM . The free or loosely bound iron concentration in cells is not known with certainty but has been estimated using calcein to be 1.3 μM in mouse leukemia cells (Picard et al., 1998). Thus, assuming that similar concentrations of free iron were present in myocytes, a 3-h preincubation with dexrazoxane would produce more than enough ADR-925 to chelate all of the free iron in the myocytes.

In conclusion, this study showed that whereas dexrazoxane was able to protect cardiac myocytes from doxorubicin-induced damage, its **B** and ADR-925 metabolites were not. Thus, these metabolites are unlikely to be useful on their own as antioxidant drugs. The ability of **B**, ADR-925, and dexrazoxane to displace iron from intracellular trapped iron-calcein complex suggests that these metabolites were taken up into the myocyte and bound iron. The fact that the these

anionic metabolites did not protect myocytes from doxorubicin suggests either that the metabolites do not have access to the same cellular compartments that neutral dexrazoxane has, or that they are taken up too slowly to protect, or, alternatively, that dexrazoxane protects myocytes through its ability to inhibit topoisomerase II through some unknown mechanism.

References

- Barnabé N, Zastre J, Venkataram S, and Hasinoff BB (2002) Deferiprone protects against doxorubicin-induced myocyte cytotoxicity. *Free Radic Biol Med* **33**:266–275.
- Breuer W, Epsztejn S, and Cabantchik ZI (1995) Iron acquired from transferrin by K562 cells is delivered into a cytoplasmic pool of chelatable iron(II). *J Biol Chem* **270**:24209–24215.
- Buss JL and Hasinoff BB (1993) The one-ring open hydrolysis product intermediates of the cardioprotective agent ICRF-187 (dexrazoxane) displace iron from iron-anthracycline complexes. *Agents Actions* **40**:86–95.
- Buss JL and Hasinoff BB (1995) Ferrous ion strongly promotes the ring opening of the hydrolysis intermediates of the antioxidant doxorubicin cardioprotective agent ICRF-187 (dexrazoxane). *Arch Biochem Biophys* **317**:121–127.
- Buss JL and Hasinoff BB (1997) Metal ion-promoted hydrolysis of the antioxidant cardioprotective agent dexrazoxane (ICRF-187) and its one-ring open hydrolysis products to its metal-chelating active form. *J Inorg Biochem* **68**:101–108.
- Cabantchik ZI, Glickstein H, Milgram P, and Breuer W (1996) A fluorescence assay for assessing chelation of intracellular iron in a membrane model system and in mammalian cells. *Anal Biochem* **233**:221–227.
- Dawson KM (1975) Studies on the stability and cellular distribution of dioxopiperazines in cultured BHK-21S cells. *Biochem Pharmacol* **24**:2249–2253.
- Diop NK, Vitellaro LK, Arnold P, Shang M, and Marusak RA (2000) Iron complexes of the cardioprotective agent dexrazoxane (ICRF-187) and its desmethyl derivative, ICRF-154: solid state structure, solution thermodynamics and DNA cleavage activity. *J Inorg Biochem* **78**:209–216.
- Esposito BP, Epsztejn S, Breuer W, and Cabantchik ZI (2002) A review of fluorescence methods for assessing labile iron in cells and biological fluids. *Anal Biochem* **304**:1–18.
- Gianni L, Corden BJ, and Myers CE (1983) The biochemical basis of anthracycline toxicity and anti-tumor activity. *Rev Biochem Toxicol* **5**:1–82.
- Hasinoff BB (1994a) An HPLC and spectrophotometric study of the hydrolysis of ICRF-187 (dexrazoxane, (+)-1,2-bis(3,5-dioxopiperazinyl-1-yl)propane) and its one-ring opened intermediates. *Int J Pharm* **107**:67–76.
- Hasinoff BB (1994b) Pharmacodynamics of the hydrolysis-activation of the cardioprotective agent (+)-1,2-bis(3,5-dioxopiperazinyl-1-yl)propane. *J Pharm Sci* **83**:64–67.
- Hasinoff BB (1998) Chemistry of dexrazoxane and analogues. *Semin Oncol* **25** (Suppl 10):3–9.
- Hasinoff BB (2002) Dexrazoxane (ICRF-187) protects cardiac myocytes against hypoxia-reoxygenation damage. *Cardiovasc Toxicol* **2**:111–118.
- Hasinoff BB, Abram ME, Barnabé N, Khelifa T, Allan WP, and Yalowich JC (2001) The catalytic DNA topoisomerase II inhibitor dexrazoxane (ICRF-187) induces differentiation and apoptosis in human leukemia K562 cells. *Mol Pharmacol* **59**:453–461.
- Hasinoff BB, Hellmann K, Herman EH, and Ferrans VJ (1998) Chemical, biological and clinical aspects of dexrazoxane and other bisdioxopiperazines. *Curr Med Chem* **5**:1–28.
- Hasinoff BB, Kuschak TI, Yalowich JC, and Creighton AM (1995) A QSAR study comparing the cytotoxicity and DNA topoisomerase II inhibitory effects of bisdioxopiperazine analogs of ICRF-187 (dexrazoxane). *Biochem Pharmacol* **50**:953–958.
- Hasinoff BB, Schnabl KL, Marusak RA, Patel D, and Huebner E (2003) Dexrazoxane (ICRF-187) protects cardiac myocytes against doxorubicin by preventing damage to mitochondria. *Cardiovasc Toxicol*, in press.
- Hasinoff BB, Venkataram S, Singh M, and Kuschak TI (1994) Metabolism of the cardioprotective agents dexrazoxane (ICRF-187) and levrazoxane (ICRF-186) by the isolated hepatocyte. *Xenobiotica* **24**:977–987.
- Herman EH, El-Hage AN, Creighton AM, Witiak DT, and Ferrans VJ (1985) Comparison of the protective effect of ICRF-187 and structurally related analogues against acute daunorubicin toxicity in Syrian golden hamsters. *Res Commun Chem Pathol Pharmacol* **48**:39–55.
- Hershko G, Link G, Tzahor M, Kaltwasser JP, Athias P, Grynberg A, and Pinson A (1993) Anthracycline cytotoxicity is potentiated by iron and inhibited by deferoxamine: studies in rat heart cells in culture. *J Lab Clin Med* **122**:245–251.
- Hochster H, Liebes L, Wadler S, Oratz R, Wernz JC, Meyers M, Green M, Blum RH, and Speyer JL (1992) Pharmacokinetics of the cardioprotector ADR-529 (ICRF-187) in escalating doses combined with fixed-dose doxorubicin. *J Natl Cancer Inst* **84**:1725–1730.
- Huang Z-X, May PM, Quinlan KM, Williams DR, and Creighton AM (1982) Metal binding by pharmaceuticals. Part 2. Interactions of Ca(II), Cu(II), Fe(II), Mg(II), Mn(II) and Zn(II) with the intracellular hydrolysis products of the antitumor agent ICRF-159 and its inactive homologue ICRF-192. *Agents Actions* **12**:536–542.
- Kakhlon O and Cabantchik ZI (2002) The labile iron pool: characterization, measurement and participation in cellular processes. *Free Radic Biol Med* **33**:1037–1046.
- Meyers CE (1998) The role of iron in doxorubicin-induced cardiomyopathy. *Semin Oncol* **25** (Suppl 10):10–14.
- Petrat F, de Groot H, Sustmann R, and Rauen U (2002) The chelatable iron pool in living cells: a methodically defined quantity. *Biol Chem* **383**:489–502.

