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# METABOLISM OF THE CARDIOPROTECTIVE DRUG DEXRAZOXANE AND ONE OF ITS METABOLITES BY ISOLATED RAT MYOCYTES, HEPATOCYTES AND BY BLOOD

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Running title: DEXRAZOXANE METABOLISM BY MYOCYTES, HEPATOCYTES AND BLOOD

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[1] Abbreviations used: ADR-925, N,N’ -[(1S)-1-methyl-1,2-ethanediyl]bis[(N-(2-amino-2-oxoethyl)]glycine; **B**, N-(2-amino-2-oxoethyl)-N-[(1S)-2-(3,5-dioxo-1-piperazinyl)-1-methylethyl]glycine; **C**, N-(2-amino-2-oxoethyl)-N-[(2S)-2-(3,5-dioxo-1-piperazinyl)propyl]glycine; DHPase, dihydropyrimidine amidohydrolase or dihydropyrimidinase; DHOase, dihydroorotase; DTPA, diethylenetriaminepentaacetic acid; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; HPLC, high-pressure liquid chromatography; $t_{½}$, half-time.

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## **ABSTRACT**:

The metabolism of the antioxidant cardioprotective agent dexrazoxane (ICRF-187) and one of its one-ring open metabolites to its active metal ion binding form ADR-925 has been investigated in neonatal rat myocyte and adult rat hepatocyte suspensions, and in human and rat blood and plasma with a view to characterizing their hydrolysis-activation. Dexrazoxane is clinically used to reduce the iron-based oxygen free radical-mediated cardiotoxicity of the anticancer drug doxorubicin. Dexrazoxane may act through its hydrolysis product ADR-925 by removing iron from the iron-doxorubicin complex, or binding free iron, thus preventing oxygen radical formation. Our results indicate that dexrazoxane underwent partial uptake and/or hydrolysis by myocytes. A one-ring open metabolite of dexrazoxane underwent nearly complete dihydroorotase-catalyzed metabolism in a myocyte suspension. Hepatocytes that contain both dihydropyrimidinase and dihydroorotase, completely hydrolyzed dexrazoxane to ADR-925 and released it into the extracellular medium. Thus, in hepatocytes the two liver enzymes acted in concert, and sequentially on dexrazoxane, to first produce the two ring-opened metabolites, and then to produce the metabolite ADR-925. We also showed that the hydrolysis of one of these metabolites was promoted by $Ca^{2+}$ and $Mg^{2+}$ in plasma, and thus further metabolism of these intermediates likely occurs in the plasma after they are released from the liver and kidney. In conclusion these studies provide a nearly complete description of the metabolism of dexrazoxane by myocytes and hepatocytes to its presumably active form ADR-925.

## Introduction

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 (Malisza and Hasinoff, 1995; Meyers, 1998) on the relatively unprotected cardiac muscle. Neutral dexrazoxane, which is permeable to cells (Dawson, 1975), may act through its rings-opened hydrolysis product ADR-925[1] (Fig. 1), which can either remove iron from the iron-doxorubicin complex (Buss and Hasinoff, 1993), or bind free iron, thus preventing iron-based oxygen radical formation. Thus, dexrazoxane can be considered a pro-drug analog of EDTA that is activated upon hydrolysis to its one-ring open intermediates **B** and **C**, and then to its fully rings-opened form ADR-925 according to the scheme in Fig. 1 (Hasinoff, 1990; 1994b; 1994a; 1998; Hasinoff et al., 1998).

Our previous spectrophotometric and HPLC studies (Hasinoff, 1994b; 1994a) showed that under physiological conditions (37°C and pH 7.4) dexrazoxane is only slowly hydrolyzed to **B** and **C** ($t_{½}$ of 9.3 h), and to the final hydrolysis product ADR-925 ($t_{½}$ of 23 h) according to the kinetic scheme shown in Fig. 1. Given the slow rate at which dexrazoxane hydrolysis-activation occurs *in vitro* it is, thus, unclear how sufficient amounts of ADR-925 could be present in heart tissue to chelate iron and prevent oxygen radical damage before dexrazoxane was eliminated (β-phase $t_{½}$ of 4.2 ± 2.9 h in humans) (Hochster et al., 1992). More recently we have shown that dexrazoxane is quickly metabolized to **B** and **C**, and then to ADR-925 in humans (Schroeder et al., 2003) and in a rat model (Schroeder and Hasinoff, 2002). The rapid rate of hydrolysis of dexrazoxane and the rapid appearance of ADR-925 in plasma *in vivo* suggests that first dexrazoxane, and then **B** and **C**, are all enzymatically metabolized.

We previously showed that **B** and **C** are rapidly formed from dexrazoxane in a primary rat hepatocyte suspension (Hasinoff et al., 1994). These results were both consistent with dexrazoxane being metabolized by the zinc hydrolase DHPase (EC 3.5.2.2). Furthermore, we have shown that pure DHPase enzymatically hydrolyzed dexrazoxane to **B** and **C**, but did not enzymatically hydrolyze **B** and **C** to ADR-925 (Hasinoff et al., 1991; Hasinoff, 1993). We have also shown that another zinc hydrolase DHOase (EC 3.5.2.3) is able to enzymatically hydrolyze **B** and **C** to ADR-925, but does not act on dexrazoxane (Schroeder et al., 2002). Thus, DHPase and DHOase may act sequentially and in concert to effect the full metabolism of dexrazoxane to its active metal ion chelating form ADR-925. While DHOase is present in a variety of tissues including the heart, liver, and kidney (Kennedy, 1974) and in erythrocytes and leukocytes (Smith and Baker, 1959), DHPase is only present in the liver and the kidney, but not in the heart (Dudley et al., 1974; Hasinoff et al., 1991; Hamajima et al., 1996). Because dexrazoxane acts as a cardioprotective agent we investigated the metabolism of dexrazoxane and its one-ring open intermediate **C** to ADR-925 in neonatal rat myocytes, in adult rat hepatocytes and in blood with a view to gaining a complete description of its metabolic hydrolysis-activation.

