DMD Fast Forward. Published on June 24, 2005 as doi:10.1124/dmd.105.005546
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DMD #5546

METABOLISM OF THE ONE-RING OPEN METABOLITES OF THE CARDIOPROTECTIVE DRUG DEXRAZOXANE TO ITS ACTIVE METAL CHELATING FORM IN THE RAT

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Running title: METABOLISM OF THE DEXRAZOXANE METABOLITES

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Number of text pages: 15

Number of Tables: 2

Number of Figures: 3

Number of References: 32

Number of words in Abstract: 250/250

Number of words in Introduction: 556/750

Number of words in Discussion: 1340/1500

[1] Abbreviations used: ICRF-187, dexrazoxane; 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; HPLC, high-pressure liquid chromatography.

ABSTRACT:

Dexrazoxane (ICRF-187) is clinically used as a doxorubicin cardioprotective agent and may act by preventing iron-based oxygen free radical damage through the iron chelating ability of its fully hydrolyzed metabolite ADR-925. Dexrazoxane undergoes initial metabolism to its two one-ring open intermediates, and is then further metabolized to its active metal ion binding form ADR-925. The metabolism of these intermediates to the rings-opened metal-chelating product ADR-925 has been determined in a rat model in order to identify the mechanism by which dexrazoxane is activated. The plasma concentrations of both intermediates rapidly decreased after their *i.v.* administration to rats. A maximum concentration of ADR-925 was detected 2 min after *i.v.* bolus administration, indicating that these intermediates were both rapidly metabolized *in vivo* to ADR-925. The kinetics of the initial appearance of ADR-925 was consistent with formation rate-limited metabolism of the intermediates. After administration of dexrazoxane or its two intermediates ADR-925 was detected in significant levels in both heart and liver tissue, but was undetectable in brain tissue. The rapid rate of metabolism of the intermediates was consistent with their hydrolysis by tissue dihydroorotase. The rapid appearance of ADR-925 in plasma may make ADR-925 available to be taken up by heart tissue and bind free iron. These studies showed that the two one-ring open metabolites of dexrazoxane were rapidly metabolized in the rat to ADR-925 and thus these results provide a mechanism by which dexrazoxane is activated to its active metal-binding form.

Introduction

Dexrazoxane (ICRF-187, Zinecard®, Fig. 1) is clinically used to reduce doxorubicin-induced cardiotoxicity (Hasinoff, 1998; Hasinoff et al., 1998; Cvetkovic and Scott, 2005). 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. 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[1] according to the scheme in Fig. 1 (Hasinoff, 1990; 1994b; 1994a; 1998; Schroeder et al., 2005). Thus, dexrazoxane may act through its rings-opened hydrolysis product ADR-925 (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.

Under physiological conditions (37ºC 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 (Hasinoff, 1994b; 1994a). 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). It is, however, observed that in both humans (Schroeder et al., 2003) and the rat (Schroeder and Hasinoff, 2002) dexrazoxane is quickly metabolized to **B** and **C**, and then to ADR-925. The rapid rate of hydrolysis of dexrazoxane to **B** and **C** and the rapid appearance of ADR-925 in plasma *in vivo* suggested that first dexrazoxane, and then **B** and **C**, were all enzymatically

metabolized.

We previously showed that the dexrazoxane metabolic intermediates **B** and **C** are rapidly formed from dexrazoxane in a primary rat hepatocyte suspension (Hasinoff et al., 1994), a result that is consistent with dexrazoxane being metabolized by the zinc hydrolase DHPase (EC 3.5.2.2). However, while pure DHPase enzymatically hydrolyzes dexrazoxane to **B** and **C**, it is unable to enzymatically hydrolyze **B** and **C** to ADR-925 (Hasinoff et al., 1991; Hasinoff, 1993). In other work with purified DHOase (EC 3.5.2.3) we showed that this zinc hydrolase is able to enzymatically hydrolyze **B** and **C** to ADR-925, but cannot act on dexrazoxane (Schroeder and Hasinoff, 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), 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 its one-ring open intermediates **B** and **C** to ADR-925 in adult rats with a view to gaining a complete description of the metabolic hydrolysis-activation of dexrazoxane. More recently we showed that **C** is rapidly metabolized by DHOase in both rat myocyte and hepatocyte suspensions (Schroeder et al., 2005). We also showed that **C** underwent a slower Ca^{2+}- and Mg^{2+}-promoted hydrolysis in both human and rat plasma (Schroeder et al., 2005).

