THE DIHYDROOROTASE INHIBITOR 5-AMINOOROTIC ACID INHIBITS THE
METABOLISM IN THE RAT OF THE CARDIOPROTECTIVE DRUG DEXRAZOXANE
AND ITS ONE-RING OPEN METABOLITES

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Running title: METABOLISM OF DEXRAZOXANE

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Number of text pages: 16
Number of Tables: 1
Number of Figures: 4
Number of References: 35
Number of words in Abstract: 233
Number of words in Introduction: 496
Number of words in Discussion: 1145

ABBREVIATIONS: $AUC_{5-120}$, area-under-the-curve of concentration-time data for dexrazoxane or ADR-925 from 5 to 120 min; 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; DHOase, dihydroorotase; DHPase, dihydropyrimidine amidohydrolase or dihydropyrimidinase; HPLC, high-pressure liquid chromatography; LDH, lactate dehydrogenase; $t_{1/2}$, half-life.
ABSTRACT:

Dexrazoxane (ICRF-187) is clinically used as a doxorubicin cardioprotective agent and to prevent anthracycline extravasation injury. It may act by preventing iron-based oxygen free radical damage through the iron chelating ability of its metabolite ADR-925. Dexrazoxane undergoes an initial metabolism to its two one-ring open intermediates (B and C), and is then further metabolized to its presumably active metal-chelating form ADR-925. We previously showed that the first ring opening reaction is catalyzed by dihydropyrimidinase and the second by dihydroorotase (DHOase), but not vice versa. In order to determine if DHOase was important in the metabolism of dexrazoxane its metabolism and that of B and C to ADR-925 was measured in rats that were pretreated with the DHOase inhibitor 5-aminoorotic acid. In rats pretreated with 5-aminoorotic acid the area-under-the-curve concentration of ADR-925 was reduced 5.3-fold. In rats treated with a mixture of B and C the maximum concentration of ADR-925 in the plasma was significant decreased in rats pretreated with 5-aminoorotic acid which indicates that DHOase directly metabolized B and C. Both heart and liver tissue levels of ADR-925 in rats were also greatly reduced by pretreatment with 5-aminoorotic acid. Together these results indicate that the metabolism of dexrazoxane and of B and C is mediated by DHOase. These results provide a mechanistic basis for the antioxidant cardioprotective activity of dexrazoxane.
Dexrazoxane (ICRF-187, Zinecard™, Totect™, Savene™, Fig. 1) is clinically used to reduce doxorubicin-induced cardiotoxicity (Hasinoff, 1998; Hasinoff et al., 1998; Cvetkovic and Scott, 2005; Hasinoff and Herman, 2007) and has just been approved in the U.S. for the prevention of anthracycline-induced extravasation injury (Hasinoff, 2008). There is now considerable evidence to indicate that the cardiotoxicity of doxorubicin may be due to iron-dependent oxygen free radical formation (Malisza and Hasinoff, 1995; Meyers, 1998; Hasinoff and Herman, 2007) on the relatively unprotected cardiac muscle. Dexrazoxane may act through its metal chelating 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 (Diop et al., 2000), 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; 1994a; 1994b; 1998; Hasinoff and Herman, 2007).