- Picard V, Epsztejn S, Santambrogio P, Cabantchik ZI, and Beaumont C (1998) Role of ferritin in the control of the labile iron pool in murine erythroleukemia cells. *J Biol Chem* **273**:15382–15386.
- Sarvazyan N (1996) Visualization of doxorubicin-induced oxidative stress in isolated cardiac myocytes. *Am J Physiol* **271**:H2079–H2085.
- Sawyer DB, Fukazawa R, Arstall MA, and Kelly RA (1999) Daunorubicin-induced apoptosis in rat cardiac myocytes is inhibited by dexrazoxane. *Circ Res* **84**:257–265.
- Schroeder PE, Davidson JN, and Hasinoff BB (2002) Dihydroorotase catalyzes the ring-opening of the hydrolysis intermediates of the cardioprotective drug dexrazoxane (ICRF-187). *Drug Metab Dispos* **30**:1431–1435.
- Schroeder PE and Hasinoff BB (2002) The doxorubicin-cardioprotective drug dexrazoxane undergoes metabolism in the rat to its metal ion-chelating form ADR-925. *Cancer Chemother Pharmacol* **50**:509–513.
- Schroeder PE, Jensen PB, Sehested M, Hofland KF, Langer SW, and Hasinoff BB (2003) Metabolism of dexrazoxane (ICRF-187) used as a rescue agent in cancer patients treated with high-dose etoposide. *Cancer Chemother Pharmacol*, in press.
- Sheppard LN and Kontoghiorghes GJ (1993) Competition between deferiprone, desferrioxamine and other chelators for iron and the effect of other metals. *Arzneim-Forsch* **43**:659–663.
- Sokolove PM and Shinaberry RG (1988) Na⁺-independent release of Ca²⁺ from rat heart mitochondria. *Biochem Pharmacol* **37**:803–812.
- Swift LM and Sarvazyan N (2000) Localization of dichlorofluorescein in cardiac myocytes: implications for assessment of oxidative stress. *Am J Physiol* **278**:H982–H990.
- Tsuji A, Terasaki T, Tamai I, and Takeda K (1990) In vivo evidence for carrier-mediated uptake of beta-lactam antibiotics through organic anion transport systems in rat kidney and liver. *J Pharmacol Exp Ther* **253**:315–320.
- Zanninelli G, Glickstein H, Breuer W, Milgram P, Brissot P, Hider RC, Konijn AM, Libman J, Shanzer A, and Cabantchik ZI (1997) Chelation and mobilization of cellular iron by different classes of chelators. *Mol Pharmacol* **51**:842–852.

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