Methods

Materials. Dexrazoxane hydrochloride and ADR-925 were gifts from Adria Laboratories (Columbus, OH) and were used as supplied. HPLC-grade methanol was from Fisher (Nepean, Canada). Catalase (C-30), Tris, Chelex resin, 1-heptanesulfonic acid and 1-octanesulfonic acid were from Sigma (St. Louis, MO). Calcein ("high purity") was from Molecular Probes (Eugene, OR). Pharmacokinetic analyses were carried out using WinNonlin 4.0 (Pharsight, Mountain View, CA). Errors where cited or plotted are SEs.

Analysis of B, C and ADR-925. The HPLC analysis of dexrazoxane **B** and **C** 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; Schroeder et al., 2005). We have previously described a calcein fluorescence flow-injection analysis determination of ADR-925 on an HPLC apparatus (Schroeder et al., 2002; Schroeder et al., 2003; Schroeder et al., 2005). In this study ADR-925 was determined using the same method except that the fluorescence was determined on a BMG (Durham, NC) Fluostar Galaxy 96-well fluorescence plate reader (λ_{ex} 485 nm, λ_{em} 520 nm).

DHPase-mediated biosynthesis of the B/C mixture. Milligram quantities of a mixture of **B** and **C** were biosynthetized using the dexrazoxane hydrolyzing activity of DHPase. We previously showed that DHPase rapidly hydrolyzes dexrazoxane to **B** and **C**, but not to ADR-925 (Hasinoff et al., 1991; Hasinoff, 1993). DHPase is present as a minor protein contaminant in C-30 Sigma bovine catalase. We and others have previously used it as a source to obtain pure DHPase (Hasinoff et al., 1991; Hasinoff, 1993). Four ml of a crystalline suspension of catalase (35 mg protein/ml) were dialyzed (molecular weight cut-off of 12,000) for 24 h at 4ºC with 4 l

of distilled water to remove preservative. Two ml of dexrazoxane hydrochloride in water (30 mg/ml) were titrated with 5 M NaOH to a pH 7, and then added to the dialyzed catalase/DHPase mixture such that the final dexrazoxane solution concentration was 10 mg/ml. The reaction was carried out at room temperature and was monitored by HPLC and typically reached completion in 45 to 60 min. The reaction was deemed complete when the dexrazoxane levels were less then 10 μM (or less then 0.03 mol % of the initial dexrazoxane concentration). Acetonitrile (2:1, v/v) was used to precipitate proteins from the reaction mixture. After centrifugation to remove precipitated protein the **B**/**C** mixture was dried under nitrogen and reconstituted in sterile saline (0.9% NaCl w/v) just before use. Under these reaction conditions, dexrazoxane was hydrolyzed to **B** and **C** with a **B**:**C** ratio of 4.1:1, which was consistent with our previous published enzyme kinetic reports of DHPase-mediated hydrolysis of dexrazoxane (Hasinoff, 1993). The **B**/**C** mixture obtained was also analyzed for ADR-925 and was found to contain less then 0.5 mol % ADR-925.

Preparation of ADR-925 from dexrazoxane. ADR-925 used for the animal experiments was prepared by adding 50 μl of 5 M NaOH per ml to 10 mg/ml dexrazoxane hydrochloride solution for 48 h. We previously showed that the NaOH-catalyzed hydrolysis of dexrazoxane gave ADR-925 (Hasinoff, 1994a). At 48 h, the reaction was quenched with 45 μl of 5 M HCl and was titrated to pH 7. ADR-925 prepared in this fashion contained less then 0.003, 0.006, and 0.006 mol % of dexrazoxane, **B** and **C**, respectively. The ADR-925 was filter sterilized prior to animal use.