We previously showed that dexrazoxane is quickly metabolized to B and C, and then to ADR-925 in humans (Schroeder et al., 2003) and in rats (Schroeder and Hasinoff, 2002). The rapid rate of hydrolysis of dexrazoxane and the rapid appearance of ADR-925 in plasma in vivo suggests that dexrazoxane first, and then B and C, are all enzymatically metabolized. We also showed that rats rapidly metabolized a mixture of B and C to ADR-925 which suggested that these metabolites were themselves enzymatically metabolized (Schroeder and Hasinoff, 2005). We have shown that pure DHPase (EC 3.5.2.2) enzymatically hydrolyzes dexrazoxane to B and C, but cannot 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 and Hasinoff, 2002). Thus, it is hypothesized that DHPase and DHOase act sequentially, and in concert, to effect the full metabolism of dexrazoxane to its active metal ion chelating form ADR-925. We also previously showed that B and C are rapidly formed from dexrazoxane in a primary rat hepatocyte suspension (Schroeder et al., 2005) which was consistent with dexrazoxane being metabolized by DHPase. We also showed that C underwent DHOase-catalyzed metabolism in a myocyte suspension (Schroeder et al., 2005). 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). However, while DHPase is present in the liver and the kidney, it is not present in the heart (Dudley et al., 1974; Hasinoff et al., 1991; Hamajima et al., 1996). In order to determine the importance of DHOase-mediated metabolism of dexrazoxane, the metabolism of dexrazoxane and B and C were studied in rats that had been pretreated with the potent DHOase inhibitor 5-aminoorotic acid (Christopherson and Jones, 1980).
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 was from Molecular Probes (Eugene, OR). Pharmacokinetic analyses were carried out using WinNonlin 4.0 (Pharsight, Mountain View, CA) using an empirical, non-compartmental method for an i.v. bolus infusion or a one-compartment method as appropriate. The effect of DHOase inhibition on pharmacokinetic parameters for dexrazoxane, B, C and ADR-925 were compared using an independent-samples t-test where p is the statistical significance (Jones, 2002). Errors, where cited or plotted, are the SEM.

Analysis of B, C and ADR-925. The HPLC analysis of dexrazoxane, B and C using an ion-pair reagent with the reversed phase C₁₈-column (detection wavelength 205 nm) has been described (Schroeder et al., 2002; Schroeder et al., 2003; Schroeder and Hasinoff, 2005). ADR-925 was determined on a BMG (Durham, NC) Fluostar Galaxy 96-well fluorescence plate reader (λ_{ex} 485 nm, λ_{em} 520 nm) using the calcein fluorescence assay method we previously described (Schroeder and Hasinoff, 2005). The limit of quantitation for dexrazoxane, B, C and ADR-925 in plasma was 0.5, 1, 1 and 10 µM, respectively.

DHPase-catalyzed biosynthesis of the B/C mixture. Milligram quantities of the B/C mixture were biosynthesized using DHPase as we previously described (Schroeder and Hasinoff, 2005). DHPase rapidly hydrolyzes dexrazoxane to B and C, but not to ADR-925 (Hasinoff et al., 1991; Hasinoff, 1993; Schroeder and Hasinoff, 2005). Dexrazoxane and ADR-925 levels in the
treatment mixture were less than 0.03 and 0.1 mol %, respectively, of the initial dexrazoxane concentration. Under these reaction conditions, dexrazoxane was hydrolyzed to B and C with a B:C ratio of 4.1:1, which is consistent with previous published enzyme kinetic reports of DHPase-mediated hydrolysis of dexrazoxane (Hasinoff, 1993; Schroeder and Hasinoff, 2005). Because of the amounts of B and C required for the rat studies it was not practical to separate the chemically very similar B and C (Fig. 1).

Dosing and sample collection. Treatment of the rats (male Sprague-Dawley, 300 - 350 g) with the drugs, blood sampling and sample handling were done as previously described (Schroeder and Hasinoff, 2005). A 10 mg/ml solution of 5-aminoorotic acid was prepared just before use in sterile saline (0.9 % NaCl, w/v) by adding NaOH to dissolve it and it was then titrated to pH 7 and stored at 37°C until used. The 5-aminoorotic acid-treated rats were treated with an i.v. bolus infusion (2 ml/min) through the tail vein with 5-aminoorotic acid (20 mg/kg) 5 min prior to treatment with either dexrazoxane hydrochloride (40 mg/kg) or the B/C (20 mg/kg) mixture. The animal protocol was approved by the University of Manitoba Animal Care Committee.