## Methods

**Materials.** Dexrazoxane hydrochloride and ADR-925 were gifts from Adria Laboratories (Columbus, OH) and were used as supplied. As previously reported (Schroeder et al., 2002) dexrazoxane was assayed by HPLC and was found to contain 0.98, 0.41, and 0.08 mol% **B**, **C**, and ADR-925, respectively. HPLC-grade methanol was from Fisher (Nepean, Canada). Tris, Chelex resin, 5-aminoorotic acid, furosemide, L-dihydroorotic acid, 1-heptanesulfonic acid and 1-octanesulfonic acid were from Sigma (St. Louis, MO). 4-Chlorobenzenesulfonamide was from Aldrich (Milwaukee, WI). α-MEM was from Invitrogen (Burlington, Canada). Trypsin, collagenase, and DNase were from Worthington Biochemicals (Lakewood NJ). Calcein was from Molecular Probes (Eugene, OR). Microgram quantities of **C** were prepared by NaOH treatment of dexrazoxane and column purified as described (Hasinoff, 1994a; Schroeder et al., 2002). The purified **C** contained less than 0.001 mol% dexrazoxane, 0.1 mol% **B** and 0.05 mol% ADR-925, respectively. Experiments were only carried out with intermediate **C** as we previously showed that DHOase hydrolyzes **B** and **C** at approximately equal rates under non-saturating Michaelis-Menten conditions (Schroeder et al., 2002), and thus the metabolism of these structurally similar intermediates (Fig. 1) is likely very similar.

The α-MEM cell suspension medium contained 40 mM HEPES in order to maintain tight pH control (pH 7.4). The Tris/NaCl control buffer (pH 7.4) contained 100 mM Tris and 0.9% (w/v) NaCl and was stirred with Chelex for 24 h prior to use to deplete heavy metal ions known to promote hydrolysis (Buss and Hasinoff, 1997). In order to study the metabolism of dexrazoxane to ADR-925 an hepatocyte suspension buffer (pH 7.4) was used to avoid fluorescing interferences from α-MEM. It contained 137 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 0.64 mM $MgCl_2$, 1.1 mM $KH_2PO_4$, 0.7 mM $Na_2SO_4$, and 34 mM HEPES as described (Hasinoff et al., 1994). Artificial plasma (pH 7.4), contained 130 mM NaCl, 4.9 mM KCl, 2.5

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mM $CaCl_2$, 1.2 mM $MgCl_2$, 5 mM $KH_2PO_4$, 34 mM HEPES as described (Luquita et al., 2001). Where rates of drug loss from the solutions were compared, a *t*-test comparison of the slopes (Jones, 2002) was carried out.

**Analysis of C, ADR-925 and dihydroorotic acid.** The HPLC analysis of dexrazoxane, **C**, and dihydroorotic acid using an ion-pair reagent with the reversed phase $C_{18}$-column (detection wavelength 205 nm) has been described (Schroeder et al., 2002; Schroeder et al., 2003). The calcein fluorescence flow-injection analysis determination of ADR-925 on an HPLC apparatus has also been described (Schroeder et al., 2002; Schroeder et al., 2003).

**Myocyte isolation and dosing.** Ventricular myocytes were isolated from 1-3 day old Sprague-Dawley rats as described (Hasinoff et al., 2003). Briefly, minced ventricles were serially digested with collagenase and trypsin in PBS/1% (wt/v) glucose at 37°C in the presence of DNase and preplated for 1 h in large petri dishes to remove fibroblasts. The myocytes remaining in the supernatant were collected and centrifuged for 5 min at 400 *g* and then resuspended in α-MEM medium at a density of $2 \times 10^6$ cells/ml (1 ml/well) in a 24-well plate. The myocytes were used immediately following the isolation procedure. The preparation was typically greater than 90% viable by the trypan blue exclusion method. After addition of drug to the cell suspensions, samples of 150 μl were removed at the times indicated. After centrifugation the supernatant (100 μl) was removed and acidified to pH 3.0 with HCl and stored at -80°C until analyzed (Hasinoff, 1994b), typically within 1-3 d.

The plates containing myocytes, hepatocytes, whole blood or plasma were constantly and gently stirred on a gyratory platform shaker in an incubator at 37°C to maintain the cells in suspension. Samples were taken from duplicate wells and duplicate HPLC or calcein analyses were carried out on each sample. Where the cell suspensions were also treated with inhibitors the cells were preincubated with inhibitors for 30 min prior to addition of dexrazoxane, dihydroorotic acid or **C**. In the calculation of the percentage inhibition of hydrolysis of **C** the rates of hydrolysis were corrected for the background rate of hydrolysis of **C**.

**Hepatocyte isolation.** Hepatocytes were isolated from female Sprague-Dawley rats (200-250 g) by a collagenase perfusion method previously described (Burczynski and Cai, 1994; Hasinoff et al., 1994). The isolated hepatocytes, which comprised greater than 95% of the isolated cell population, were stored at room temperature and used within 1 h of the completed perfusion. Cell viability was greater than 90% by the trypan blue exclusion method. All animal studies were performed in accordance with the principles and guidelines of the Canadian Council on Animal Care and the University of Manitoba Animal Care Committee.