Dosing and sample collection. The rats (male Sprague-Dawley, 300-350 g) were allowed

food and water *ad libitum* before the study. The rats were anaesthetized *i.p.* with a combination of ketamine (90 mg/ml) and xylazine (10 mg/kg) and the left jugular vein was cannulated. The **B**/**C** mixture and ADR-925 were administered at a dose of 20 mg/kg (5 mg/ml in saline) as an *i.v.* bolus infusion (2 ml/min) through the tale vein. Blood samples (300 μl) were removed from the left jugular vein as described (Schroeder and Hasinoff, 2002) from a group of 3 rats at times of 2, 15, 30, 45, 60, and 90 min. One 300 μl blood sample was drawn from each rat prior to the administration of the **B**/**C** mixture or ADR-925 as a control. The blood samples were added to heparin (10 μl of 1000 units/ml) and centrifuged for 5 min at 500 *g*. The plasma was removed, 20 μl of 5 M HCl per ml of plasma was added, and the samples were stored at -80ºC to prevent further hydrolysis (Hasinoff, 1994a). To precipitate plasma proteins acetonitrile (Fisher) was added in a 2:1 ratio (v/v). The sample was mixed, allowed to settle for 5 min and centrifuged at 8000 *g* for 10 min. The supernatant was removed, acidified to pH 3 with HCl, and evaporated to dryness under nitrogen. The samples for the ADR-925 analysis were reconstituted in water to their original volume just before analysis. Samples for dexrazoxane, **B** and **C** analysis were reconstituted in10 mM HCl/500 μM Na_2EDTA to their original volume just prior to analysis. The dexrazoxane hydrochloride (40 mg/kg) for the tissue level studies was administered as described (Schroeder and Hasinoff, 2002). The animal protocol was approved by the University of Manitoba Animal Care Committee.

ADR-925 levels in liver, heart, and brain homogenates. After the last blood collection time point (2.8 h after the infusion of dexrazoxane, or 1.5 h after the infusion of the **B**/**C** mixture or ADR-925) the heart, brain and liver, were removed and weighed. Using surgical scissors, the

organs were cut into small pieces (< 5 mm^3), washed three times in a 50 ml centrifuge tube by adding 30 ml of 10 mM HCl to stabilize dexrazoxane and **B** and **C** (Hasinoff, 1994a), and rapidly swirled for about 1 min after which the wash solution was discarded. After the third wash the wash solution was visibly clear of blood. The washed minced organs were homogenized using a Polytron (Kinematica GmbH, Switzerland) homogenizer for 5 min. The homogenate was then centrifuged at 0ºC for 2 h at 18,000 *g*. The supernatant was removed and placed in 1.5 ml microcentrifuge tube and stored at -80ºC until analyzed. For the animals treated with dexrazoxane part of the supernatant sample was treated with 250 mM NaOH for 15 h in order to convert dexrazoxane, **B** and **C** into ADR-925 (Hasinoff, 1994a) and thus have some measure of the total amount ($ADR\text{-}925_{tot}$) of all forms of dexrazoxane in the tissues.

Results

Metabolism of the B/C mixture in the rat. As shown in Fig. 2a when rats were treated with the **B**/**C** mixture both **B** and **C** underwent rapid elimination. It is clear that **B** and **C** were quickly metabolized to ADR-925 as their hydrolysis product ADR-925 was present in the plasma at its maximum level by the first time point of 2 min. Subsequently, ADR-925 itself was also quickly eliminated from the plasma.

In an initial pharmacokinetic analysis the plasma concentrations for **B**, **C** and ADR-925 over the first 30 min were each individually fitted to an *i.v.* bolus one-compartment model with a single exponential term where C_b, C_c and C_{adr} are the concentrations of **B**, **C** and ADR-925 at time *t*, respectively. C_B, C_C and C_{ADR} are their respective concentrations at time zero, and k_b, k_c

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and k_{ADR} are their respective first-order elimination rate constants in eqs. 1, 2 and 3:

$$C_b = C_B \cdot e^{-k_b \cdot t} \quad (1)$$

$$C_c = C_C \cdot e^{-k_c \cdot t} \quad (2)$$

$$C_{adr} = C_{ADR} \cdot e^{-k_{adr} \cdot t} \quad (3)$$

The average of the individually derived pharmacokinetic parameters for **B**, **C** and ADR-925 after the bolus administration of the **B**/**C** mixture are given in Table 1. From the elimination rate constants in Table 1 it can be seen that **B** and **C** were rapidly eliminated with half-lives of 4.6 and 3.2 min, respectively. ADR-925 was, likewise, rapidly eliminated with an apparent half-life of 8.3 min. The C_B:C_C ratio of 5.5 ± 1.6 (Table 1) compares well to the ratio of the analytically determined starting dose ratio in the **B**/**C** mixture of 4.1:1