ADR-925 levels in heart and liver homogenates. After the last blood collection time point (2 - 2.5 h) after the infusion of dexrazoxane, or 1.5 h after the infusion of the B/C mixture), the heart and liver were removed, weighed and treated as previously described (Schroeder and Hasinoff, 2005). Using surgical scissors, the organs were cut into small pieces (< 5 mm³), 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 homogenizer (Kinematica GmbH, Switzerland) for 5 min. The homogenate was centrifuged at 0°C for 2 h at 18,000 g to remove HCl-denatured protein. The supernatant was removed and stored at -80°C until analyzed.

**Myocyte isolation and culture.** Ventricular myocytes were isolated from 1 - 3 days old Sprague-Dawley rats as we have described (Hasinoff et al., 2003a; Schroeder et al., 2005; Hasinoff et al., 2007). 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 deplete fibroblasts which preferentially attach. Collagenase, trypsin and deoxyribonuclease were obtained from Worthington Biochemicals (Freehold, NJ). For the lactate dehydrogenase (LDH) release experiments the myocyte-rich supernatant was plated at high density on day zero in 24-well plastic culture dishes (5 x 10⁵ myocytes/well, 750 µl/well). 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 two. After addition of drug to the cell suspensions, samples were removed at the times indicated.

**LDH release assay.** LDH released into the myocyte growth medium was determined as previously described (Hasinoff et al., 2003a; 2007). For the 5-aminoorotic acid treatments the myocytes were pretreated with 5-aminoorotic acid (100 µM) for 1 h, then treated with dexrazoxane for 3 h, and then with doxorubicin (1.6 µM) for 3 h. At the end of the doxorubicin treatment period the myocytes were washed twice with (and replaced with) medium containing 5-aminoorotic acid (100 µM) and dexrazoxane. Starting on day 6 after plating, samples (80 µl) of the myocyte supernatant were collected every 24 h for 3 d after treatment and the samples frozen at -80°C and analyzed within 1 week. After the last supernatant sample was taken, the myocytes
were lysed with 250 µl of 1% (v/v) Triton X-100/2 mM EDTA/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 µl samples previously taken. The LDH activity was determined in quadruplicate in a kinetic assay in 96-well plate in a Molecular Devices (Menlo Park, CA) plate reader as previously described (Hasinoff et al., 2003a; 2007). The initial velocity of the LDH-catalyzed reaction of NAD$^+$ with lactate to produce NADH and pyruvate was determined 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). Where significance is indicated, an unpaired $t$-test was used.
Results

The effect of pretreating rats with 5-aminoorotic acid prior to dexrazoxane treatment on dexrazoxane elimination and B, C and ADR-925 formation. Data obtained for dexrazoxane elimination and B, C and ADR-925 formation, with and without 5-aminoorotic acid pretreatment, are compared in Figs. 2a and 2b. The limited sampling in the alpha (or distribution) phase of the concentration-time curve made compartmental analysis inappropriate. Thus, it was decided to compare the concentration-time curves for dexrazoxane in plasma for rats treated with the DHOase inhibitor 5-aminoorotic acid using non-compartmental area-under-the-curve ($AUC_{5-120}$) analysis of the concentration of dexrazoxane or ADR-925 from the first to the last time point (5 to 120 min). Therefore, values for the maximum plasma concentration were obtained directly from the observed data at 5 min.

The concentration-time dexrazoxane plasma levels shown in Fig. 2a for rats treated with dexrazoxane only (Fig. 2a) are similar to those previously obtained (Hasinoff and Aoyama, 1999a; Schroeder and Hasinoff, 2002). The plasma concentration of dexrazoxane at 5 min for rats treated with 5-aminoorotic acid prior to dexrazoxane treatment was 515 ± 38, which is close to the value of 529 ± 41 µM for dexrazoxane alone. Also, the $AUC_{5-120}$ of dexrazoxane for rats pretreated with 5-aminoorotic acid was not significantly different ($p > 0.5$) compared to rats treated with dexrazoxane alone (15,100 ± 970 µM•min compared to 18,600 ± 1200 µM•min, respectively) which indicated that 5-aminoorotic acid did not change the elimination kinetics of dexrazoxane, and thus the decrease in the ADR-925 $AUC_{5-120}$ values was not due to 5-aminoorotic acid-mediated changes in the systemic clearance of dexrazoxane.