**Blood collection and dosing.** Blood (15 ml) was collected from anesthetized Sprague-Dawley rats (300-350 g) as described (Schroeder and Hasinoff, 2002) into sterile centrifuge tubes containing 100 units heparin and centrifuged at 500 *g* for 5 min to obtain plasma. Blood was withdrawn from a 27 y old female into 5 ml heparinized tubes. All *in vitro* whole blood and blood plasma experiments began less then one h after collection. The blood and plasma samples were buffered with Tris (final concentration 100 mM, pH 7.4) which resulted in a dilution of the samples by 5%. The blood and plasma samples (2 ml) were contained in 6-well plastic plates in a $CO_2$ (5% (v/v)) incubator at 37°C and sampled (blood 200 μl, plasma 100 μl) at the times indicated and treated as described (Hasinoff, 1994b). The protocol was approved by the

University of Manitoba Health Research Ethics Board.

## Results

**Metabolism of dexrazoxane, C and dihydroorate by myocyte suspensions.** As shown in Fig. 2a when 10 μM dexrazoxane was added to a myocyte suspension there was an initial decrease in dexrazoxane concentration in the medium that was followed by a slower decrease in concentration that paralleled the decrease in concentration in the control experiment in medium alone. A *t*-test comparison of the slopes of all of the control data compared to the last 4 data points of the myocyte suspension data ($t > 30$ min) showed that the limiting slopes were not significantly different ($p > 0.2$). However, the intercepts were significantly different ($p < 0.001$), which indicated that the small initial decrease in concentration was statistically significantly. An experiment was also conducted in which the loss of 10 μM dexrazoxane from the medium was compared at myocyte cell densities of 2 X $10^6$ and 8 X $10^6$ cell/ml. The decrease in dexrazoxane concentration at 60 min was approximately 3-fold greater at the higher cell density, a result which suggested that some myocyte enzymatic or other process dependent on myocyte density was responsible for the loss of dexrazoxane from the medium. The results of Fig. 2a were suggestive of an enzymatic reaction in which an inhibitory product was produced halting the reaction. It can be calculated that the 1.8 μM decrease in dexrazoxane concentration from the myocyte suspension of Fig. 2a corresponded to an internal cell dexrazoxane concentration of about 0.4 mM if all of the dexrazoxane were taken up. However, because it has been shown (Dawson, 1975) that razoxane passively diffuses into and out of cells at about the same rate ($t_{1/2}$

of approximately 20 min) it seems unlikely that myocytes could accumulate this high a concentration of dexrazoxane. This calculation is based on our microscopically measured suspended myocyte diameter of 17.2 ± 0.6 μm. Assay sensitivity limitations prevented us from determining whether the neonatal myocytes metabolized dexrazoxane to ADR-925.

As shown in Fig. 2b when myocytes were treated with **C** the myocyte suspension significantly ($p < 0.001$) increased the rate of loss of **C** from the medium compared to the control. We previously showed that DHOase enzymatically hydrolyzed **B** and **C** to ADR-925 and that 5-aminoorotic acid, and furosemide inhibited the hydrolysis of **C** by purified recombinant DHOase, though 4-chlorobenzenesulfonamide did not (Schroeder et al., 2002). As shown in Fig. 2b all of these compounds inhibited the loss of **C** from the myocyte suspension medium. 5-Aminoorotic acid, which is the structurally most similar to **C**, and furosemide inhibited loss of **C** by 81 and 92%, respectively, to values that were not significantly different than myocyte control rates ($p > 0.2$ and > 0.5, respectively). 4-Chlorobenzenesulfonamide more weakly inhibited the rate of loss of **C** by 51%, to a rate different than the myocyte control rate ($p < 0.01$). 5-Aminoorotic acid is a potent inhibitor ($K_i$ of 6 μM) of mammalian DHOase-catalyzed hydrolysis of dihydroorotic acid (Christopherson and Jones, 1980). 4-Chlorobenzenesulfonamide is, however, a non-competitive inhibitor ($K_i$ 200 μM) of bacterial dihydroorotase (Pradhan and Sander, 1973) and may inhibit mammalian DHOase as well(Pradhan and Sander, 1973).

In order to determine if DHOase was responsible for the loss of **C** from the myocyte suspension, and if in fact our myocytes even contained DHOase, experiments were also carried

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out in which the loss of the DHOase endogenous substrate dihydroorotic acid was determined in the presence of inhibitors (Fig. 2c). As shown in Fig. 2c the myocyte suspension significantly ($p < 0.001$) increased the rate of loss of dihydroorotic acid from the medium compared to the control medium. Pretreatment with 5-aminoorotic acid inhibited the rate of loss of dihydroorotic acid by 88%, to a value that was not significantly different than the control value ($p > 0.1$). 4-Chlorobenzenesulfonamide, and furosemide inhibited loss of dihydroorotic acid by 62 and 41%, respectively, to rates that were different than control rates ($p < 0.001$ for each, respectively). Together the results of Fig. 2b and 2c show that myocytes contain DHOase and that the DHOase was, a least in part, responsible for the hydrolysis of **C**.

**Metabolism of dexrazoxane and C by hepatocyte suspensions.** We previously showed that adult rat hepatocytes were able to metabolize dexrazoxane to **B** and **C** through a DHPase-catalyzed reaction (Hasinoff et al., 1994). This study showed that metabolites **B** and **C** were produced in the dexrazoxane-treated hepatocyte suspension with half-times of approximately 1 h, and then leveled off at 3 - 4 h at a **B** to **C** ratio of about 3.5:1. Because the liver contains both DHPase and DHOase, we decided to investigate if a hepatocyte suspension could serially hydrolyze dexrazoxane all the way to ADR-925. As shown in Fig. 3a the concentration of ADR-925 was greatly increased (e.g. 14-fold at 2 h) in the hepatocyte suspension compared to the control experiment. These results indicated that hepatocytes had the capability to completely metabolize dexrazoxane to ADR-925. Also the appearance of ADR-925 in the medium indicated that ADR-925 effluxed from the hepatocyte after its formation. In order to rule out the possibility that dexrazoxane hydrolysis was caused by enzymes released into the medium due to a loss in viability of the hepatocytes during the course of the experiment, supernatant medium from a 3 h-old hepatocyte suspension was tested for its ability to hydrolyze dexrazoxane. The rate of dexrazoxane hydrolysis in this medium was not significantly different than the control rate ($p > 0.1$) indicating that all of the hydrolysis observed in Fig. 3a was intracellular in origin.