Because ADR-925 achieved its maximum plasma concentration at the first time point of 2 min, this result suggested that it was produced from the rapid metabolism of **B** and **C**. At 2 min the plasma concentration of ADR-925 reached a value of 114 ± 15 μM, which was already about one-third the value of the concentration of **B** with a value of 315 ± 42 μM at this time. Inspection of the initial concentration-time data in Fig. 2b plotted for times up to 30 min also showed that the initial apparent elimination of ADR-925 paralleled that of **B** and **C**. Together these results suggest that the appearance of ADR-925 was formation-rate limited by the rapid *in vivo* metabolism of **B** and **C**. Given these observations it was decided to attempt a more complete pharmacokinetic analysis that would contain terms that described the rates at which both **B** and **C** were metabolized. Thus, the initial elimination of **B** and **C** was modeled assuming that they were eliminated solely through metabolism via a first-order process, and that the ADR-

925 metabolite formed was itself eliminated via a first-order process with a rate constant k_m. Thus, with these assumptions the elimination of **B** and **C** and the first-order formation of ADR-925 for times up to 30 min, can be described by eqs. 4, 5 and 6 (Jackson et al., 2004):

$$C_m = k_{fb} \cdot C_B \, (e^{-k_m \cdot t} - e^{-k_{fb} \cdot t})/(k_{fb} - k_m) + k_{fc} \cdot C_C \, (e^{-k_m \cdot t} - e^{-k_{fc} \cdot t})/(k_{fc} - k_m) \quad (4)$$

$$C_b = C_B \cdot e^{-k_{fb} \cdot t} \quad (5)$$

$$C_c = C_C \cdot e^{-k_{fb} \cdot t} \quad (6)$$

where C_m, C_b and C_c are the concentrations of the metabolite ADR-925, **B** and **C**, respectively, at time t, and k_{fb} and k_{fc} are the metabolic formation rate constants for conversion of **B** and **C**, respectively, to ADR-925. C_B and C_C are the concentrations of **B** and **C** at time zero as before. The whole concatenated concentration-time data set for times up to 30 min (Fig. 2b) for all 3 rats was simultaneously fit using WinNonlin to eqs. 4, 5 and 6, weighted with the reciprocal of the calculated concentrations. The best fit pharmacokinetic parameters obtained are listed in Table 1. The smooth lines in Fig. 2b were calculated from these best-fit pharmacokinetic parameters. As might be expected k_{fb} and k_{fc} are close to the values obtained for k_b and k_c from the analysis of the individual **B** and **C** concentration-time data and the fit of that data to eqns. 2 and 3. It can be also be seen from Table 1 that k_{fb} and k_{fc} are similar in value to k_m, indicating that the rates of formation and elimination of ADR-925 were of the same order of magnitude, which consequently resulted in the initial apparent ADR-925 elimination nearly paralleling the initial elimination of **B** and **C**.

Elimination of ADR-925 in the rat. Experiments were also carried out to measure the plasma distribution and elimination of rats treated with 20 mg/kg of the metabolite ADR-925 (Fig. 3). A two-compartment model was used to describe the entire plasma ADR-925 concentration-time data. Thus the data was fit using WinNonlin to eq. 7.

$$C_{ADR} = A \cdot e^{-\alpha \cdot t} + B \cdot e^{-\beta \cdot t} \qquad (7)$$

Eq. 7 describes the elimination of ADR-925 where α and β are the alpha-distributive and beta-terminal phase elimination rate constants, respectively. *A* and *B* are their respective pre-exponential factors, the sum of which gives C_{max}. The average of the best fit parameters from 3 rats gave α and β values of 0.40 ± 0.20 min^{-1} and 0.024 ± 0.011 min^{-1}, respectively and a C_{max} value of 820 ± 230 μM. The smooth curve in Fig. 3 was calculated from the best fit parameters for all the data in eq. 7. The average derived overall elimination rate constant k_{10} had a value of 0.14 ± 0.06 min^{-1}.

Tissue levels of ADR-925 in rats treated with dexrazoxane, the B/C mixture or ADR-925. Because our previous studies showed that dexrazoxane was quickly metabolized in the rat (Schroeder and Hasinoff, 2002) and in humans (Schroeder et al., 2003), it was decided to determine the tissue levels of ADR-925 in heart, liver and brain tissues in order to determine if in fact ADR-925 was present in these tissues. Tissues were collected 2.8 h after administration of 40 mg/kg of dexrazoxane and 1.5 h after the administration of 20 mg/kg of the **B**/**C** mixture or ADR-925. In the dexrazoxane study part of the tissue supernatants were treated with NaOH to

convert any remaining dexrazoxane, **B** or **C** in the tissue to ADR-925. Thus the ADR-925_{total} values in Table 2 are the sum of the dexrazoxane, **B**, **C** and ADR-925 levels in the tissue. The ADR-925 assay is a sensitive fluorescence assay (Schroeder and Hasinoff, 2002) and allowed a determination of low amounts of ADR-925 in these tissues. However, low levels of dexrazoxane, **B** and **C** in these tissues precluded their direct determination by HPLC.