The intermediates B and C were both maximally observed in plasma at the earliest measured
time point (5 min) (Fig. 2b). Pretreatment with 5-aminoorotic acid did not significantly change ($p > 0.2$) the plasma concentrations of either B or C at 5 min. After dexrazoxane administration, the B and C levels both rapidly fell. The concentration of C fell below the limit of detection at times greater than 15 min for the 5-aminoorotic acid pretreatment. For the dexrazoxane alone treatment low, but detectable levels of C were observable up to 120 min. Detectable levels of B, with and without 5-aminoorotic acid pretreatment, were observed up to 120 min. Levels of B with the 5-aminoorotic acid pretreatment were above those compared to dexrazoxane alone treatment up to about 45 min, after which they were both approximately the same. The fact that after dexrazoxane treatment the levels of B and C did not change with 5-aminoorotic acid pretreatment (but that of their ADR-925 metabolite did) suggests that DHPase-mediated formation of B and C, and non-metabolic routes of elimination to ADR-925 are rate-determining processes for these two intermediate metabolites. Also the fact that ADR-925 levels in 5-aminoorotic acid treated and untreated rats are not significantly different up to 30 min, and are only different thereafter, also suggests that the DHOase-mediated conversion of B and C into ADR-925 is a slower process than the first step (Fig. 1). Due to the size and charge state of B and C, it is also likely that urinary clearance also contributes to the systemic elimination of B and C. Thus, it is not surprising that the systemic impact of enzymatic hydrolysis (metabolic clearance) is masked by this.

As shown in Fig. 2a ADR-925 was seen in the plasma of rats pretreated or not with 5-aminoorotic acid prior to treatment with dexrazoxane. However, pretreatment with 5-aminoorotic acid had a large effect on the plasma levels of ADR-925. The $AUC_{5-120}$ values for ADR-925 in rats pretreated with 5-aminoorotic acid was 5.3-fold lower ($p < 0.001$) compared to rats treated
with dexrazoxane alone (940 ± 240 µM•min compared to 4980 ± 460 to µM•min, respectively). Thus, these results indicate that 5-aminoorotic acid pretreatment strongly inhibited the appearance of ADR-925 in the plasma.

The effect of pretreatment with 5-aminoorotic acid prior to a B/C mixture treatment on B and C elimination and ADR-925 formation. The concentration-time plasma levels of B, C and ADR-925 of rats treated with a B/C mixture, with and without a 5-aminoorotic acid pretreatment, are given in Fig. 3a and 3b. The data were fit to a one-compartment pharmacokinetic model because a two-compartment model was judged inappropriate based on Akaike’s information criterion estimated from WinNonlin. The $C_{\text{max}}$ for B (Fig. 3a) for rats pretreated with 5-aminoorotic acid prior to treatment with a B/C mixture rats was not significantly different ($p$ of 0.9) compared to rats treated with a B/C mixture alone (427 ± 111 µM compared to 420 ± 81 µM, respectively). Both B and C initially rapidly decreased similarly in concentration with or without pretreatment with 5-aminoorotic acid, but levels of B appeared to be prolonged with the 5-aminoorotic acid pretreatment. Likewise, the half-life for B for rats pretreated with 5-aminoorotic acid prior to treatment with a B/C mixture was not significantly different ($p$ of 0.6) compared to rats treated with the B/C mixture alone (5.8 ± 2.9 compared to 4.8 ± 1.0 min, respectively). The fact that the pharmacokinetic parameters $C_{\text{max}}$ and the half-life for elimination of B and C were unchanged when rats were pretreated with 5-aminoorotic acid prior to treatment with the B/C mixture, compared to rats treated with the B/C mixture alone indicated that 5-aminoorotic acid did not change the elimination kinetics of B and C, and thus the decrease in the ADR-925 $C_{\text{max}}$ value was not due to 5-aminoorotic acid-mediated changes in the elimination of B and C.
The ratio of the average values of B and C (414 ± 33 and 66 ± 13 µM, respectively) observed at 2 min of 6.3 ± 1.4 can be compared to the ratio of 4.4 in the infusion mixture. Because of the lower concentration of C in the mixture and its rapid decrease to below quantifiable limits, it was not possible to carry out further analysis of the data for C.