In order to determine if hepatocytes could metabolize **C**, and if DHOase was responsible for the loss of **C** from the hepatocyte suspension, experiments were carried out in which hepatocytes were treated with **C**. As shown in Fig. 3b the hepatocyte suspension significantly ($p < 0.001$) increased the rate of loss of **C** from the medium compared to the control medium. As shown in Fig. 3b, 5-aminoorotic acid and 4-chlorobenzenesulfonamide inhibited the loss of **C** from the hepatocyte suspension medium by 68 and 52%, respectively, to values that were not significantly different than hepatocyte buffer control rates ($p > 0.2$). These results indicate that DHOase was, at least in part, responsible for the metabolic hydrolysis of **C** in the hepatocyte. A comparison of the data of Figs. 2b and 3b shows that the net hydrolysis of **C** in the hepatocyte suspension was approximately 1.5-fold higher than in the myocyte suspension. These rates also do not necessarily reflect differing levels of metabolizing enzyme in the two cells, but may reflect differing rates of uptake of **C** into the cells.

**Metabolism of dexrazoxane and C in blood and plasma.** We previously showed that dexrazoxane is not hydrolyzed in rat plasma at rates any greater than buffer control rates (Hasinoff and Aoyama, 1999b). We decided to extend these studies to human and rat whole blood and plasma to determine if they had any dexrazoxane hydrolyzing activity. The rate of dexrazoxane hydrolysis in human blood and plasma was followed over 4 h (2 separate determinations of 9 time points, data not shown). The rate of loss of dexrazoxane was no different than in the Tris/NaCl control buffer ($p > 0.5$ for each). Similarly the rates in rat blood and plasma were no different than in the Tris/NaCl control buffer ($p > 0.5$ and 0.2, respectively). Thus, it can be concluded that neither blood nor plasma have any dexrazoxane hydrolyzing activity above that of background control rates (Hasinoff, 1994b; 1994a).

It has been reported that DHOase is present in erythrocytes and leukocytes (Smith and Baker, 1959). Thus, experiments were carried out to investigate whether whole blood and plasma had any **C** hydrolyzing activity. We also previously reported that $Ca^{2+}$ and $Mg^{2+}$, which are present in plasma at millimolar concentrations, promoted the ring-opening hydrolysis of **B** and **C** between 2.5-and 18- fold (Buss and Hasinoff, 1997). The promotion of the hydrolysis of the intermediates **B** and **C** likely occurs through the formation of a metal ion complex, as we demonstrated with the $Fe^{3+}$-**B** complex (Buss and Hasinoff, 1997). The rate of loss of dihydroorotic acid concentration (which was undetectable) from the medium was not significantly different ($p > 0.5$) over 90 min in experiments in which 200 μM dihydroorotic acid was incubated with human or rat blood or plasma (data not shown) compared to a control experiment carried out in α-MEM (Fig. 2c). Thus, within the detection limits of our experiments blood or plasma had no significant DHOase activity.

The results of Figs. 4a and 4b showed that relative to a Tris/NaCl buffer control, human and rat blood and plasma all significantly ($p < 0.002$ and $< 0.001$, for rat and human, respectively) promoted the hydrolysis of **C** by 3.3- to 4.9-fold. In order to test whether the $Ca^{2+}$ and $Mg^{2+}$ present in the plasma were responsible for this increased rate of hydrolysis of **C**, experiments were carried out in which rat plasma was pretreated with DTPA. DTPA is a extremely strong $Ca^{2+}$ and $Mg^{2+}$ binding analog of EDTA (Cheng et al., 1982), and when present in solution in molar excess of $Ca^{2+}$ and $Mg^{2+}$, would reduce the free $Ca^{2+}$ and $Mg^{2+}$ to extremely low levels such that effectively none would be available to complex the weaker chelator **C**. Thus the results of Fig. 5a show that the addition of 5 mM DTPA to rat plasma inhibited the loss in concentration of **C** from plasma by 97%, to a rate that was not significantly different ($p > 0.2$) than the Tris/NaCl buffer control rate. The addition of 100 μM DTPA to rat plasma, a concentration which was sufficient to only slightly reduce the $Ca^{2+}$ and $Mg^{2+}$ concentration, caused 27% inhibition of the net rate of loss in concentration of **C**, to a value that just reached statistical significance ($p < 0.05$) compared to plasma alone. Thus, these results indicated that the $Ca^{2+}$ and $Mg^{2+}$ in the plasma were responsible for increasing the rate of hydrolysis of **C**. The fact that 100 μM DTPA also caused a significant decrease in the rate of loss of **C** from plasma raises the possibility that there was another component contributing to the loss of **C** from plasma. DTPA is an extremely strong chelator of the di-and trivalent metal ions $Fe^{2+}$, $Fe^{3+}$, $Cu^{2+}$, and $Zn^{2+}$ (Cheng et al., 1982), and because we have shown that these metal ions all strongly promote the ring-opening hydrolysis of **C** (Buss and Hasinoff, 1997), the possibility arises that small amounts of these metal ions, either free or bound to biological components in the plasma, may be promoting hydrolysis of **C**.