The results in Table 2 show that after administration of 40 mg/kg of dexrazoxane both the heart and liver ADR-925 levels were approximately one-half of the total amount of all forms of dexrazoxane and its 3 metabolites (ADR-925_{total}). The presence of ADR-925 in the heart and liver indicate that dexrazoxane was metabolized *in vivo* to ADR-925. It is also noteworthy that ADR-925 levels in the brain were undetectable (< 2 μmol/kg wet tissue), indicating that the polar dexrazoxane and its three polar metabolites were unable to detectably cross the blood-brain barrier. We previously showed that dexrazoxane, **B**, **C** and ADR-925 were all detectable in the plasma (49, 13, 3.2 and 30 μM, respectively) of similarly dexrazoxane-treated rats at 3 h (Schroeder and Hasinoff, 2002), and thus it is not surprising that significant levels of ADR-925 were still present in the heart and liver at 2.8 h after dexrazoxane administration.

Tissue levels of ADR-925 1.5 h after administration of a 20 mg/kg **B**/**C** mixture were also determined (Table 1). Levels in the heart and liver were approximately one-half of those found for the two-fold higher dose of dexrazoxane. These results also indicated that **B** and **C** were metabolized *in vivo* to ADR-925. ADR-925 levels in brain tissue, similar to the dexrazoxane treatment, were also undetectable (< 2 μmol/kg wet tissue). ADR-925 was also detected in the heart and liver of animals treated with ADR-925, though at levels that were about 6-10 fold

lower than the dexrazoxane-treated animals. This result is consistent with the polar ADR-925 being much less permeable to these tissues.

Discussion

The ability of the metal chelating metabolite ADR-925 (Fig. 1) to either remove iron from the iron-doxorubicin complex (Buss and Hasinoff, 1993), or strongly bind free Fe^{2+} (K_f $10^{10.0}$ M^{-1}) (Huang et al., 1982) or Fe^{3+} (K_f $10^{18.2}$ M^{-1}) (Diop et al., 2000), is likely the basis of the mechanism by which dexrazoxane prevents iron-based doxorubicin-mediated free radical oxidative stress on the heart muscle. Our previous studies on the metabolism of dexrazoxane in humans (Schroeder et al., 2003) and in the rat (Hasinoff and Aoyama, 1999b; 1999a; Schroeder et al., 2002) showed that **B**, **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 rapidly metabolized to ADR-925. Thus, these results suggested that rats treated with a **B**/**C** mixture might also metabolize **B** and **C** to ADR-925.

We also previously showed that DHPase, which is present in the liver and the kidney, can efficiently hydrolyze dexrazoxane (Hasinoff et al., 1991; Hasinoff, 1993; 1994c; Hasinoff et al., 1994; Hasinoff and Aoyama, 1999b), and is likely the enzyme that is primarily responsible for the metabolism of dexrazoxane to **B** and **C**. We have also shown that DHPase is unable to convert **B** and **C** into ADR-925 (Hasinoff et al., 1991). Likewise we have shown that DHOase is able to enzymatically hydrolyze **B** and **C** to ADR-925, but is unable to hydrolyze dexrazoxane (Schroeder and Hasinoff, 2002).