As can be seen from the data in Fig. 3b ADR-925 rapidly appeared in plasma at its maximally observed concentration at the first time point of 2 min and then decreased with time. The C_max for ADR-925 for rats pretreated with 5-aminoorotic acid prior to treatment with a B/C mixture was significantly decreased (p of 0.018) compared to rats treated with a B/C mixture alone (49 ± 2 µM compared to 137 ± 38 µM, respectively). While the half-life for ADR-925 for rats pretreated with 5-aminoorotic acid prior to treatment with a B/C mixture rats did increase, the increase was not quite significant (p of 0.053) compared to rats treated with a B/C mixture alone (25 ± 9 min compared to 9 ± 4 min, respectively). Also, the plasma level of ADR-925 in either treatment group was not significantly different at 1.5 h (p of 0.4).

The effect on ADR-925 tissue levels of pretreatment with the DHOase inhibitor 5-aminoorotic acid prior to treatment with dexrazoxane or the B/C mixture. ADR-925 levels in the 18,000 g supernatant fraction of heart and liver tissue homogenates of rats treated with 5-aminoorotic acid prior to treatment with dexrazoxane (40 mg/kg) or treatment with the B/C mixture (20 mg/kg) are shown in Table 1. When the dexrazoxane-treated rats were pretreated with 5-aminoorotic acid, ADR-925 levels in both the heart and the liver were significantly reduced (5.3- and 9.7-fold, p < 0.001 and 0.005, respectively). In contrast, however, when the B/C mixture-treated rats were pretreated with 5-aminoorotic acid the ADR-925 levels in neither the heart nor the liver were significantly reduced (p of 0.7 and 0.5, respectively). In the case of
the dexrazoxane treatment these results showed that 5-aminoorotic acid greatly inhibited the conversion of B and C to ADR-925, resulting in reduced heart and tissue levels. It is not known how quickly 5-aminoorotic acid is distributed in the heart and liver (and other) tissues that contain DHOase, and whether inhibitory levels of 5-aminoorotic acid are achieved in DHOase-containing tissues. The values we obtained can be compared to total $^{14}$C-labeled dexrazoxane levels determined at 5 min in heart and liver of 320 and 640 $\mu$mol/kg in heart and liver, respectively for mice administered 100 mg/kg of dexrazoxane (Mhatre et al., 1982).

Effect of 5-aminoorotic acid on dexrazoxane-mediated protection from doxorubicin-induced LDH release in myocytes. LDH release is a widely used measure of drug-induced damage to myocytes (Adderley and Fitzgerald, 1999; Hasinoff et al., 2003a; 2007). Using the LDH release assay we examined the ability of 5-aminoorotic acid to inhibit dexrazoxane-mediated protection from doxorubicin-induced LDH release to determine if inhibition of DHOase reduced the ability of dexrazoxane to protect myocytes from doxorubicin-induced damage. As shown in Figs. 4a and 4b, 5, 20 and 100 $\mu$M dexrazoxane significantly reduced LDH release in a concentration dependent manner from myocytes ($p$ of 0.021, 0.004 and < 0.001 at 24 h; and $p$ of 0.008, 0.003 and < 0.001 at 72 h, respectively) from doxorubicin-induced LDH release. Doxorubicin significantly increased LDH release compared to untreated control myocytes ($p$ of < 0.001) at both times. Pretreatment of myocytes with 5-aminoorotic acid compared to untreated control myocytes did not significantly affect LDH release. Pretreatment of myocytes with 5-aminoorotic acid did not significantly affect the ability of dexrazoxane to protect myocytes from doxorubicin-induced LDH release at both 24 and 72 h.
Discussion