The results of Fig. 5b, in which 5 mM DTPA was added to artificial plasma that contained plasma concentrations of $Ca^{2+}$ and $Mg^{2+}$, showed that DTPA significantly ($p < 0.001$) reduced the rate of hydrolysis compared to artificial plasma. Thus, these results confirm that $Ca^{2+}$ and

$Mg^{2+}$ make a significant contribution to the hydrolysis of **C** in plasma.

## Discussion

Our previous studies on the metabolism of dexrazoxane in humans (Schroeder et al., 2003) and in the rat (Hasinoff and Aoyama, 1999a; Schroeder and Hasinoff, 2002) showed that **B** and **C** and ADR-925 quickly appeared in the plasma after bolus *i.v.* administration, a result which suggested that dexrazoxane, and **B** and **C**, were metabolized. While dexrazoxane does undergo hydrolysis to ADR-925, this reaction is slow, with dexrazoxane hydrolyzing to **B** and **C** with a $t_{½}$ of 9.3 h at 37°C and pH 7.4, and to the final hydrolysis product ADR-925 with a $t_{½}$ of 23 h (Fig. 1) (Hasinoff, 1994b; 1994a). The fact that the monoanionic **B** and **C** and dianionic ADR-925 are present in human and rat plasma at relatively high concentrations (Hasinoff and Aoyama, 1999a; Schroeder and Hasinoff, 2002) also suggested that it was being released from the cells in which it was formed, as blood itself does not promote the hydrolysis of dexrazoxane. The metal chelating ADR-925 (Fig. 1), which can either remove iron from the iron-doxorubicin complex (Buss and Hasinoff, 1993), or strongly bind free iron (Diop et al., 2000), thus preventing iron-based oxygen radical formation, is thought to be the active form of dexrazoxane.

The results of Fig. 2a suggest that dexrazoxane was metabolized by myocytes in manner that involved product inhibition. The enzyme responsible for this process is unknown, but was unlikely to be DHPase as it is not found in the heart (Dudley et al., 1974; Hasinoff et al., 1991; Hamajima et al., 1996). Homologs of DHPase of unknown function and activity, called DRP-2 and DRP-3 (DHPase-related-protein), which have 58-59% homology with human DHPase, are found in the heart (Hamajima et al., 1996).While we did not find dexrazoxane hydrolyzing activity in the supernatant of porcine heart homogenate (Hasinoff et al., 1991), it is not possible to completely rule out DRP-2 and DRP-3 being responsible for the results of Fig. 2a due to possible inactivation of these enzymes in the homogenate supernatant. The enzyme responsible also cannot be DHOase, as we have shown that it has no dexrazoxane-hydrolyzing activity (Schroeder et al., 2002). The results of Fig. 2a are somewhat consistent with published reports using adult rat heart myocytes that showed that dexrazoxane is rapidly taken up (Doroshow, 1995) and rapidly metabolized to ADR-925 (Doroshow et al., 1991). Efflux of both dexrazoxane and ADR-925 from myocytes is also equally rapid (Doroshow et al., 1991; Doroshow, 1995). However, we previously showed that dexrazoxane treatment of myocytes only resulted in a slow displacement of iron from an intracellular fluorescence-quenched iron-calcein complex (Hasinoff et al., 2003), a result which suggests that myocytes do not quickly metabolize dexrazoxane to ADR-925. Also, the rapid efflux of dexrazoxane and ADR-925 (Doroshow et al., 1991; Doroshow, 1995) that was seen may have been from a sub-population of non-viable myocytes as the efflux of dexrazoxane from BHK-21S cells was not previously reported to be fast ($t_{1/2}$ of approximately 20 min) (Dawson, 1975).

Myocytes, however, do contain DHOase (Kennedy, 1974) and we previously showed that DHOase is able hydrolyze **B** and **C** to ADR-925 (Schroeder et al., 2002). The results of Fig. 2b and 2c demonstrate that myocytes can effectively hydrolyze both **C** and the DHOase endogenous substrate dihydroorotic acid, and that this hydrolysis was nearly completely inhibited by the potent and specific DHOase inhibitor 5-aminoorotic acid (Christopherson and Jones, 1980).

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While it is possible that these compounds may be blocking cellular uptake rather than inhibiting metabolism, we feel that this is not likely given the diversity in the chemical structures of these compounds. These results also demonstrated that **C** was taken up by myocytes and thus, the metabolism of dexrazoxane by DHPase by liver cells, and the release of **B** and **C** into the plasma where they may be taken up by myocytes, provide a mechanism by which ADR-925 can be formed in the heart and exert its metal chelating antioxidant protective effects.

The data of Fig. 2b and 2c show that the myocyte suspension increased the rate of hydrolysis of 50 μM **C** or dihydroorotic acid by about 200 nM/min each. From our previously determined (Schroeder et al., 2002) values of $V_{\max}$ and $K_m$ for **C** and dihydroorotic acid (at 15°C) it is possible to estimate that at 50 μM substrate concentration, DHOase in solution would hydrolyze dihydroorotic acid approximately 6-fold faster than **C**. Thus, the fact that rate of hydrolysis of the **C** and dihydroorotic acid in the myocyte suspension were nearly the same suggests that **C** was taken up by the myocytes more quickly than dihydroorotic acid.