The results of this study showed that the **B**/**C** mixture was very rapidly metabolized to ADR-925, a result that suggested enzyme-mediated metabolism. The peak plasma concentration of dexrazoxane of 530 μM seen in our previous rat study (Schroeder and Hasinoff, 2002), after administration of 40 mg/kg of dexrazoxane, can be compared to peak plasma concentrations of **B** and **C** of 442 and 76 μM, respectively, for a 20 mg/kg dose of the **B**/**C** mixture (Table 1). In fact ADR-925 was maximally present in plasma at 2 min when the first blood sample was taken. The rapid appearance of ADR-925 suggested that the initial pharmacokinetics could be analyzed by a model in which the initial concentrations of the ADR-925 metabolite were largely determined by metabolism of **B** and **C**. Kinetic analysis of the data (Fig. 2b) in this formation rate-limited model showed that the initial ADR-925 elimination rate constant of 0.32 min^{-1} was in fact similar in magnitude to the **B** and **C** metabolite formation rate constants k_{fb} and k_{fc} of 0.13 and 0.20 min^{-1}, respectively. This simplified model, which only analyzed the data for **B** and **C** and ADR-925 up to 30 min, of necessity, involved several simplifying assumptions. It was assumed that **B** and **C**, at least initially, were only eliminated through metabolism to ADR-925. It was also assumed that the initial formation rate-limited elimination of **B** and **C** occurred through a non-saturating processes. The rapid metabolism of **B** and **C** was also a limitation on obtaining a more accurate formation-limited pharmacokinetic analysis. The elimination of ADR-925, when ADR-925 was administered alone, was biphasic and gave an average α-phase distributive phase rate constant of 0.40 min^{-1} (Fig. 3). This fast distributive phase precluded a valid comparison with k_m in the formation-limited model (Table 1) because the ADR-925 was produced from metabolism of **B** and **C** and these intermediates would already have undergone

their own prior distributive phase. This study did not directly address the question of whether or not doxorubicin administered after dexrazoxane would affect the metabolite profile or clearance of the intermediates of dexrazoxane or its metabolites as the animals were not treated with doxorubicin. Thus, we cannot rule out the possibility that doxorubicin treatment may affect the metabolism and the pharmacokinetics of dexrazoxane or it metabolites

We previously used the ratio of the initial plasma concentrations of **B** to **C**, compared to that produced by purified DHPase of 4:1 (Hasinoff, 1993), after the administration of dexrazoxane to either rats or humans, as evidence that DHPase was involved in the metabolism of dexrazoxane to **B** and **C** (Hasinoff and Aoyama, 1999a; Schroeder et al., 2003). We previously showed that purified DHOase, which is present in the liver, kidney and heart (Kennedy, 1974), is able to hydrolyze **B** and **C** to ADR-925 (Schroeder et al., 2002). Thus, if DHOase were the enzyme solely responsible for the metabolism of **B** and **C** *in vivo*, DHOase would be acting on **B** and **C** under non-saturating conditions, given that the K_m values for **B** and **C** are 4.8 and 11 mM, respectively (Schroeder et al., 2002). Thus, under these conditions DHOase would hydrolyze **B** and **C** at a rate ratio equal to the ratio of their V_{max}/K_m values of 0.9 (Schroeder et al., 2002). It can be seen from the data of Table 1 that the initial formation rate constant ratio k_{fb}/k_{fc} had a value of 0.68 ± 0.15, a value that compares reasonably well to 0.9 for the pure enzyme. Thus, these results are consistent with DHOase being largely responsible for the *in vivo* metabolism of the **B**/**C** mixture. We also previously reported that plasma, blood and plasma concentrations of Ca^{2+} and Mg^{2+} were able to promote the hydrolysis of **B** and **C** 3- to 5-fold over its background rate of hydrolysis (Buss and Hasinoff, 1997; Schroeder et al., 2005). Thus these metal ions may

partially contribute, along with DHOase, to the metabolism of **B** and **C**.

We have also shown in this study that rats treated either with dexrazoxane or the **B**/**C** mixture yield tissue levels that are relatively high in ADR-925. The values we obtained can be compared to total of all metabolite forms of ^{14}C-labeled dexrazoxane levels determined at 5 min in heart and liver of 320 and 640 μmol/kg respectively, for mice administered 100 mg/kg of dexrazoxane (Mhatre et al., 1982). Because the liver contains both DHPase and DHOase, which may act sequentially to form ADR-925, it was not unexpected that these tissues contained ADR-925. However, the fact that the heart, which does not contain DHPase (Hamajima et al., 1996), and because the supernatant of a heart homogenate does not have dexrazoxane-hydrolyzing activity (Hasinoff et al., 1991), this suggests that the ADR-925 found in the heart was from uptake of circulating **B** and **C** or ADR-925. In support of this conclusion we did show that isolated neonatal myocytes were able to slowly take up **B**, **C** and ADR-925 and displace iron from an intracellular fluorescence-quenched iron-calcein complex (Hasinoff et al., 2003). The much lower levels of ADR-925 in heart and liver when the rats were treated with ADR-925 reflects the low cellular permeability of the highly polar dianionic ADR-925.