The effect of DHOase inhibition on dexrazoxane metabolism in the rat. Our previous studies on the metabolism of dexrazoxane in humans (Schroeder et al., 2003) and in the rat (Hasinoff and Aoyama, 1999b; 1999a; Schroeder and Hasinoff, 2005) showed that B, C and ADR-925 quickly appeared in the plasma after i.v. administration, a result which suggested that dexrazoxane, B and C, were enzymatically metabolized to ADR-925. We previously showed (Schroeder et al., 2002) using purified DHOase that 5-aminoorotic acid, which is a strong inhibitor of DHOase with a inhibition constant $K_i$ of 6 µM (Christopherson and Jones, 1980), inhibited the DHOase-catalyzed hydrolysis of C. The large and significant decreases in ADR-925 plasma $AUC_{5-120}$ values and plasma levels (Fig. 2b) in rats pretreated with 5-aminoorotic acid prior to dexrazoxane treatment, relative to rats not treated, indicated that DHOase played a major role in the overall metabolism of dexrazoxane to ADR-925.

The effect of DHOase inhibition on B/C metabolism to ADR-925. The initial plasma levels (apparent $C_{\text{max}}$) of ADR-925 were significantly decreased (Fig. 3b) when rats were pretreated with 5-aminoorotic acid prior to treatment with the B/C mixture, compared to rats treated with the B/C mixture alone. This result also supports the conclusion that DHOase is important in the metabolism of B and C. The rapid appearance of ADR-925 is likely due to a combination of DHOase-mediated enzymatic hydrolysis in tissue, chemical instability, and Ca$^{2+}$- and Mg$^{2+}$-promoted conversion to ADR-925 by these metal ions present plasma (Buss and Hasinoff, 1997; Schroeder et al., 2005). In rat blood hydrolysis of C occurs with a half-life of about 50 min (Schroeder et al., 2005). Thus the lack of effect on $C_{\text{max}}$ is most likely due to the chemical instability of B and C in plasma. Blood and plasma stability experiments showed that
while dexrazoxane is stable \((t_{1/2} > 300 \text{ min})\), C is not \((t_{1/2} 50 \text{ min})\) (Schroeder et al., 2005). This, at least in part, provides an explanation as to why no measurable inhibitory effect on the systemic appearance of ADR-925 was seen following treatment with B/C. Thus, presumably, once B/C are in the systemic circulation, the primary route of clearance would be through chemical hydrolysis. In the tissues, we anticipate that the primary route of clearance of B and C would be through enzymatic hydrolysis. This blood instability would also be a primary route of systemic elimination for the B/C mixture once these metabolites diffuse into the systemic circulation.

The effect on ADR-925 tissue levels of pretreatment with the DHOase inhibitor 5-aminoorotic acid prior to treatment with dexrazoxane or the B/C mixture. Because the liver contains both DHPase and DHOase which can act sequentially to form ADR-925, it is not unexpected that this tissue would contain ADR-925 (Table 1) (Schroeder and Hasinoff, 2005). However, the fact that ADR-925 is found in the heart (which does not contain DHPase (Hamajima et al., 1996)), either when treated with dexrazoxane or the B/C mixture, suggests that B and C formed in the liver are taken up by the heart and metabolized there. In support of this conclusion we showed that isolated neonatal rat myocytes were able to take up B, C and ADR-925 and displace iron from an intracellular fluorescence-quenched iron-calcein complex (Hasinoff et al., 2003b).