The liver and kidney contain both DHOase and DHPase (Dudley et al., 1974; Kennedy, 1974; Hasinoff et al., 1991; Hamajima et al., 1996) and thus potentially have the capability to hydrolyze dexrazoxane to its fully hydrolyzed product ADR-925. We previously showed that hepatocytes were able to hydrolyze dexrazoxane to **B** and **C** (Hasinoff et al., 1994). The results of Fig. 3a show that hepatocytes were also able to effectively convert dexrazoxane to ADR-925, and that the ADR-925 produced effluxed from the hepatocytes. Hepatocytes were also able to hydrolyze **C**, and this hydrolysis was largely inhibited by the specific DHOase inhibitor 5-aminoorotic acid (Christopherson and Jones, 1980) (Fig. 3b), a result that indicates that DHOase was mainly responsible for the metabolism of **C** in hepatocytes. While the analysis of the data shown in Fig. 3b indicated that that the rates in the presence of both inhibitors was not significantly different than the control, the rate for both inhibitors was, nonetheless, faster than the control. However, because the intracellular concentration of free $Mg^{2+}$ in hepatocytes is quite high (Gasbarrini et al., 1992) (approximately 0.5 mM, and about one-half that in serum), $Mg^{2+}$-promoted hydrolysis of **B** and **C** (Buss and Hasinoff, 1997) may partially contribute to their metabolism to ADR-925. We also previously showed that myocytes were able to slowly take up **B** and **C** and ADR-925 and displace iron from an intracellular fluorescence-quenched iron-calcein complex (Hasinoff et al., 2003).

The results of Fig. 4a and 4b indicated that both blood and plasma promoted the hydrolysis of **C**. However, even though a previous report had indicated that DHOase was present in erythrocytes and leukocytes (Smith and Baker, 1959), we were able to show that neither blood nor plasma were able to significantly hydrolyze dihydroorotic acid. Thus, the increased rate of **C** hydrolysis seen cannot have any significant contribution from blood or plasma DHOase. We previously reported that $Ca^{2+}$ and $Mg^{2+}$ were able to significantly promote hydrolysis of **B** and **C** several fold over its background rate of hydrolysis (Buss and Hasinoff, 1997). The experiments of Fig. 5a in plasma and in artificial plasma containing physiological concentrations of $Ca^{2+}$ and $Mg^{2+}$, and in which a molar excess of the strong metal chelator DTPA was shown to nearly completely (97%) inhibit hydrolysis of **C**, indicated that plasma $Ca^{2+}$ and $Mg^{2+}$ were responsible for promotion of the hydrolysis of **C**. Thus, these results show that **B** and **C** are likely undergoing $Ca^{2+}$- and $Mg^{2+}$-promoted metabolism in the circulating plasma. It is not possible to predict whether the metabolism occurring in plasma contributes to or reduces the antioxidant effects of dexrazoxane, as this would depend upon factors such as how long effective circulating levels of **B** and **C** are maintained, and upon the relative rates of uptake of **B** and **C** and ADR-925 into the heart tissue where they could exert their antioxidant effects. However, it should be noted that we were unable to protect myocytes from doxorubicin-induced damage by **B** or ADR-925 (Hasinoff et al., 2003). Thus ADR-925 produced in the hepatocyte and released into the plasma may play only a small part to the antioxidant activity of dexrazoxane.

In summary the results of this study have shown that dexrazoxane underwent partial uptake and/or hydrolysis in myocytes by some unknown enzyme or process. The metabolite **C** of dexrazoxane underwent metabolism in myocytes by DHOase. Hepatocytes that contain both DHPase and DHOase, were able to completely hydrolyze dexrazoxane to ADR-925 and released it into the extracellular medium. Thus, these two liver enzymes acted in concert and sequentially on dexrazoxane and then **B** and **C** to produce ADR-925. They act sequentially because DHPase only acts on dexrazoxane, and not **B** and **C**, and DHOase only acts on **B** and **C**, and not dexrazoxane. We also showed that hydrolysis of **C** was promoted by the $Ca^{2+}$ and $Mg^{2+}$ in plasma, and thus further metabolism of **B** and **C** likely occurs in the plasma after these intermediates are released from the liver and kidney. In conclusion these studies provide a nearly complete description of the metabolism of dexrazoxane by myocytes and hepatocytes to its presumably active form ADR-925 and how it may be taken up and metabolized to exert its cardioprotective effects in the heart.

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## Footnotes

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[1] Abbreviations used are: ADR-925, N,N’ -[(1S)-1-methyl-1,2-ethanediyl]bis[(N-(2-amino-2-oxoethyl)]glycine; **B**, N-(2-amino-2-oxoethyl)-N-[(1S)-2-(3,5-dioxo-1-piperazinyl)-1-methylethyl]glycine; **C**, N-(2-amino-2-oxoethyl)-N-[(2S)-2-(3,5-dioxo-1-piperazinyl)propyl]glycine; DHPase, dihydropyrimidine amidohydrolase or dihydropyrimidinase; DHOase, dihydroorotase; DTPA, diethylenetriaminepentaacetic acid; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; HPLC, high-pressure liquid chromatography; $t_{½}$, half-time.

## Figure Captions

Fig. 1. Scheme for the hydrolysis of dexrazoxane (ICRF-187) to metabolites **B** and **C**, and its strongly metal ion chelating form ADR-925. Where indicated, only DHPase catalyzes the conversion of dexrazoxane into either **B** or **C**, and where indicated, only DHOase catalyzes the conversion of either **B** or **C** into ADR-925.

Fig. 2. a, Decrease in concentration of dexrazoxane in medium containing suspended myocytes (●), or in medium alone (○). b, Decrease in concentration of **C** in medium containing suspended myocytes (●), or myocytes in medium with 1 mM 5-aminoorotic acid (Δ), or myocytes in medium with 1 mM 4-chlorobenzenesulfonamide (▿), or myocytes in medium with 1 mM furosemide (◊), or in medium alone (○). c, Loss of dihydroorotic acid concentration in the medium containing suspended myocytes (●), or myocytes in medium with 1 mM 5-aminoorotic acid (Δ), or myocytes in medium with 1 mM 4-chlorobenzenesulfonamide (▿), or myocytes in medium with 1 mM furosemide (◊), or in medium alone (○).