The lack of detectable ADR-925 in brain tissues is consistent with the polar dexrazoxane and its three metabolites not being able to cross to the blood-brain barrier. Dexrazoxane is currently in clinical trials as a extracerebral rescue agent in cancer patients with brain metastases treated with high-dose etoposide (Holm et al., 1998; Schroeder et al., 2003; Schroeder et al., 2004). Because neither dexrazoxane nor its metabolites were found in brain tissue, these results suggest that dexrazoxane will not antagonize the topoisomerase II poisoning activity of etoposide

(Sehested et al., 1993) in cancerous brain tissue.

This study did not directly address how dexrazoxane exerted its cardioprotective effects in the heart. However, in conclusion these studies showed that the one-ring metabolites **B** and **C** of dexrazoxane were rapidly metabolized in the rat to ADR-925. Their rapid *in vivo* hydrolysis provides a mechanism by which **B** and **C** can be activated to its iron-binding form ADR-925 that has antioxidant activity. The initial plasma levels of ADR-925 that were observed were consistent with the formation-rate limited enzyme-mediated metabolism of **B** and **C**. Some of the metabolism of **B** and **C** may take place in the heart which contains the enzyme DHOase that can metabolize **B** and **C** to ADR-925.

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Footnotes

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 Brian Hasinoff. Patricia Schroeder was supported by a Manitoba Health Research Council studentship and Canadian Institutes of Health Research studentship.

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[1] Abbreviations used: ICRF-187, dexrazoxane; 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; HPLC, high-pressure liquid chromatography.

Figure Legends

Fig. 1. Hydrolytic metabolism of dexrazoxane (ICRF-187) to its one-ring open intermediate metabolites **B** and **C**, and ultimately to its presumably active 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. Average rat plasma concentrations of **B**, **C** and ADR-925 after a 20 mg/kg dose (*i.v.* bolus) of a 4.1:1 mixture of **B** and **C**.

The straight lines through the **B** (Δ), **C** (▽) and ADR-925 (●) data were calculated from a one-compartment fit model described by eqs. 1, 2 and 3 and gave average half-lives of 4.8, 3.3, and 8.3 min respectively. The data plotted are averages from 3 rats except for the **B** datum at 45 min which was from 2 rats. b. Average rat plasma concentrations of **B**, **C** and ADR-925, but replotted for times up to 30 min only, for fitting to an ADR-925 formation-rate limited model. The smooth lines (solid lines for **B** and **C** and a dashed line for ADR-925) were calculated from simultaneously fitting the whole concatenated data set to eqs. 4, 5 and 6. These equations describe a model in which ADR-925 is formation rate-limited by its rapid *in vivo* metabolism.

Fig. 3. Average rat plasma concentrations of ADR-925 after administration of a 20 mg/kg (i.v. bolus) of ADR-925. The data plotted were averages from 3 rats (with the exception at 90 min, which is from 2 rats). The smooth line was calculated from a fit of all the data to the biphasic

elimination eq. 7.

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TABLE 1

*Pharmacokinetic parameters individually modeled and modeled using a concatenated data set for **B** and **C** and ADR-925 upon the administration to rats of a 20 mg/kg bolus dose of a **B**/**C** mixture*

The individual pharmacokinetic parameters for **B** and ADR-925 were determined from the concentration-time data for the first 30 min, whereas those for **C** were determined from the data for the first 20 min. All values used in the analyses are averages from 3 rats. The parameters for the whole concatenated data set were obtained from fitting all of the concentration-time data to eqs. 4, 5 and 6 simultaneously.

One-compartment individual pharmacokinetic analyses		Metabolite pharmacokinetic analysis of concatenated data	
Parameter	average ± SE	Parameter	value ± SE
k_b (min^{-1})	0.15 ± 0.02	k_{fb} (min^{-1})	0.13 ± 0.01
C_B (μM)	420 ± 47	C_B (μM)	442 ± 40
k_c (min^{-1})	0.22 ± 0.04	k_{fc} (min^{-1})	0.20 ± 0.05
C_C (μM)	76 ± 18	C_C (μM)	76 ± 22
k_{ADR} (min^{-1})	0.084 ± 0.020	k_m (min^{-1})	0.32 ± 0.04
C_{ADR} (μM)	134 ± 10	-	-

TABLE 2

*Tissue levels of ADR-925 in rats treated with either dexrazoxane, a **B**/**C** mixture or ADR-925*

The average tissue level of ADR-925 was measured per kg of wet tissue harvested from 7 rats 2.8 h after dexrazoxane was administered (40 mg/kg), or the levels 1.5 h after the **B**/**C** mixture or ADR-925 was administrated (20 mg/kg) to 3 rats. ADR-925_{tot} is the total amount of ADR-925 measured after treatment of the homogenate with NaOH to convert all forms of dexrazoxane into ADR-925. The limit of detection was 2 μmol/kg.