Pretreatment with 5-aminoorotic acid caused a large and significant decrease of ADR-925 heart and liver tissue levels in dexrazoxane-treated rats. The dexrazoxane results are consistent with the DHOase inhibitor 5-aminoorotic acid partially inhibiting the conversion of B and C to ADR-925, resulting in reduced heart and liver tissue levels. It is, however, unknown how quickly
5-aminoorotic acid is distributed in the heart and liver (and other) tissues that contain DHOase, and thus whether inhibitory levels of 5-aminoorotic acid were achieved in DHOase-containing tissues.

In the case where rats were treated with the B/C mixture, it is unclear why the heart and liver tissue levels of ADR-925 were not affected by the 5-aminoorotic acid pretreatment. The reason cannot be that 5-aminoorotic acid did not enter the tissue because we showed that 5-aminoorotic acid inhibited the metabolism of C in both myocyte and hepatocyte suspensions (Schroeder et al., 2005). From the data of Fig. 3b it can be seen that at 1.5 h, when the tissues were collected, the plasma levels of ADR-925 were not significantly different in either treatment group. Thus, it is perhaps not surprising that the tissue levels may also have achieved equal levels. Also neutral dexrazoxane may have been distributed into the tissues more completely and quickly than the anionic and presumably less permeable B and C. If this were the case the metabolism of B and C in heart and liver tissues would be less important.

**Pretreatment of myocytes with 5-aminoorotic acid did not significantly affect the ability of dexrazoxane to protect myocytes from doxorubicin-induced LDH release.** The results shown in Figs. 4a and 4b show that inhibiting the DHOase-mediated metabolism of B and C in myocytes did not affect the ability of dexrazoxane to protect myocytes from doxorubicin-induced damage. We know that 5-aminoorotic acid enters myocytes because we previously showed that 5-aminoorotic acid inhibits the hydrolysis of C and of dihydroorotic acid, the natural substrate of DHOase (Schroeder et al., 2005) in a myocyte suspension. The DMEM/F12 myocyte culture medium contains 1.0 mM Ca$^{2+}$ and 0.9 mM Mg$^{2+}$. Thus the lack of an effect may be because in the medium B and C also undergo a Ca$^{2+}$- and Mg$^{2+}$-promoted conversion ($t_{1/2} \sim 0.9$ h) to ADR-
925 (Buss and Hasinoff, 1997; Schroeder et al., 2005) and also a slow ($t_{1/2}$ 5.7 h) non-enzymatic base-catalyzed conversion to ADR-925 (Hasinoff, 1994a; 1994b). Thus, in myocytes at least, the DHOase enzymatic pathway that yields ADR-925 (Schroeder et al., 2002) may not be critical for the activation to ADR-925. A second, though less likely, reason that 5-aminoorotic acid does not reduce the ability of dexrazoxane to protect myocytes from doxorubicin-induced damage is that dexrazoxane is cardioprotective, not through its metal chelating hydrolysis product ADR-925, but is protective through its ability to inhibit topoisomerase II (Hasinoff and Herman, 2007). Dexrazoxane is a strong inhibitor ($IC_{50}$ 10 µM) of the catalytic activity of DNA topoisomerase II (Hasinoff et al., 1995), an enzyme which catalyzes DNA strand passage events (Fortune and Osheroff, 2000). While it is possible that the cardioprotective and extravasation protective effects of dexrazoxane may be due to its ability to inhibit topoisomerase II, it is difficult to envision a mechanism by which this would occur as these tissues are not rapidly proliferating and contain only low levels of topoisomerase IIα.

The pharmacokinetics of the metabolism of dexrazoxane to B and C, and then to ADR-925 in the rat model allometrically scales well to that seen in humans (Schroeder et al., 2003; Schroeder and Hasinoff, 2005), and thus, it would appear that the kinetics of the metabolism in the two species is similar. In conclusion these studies showed that the metabolism of dexrazoxane to its presumably active metal chelating form ADR-925 in the rat was strongly inhibited by pretreatment with the DHOase inhibitor 5-aminoorotic acid. The metabolism of the one-ring open metabolites B and C were likewise inhibited in the rat by 5-aminoorotic acid pretreatment. The tissue levels of ADR-925 in the heart and the liver after dexrazoxane treatment were both strongly affected by pretreatment with 5-aminoorotic acid. Therefore it can be concluded that the
enzyme DHOase, which is present in both the heart and liver, is largely responsible for the metabolism of the B and C metabolites of dexrazoxane. Thus these results provide a mechanistic basis for the antioxidant cardioprotective activity of dexrazoxane.
References


enzymes stop being nice. Prog Nucleic Acid Res Mol Biol 64:221-253.