Myocytes (2 X $10^6$/ml) were suspended in α-MEM medium (pH 7.4) at 37°C and treated either with either 10 μM dexrazoxane (a), 50 μM **C** (b), or 50 μM dihydroorotic acid (c) in the presence or absence of a pretreatment with various DHOase inhibitors. The concentrations of dexrazoxane, **C,** or dihydroorotic acid in the medium were measured by HPLC at the time points indicated. The straight lines were least squares calculated. The error bars are standard errors from $n = 3$.

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Fig. 3. a, Hydrolysis of 100 μM dexrazoxane (●) to ADR-925 (▲) in medium containing suspended hepatocytes (7 X $10^6$/ml) in hepatocyte suspension buffer, and hydrolysis of dexrazoxane (○) to ADR-925 (Δ) in hepatocyte suspension buffer. b, Decrease in concentration of **C** in α-MEM medium containing suspended hepatocytes (●), or hepatocytes (2 X $10^6$/ml) in medium with 1 mM 5-aminoorotic acid (Δ), or hepatocytes in medium with 1 mM 4-chlorobenzenesulfonamide (▿), or in medium alone (○). The initial concentration of **C** was 50 μM.

Hepatocytes were suspended in the buffered medium (pH 7.4) at 37°C and treated with either 100 μM dexrazoxane (a), or 50 μM **C** (b), in the presence or absence of a pretreatment with various DHOase inhibitors. The concentrations of dexrazoxane or **C** in the medium were measured by HPLC at the time points indicated. The concentrations of ADR-925 were measured by a calcein fluorescence assay. The straight lines were least squares calculated. The error bars are average deviations from *n* = 2-3 for the dexrazoxane experiment and *n* = 3 for the **C** experiments.

Fig. 4. a, decrease in concentration of **C** in human blood (▲), human plasma (●), and in Tris/NaCl buffer (○); b, decrease in concentration of **C** in rat blood (▲), rat plasma (●), and in Tris/NaCl buffer (○) decrease in concentration of **C** in rat blood and plasma.

The initial concentration of **C** was 200 μM. The blood and plasma were buffered with Tris (100 mM, pH 7.4) and Tris/NaCl buffer, respectively, at 37°C. The error bars are average deviations from *n* = 2 for the human studies, and standard errors from *n* = 3 for the rat studies.

Fig. 5. a, decrease in concentration of **C**, rat plasma (●) and in rat plasma containing either 100 μM (▲) or 5 mM DTPA (◆) or in Tris/NaCl buffer (○); b, decrease in concentration of **C** in artificial plasma (○) and artificial plasma containing 5 mM DTPA (●).

The initial concentration of **C** was 200 μM. The plasma was buffered with Tris (100 mM, pH 7.4) at 37°C. The artificial plasma contained physiological concentrations of $Ca^{2+}$ (2.5 mM) and $Mg^{2+}$ (1.2 mM) at 37°C. The error bars are average deviations from $n = 2$.