Tissue	Treatment			
	dexrazoxane		**B**/**C** mixture	ADR-925
	ADR-925 (μmol/kg)	ADR-925_{tot} (μmol/kg)	ADR-925 (μmol/kg)	ADR-925 (μmol/kg)
heart	51 ± 6	100 ± 7	27 ± 6	7.4 ± 1.6
liver	97 ± 14	232 ± 26	40 ± 16	10.6 ± 0.6
brain	< 2	< 2	< 2	< 2

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Fig. 1

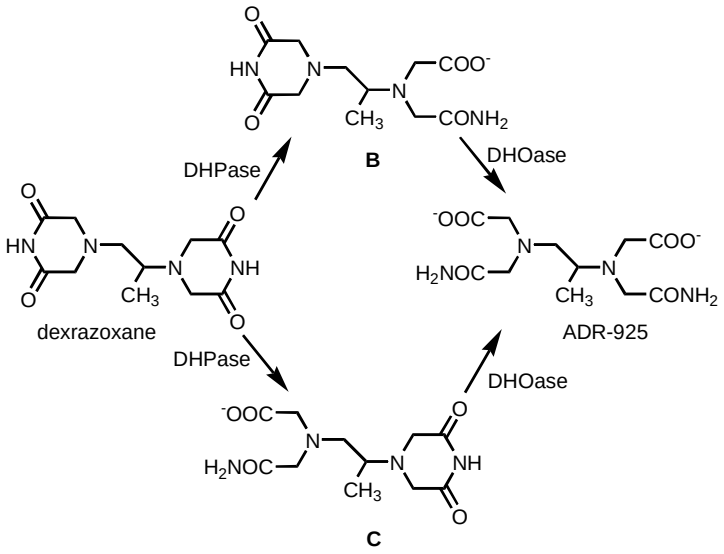

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Fig. 2

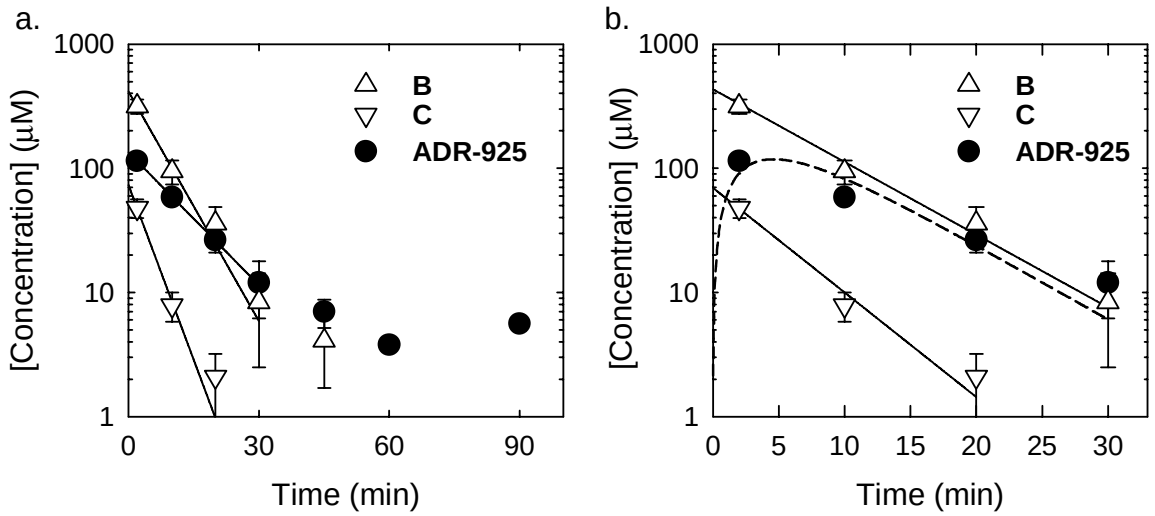

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Fig. 3