# Fig. 1

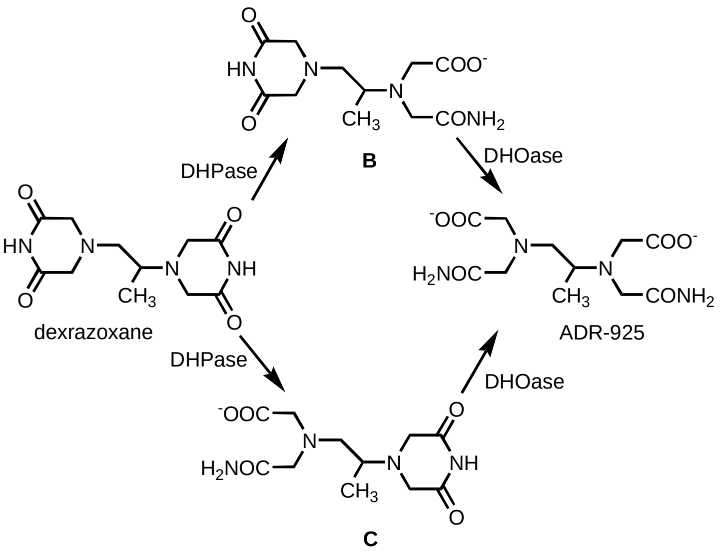

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Fig. 2

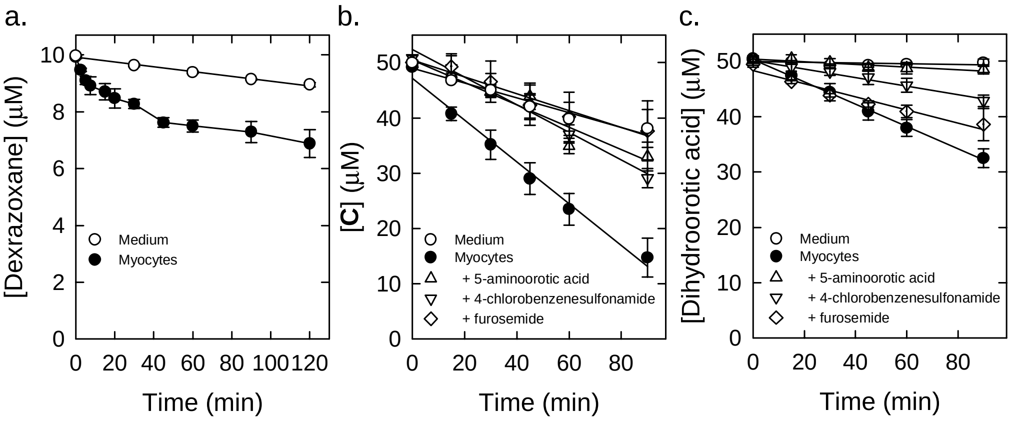

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Fig. 3

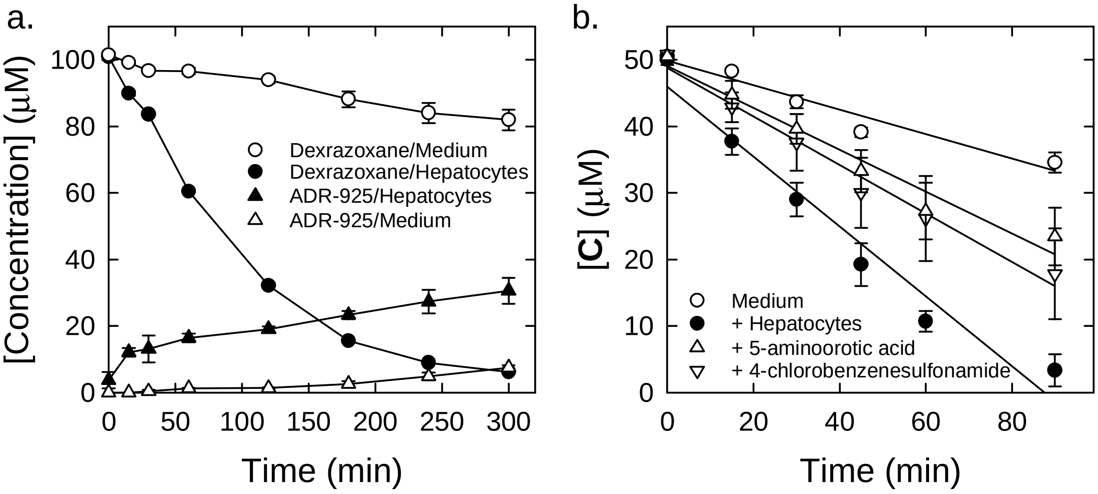


Fig. 4

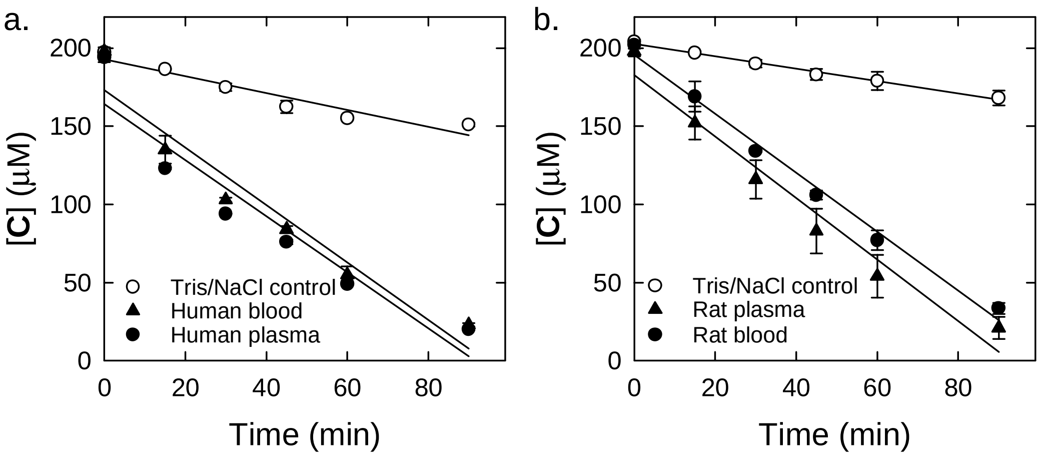
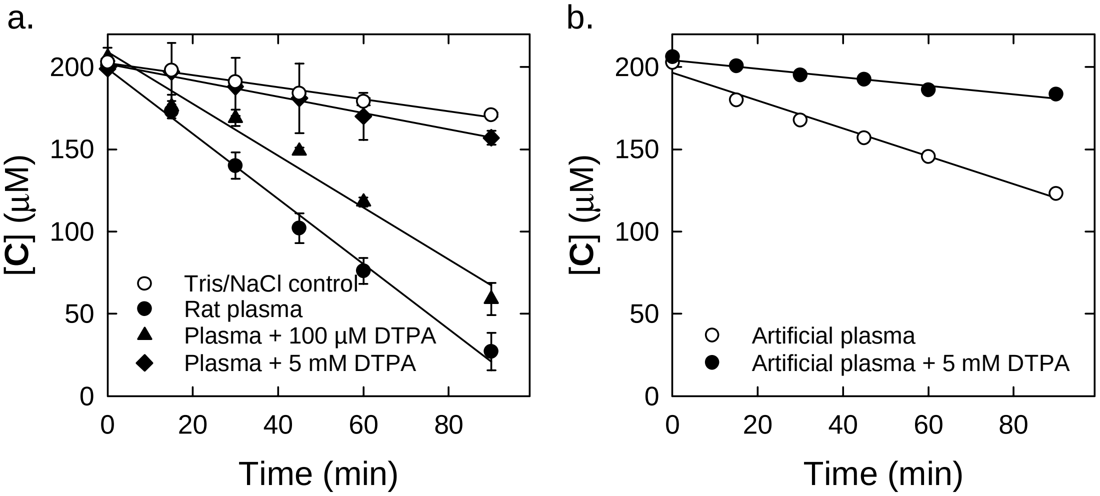

a.
[C] (µM)
200
150
100
50
0
0
20
40
60
80
Time (min)
○ Tris/NaCl control
▲ Human blood
● Human plasma
b.
[C] (µM)
200
150
100
50
0
0
20
40
60
80
Time (min)
○ Tris/NaCl control
▲ Rat plasma
● Rat blood

Fig. 5


a.
b.
[C] (µM)
0
50
100
150
200
0
20
40
60
80
Time (min)
Tris/NaCl control
Rat plasma
Plasma + 100 µM DTPA
Plasma + 5 mM DTPA
Artificial plasma
Artificial plasma + 5 mM DTPA