Footnotes

a) 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.B.H. P.E.S. was supported by a Manitoba Health Research Council studentship and Canadian Institutes of Health Research studentship.

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c)
Figure Captions

FIG. 1. Scheme for the enzymatic 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. EDTA and the DHOase inhibitor 5-aminoorotic acid are shown for comparison.

FIG. 2. a, average plasma concentrations of dexrazoxane (●) and ADR-925 (♦) for rats pretreated with 5-aminoorotic acid (20 mg/kg) 5 min prior to treatment with dexrazoxane (40 mg/kg) (n = 3) compared to average plasma concentrations of dexrazoxane (○) and ADR-925 (◇) for rats pretreated with dexrazoxane (40 mg/kg) alone (n = 6). b, average plasma concentrations of B (▲) and C (▼) for rats pretreated with 5-aminoorotic acid (20 mg/kg) 5 min prior to treatment with dexrazoxane (40 mg/kg) compared to average plasma concentrations of B (Δ) and C (∇) for rats treated with dexrazoxane (40 mg/kg) alone.

FIG. 3. a, average plasma concentrations of B (▲) and C (▼) for rats pretreated with 5-aminoorotic acid (20 mg/kg) 5 min prior to treatment with a mixture of B and C (20 mg/kg) (n = 3) compared to average plasma concentrations of B (Δ) and C (∇) for rats pretreated with a mixture of B and C (20 mg/kg) alone. Data was only reported only if all three rats had B and C values above the limit of quantitation. In the case of the 5-aminoorotic acid treated rats levels of B and C were below the limit of quantitation for two of three rats at times greater than 45 and 20 min respectively. b, average plasma concentrations of ADR-925 (♦) for rats treated with 5-
aminoorotic acid (20 mg/kg) 5 min prior to treatment with a mixture of B and C (20 mg/kg) compared to average plasma concentrations of ADR-925 (◊) for rats treated with a mixture of B and C (20 mg/kg) alone (n = 6).

FIG. 4. a, effect of pretreating neonatal rat cardiac myocytes with 5-aminoorotic acid on dexrazoxane protection of doxorubicin-induced LDH release at 24 h after treatment. The myocytes were pretreated with 5-aminoorotic acid for 1 h, then treated with dexrazoxane for 3 h, and then treated with doxorubicin for 3 h. The myocytes were then washed with media containing the concentrations of 5-aminoorotic acid and dexrazoxane indicated. The micromolar concentrations of the drugs are given below each bar. 5-aminoorotic acid pretreatment had no significant effect on dexrazoxane-mediated protection against doxorubicin-induced LDH release at any of the 3 dexrazoxane concentrations tested. b, as in a above except that LDH release was measured at 72 h. 5-aminoorotic acid pretreatment had no significant effect on dexrazoxane-mediated protection against doxorubicin-induced LDH. NS is not significant and < 0.001 is the p value.
TABLE 1

The effect of pretreatment with the DHOase inhibitor 5-aminoorotic acid on tissue levels of ADR-925 in rats treated with either dexrazoxane or with a mixture of B and C

The average tissue levels of ADR-925 were measured per kg of wet tissue harvested from 3 - 4 rats 2 - 2.5 h after either dexrazoxane was administered (40 mg/kg), or from 3 rats 1.5 h after the mixture of B and C (20 mg/kg) was administered. The limit of quantitation was 2 µmol/kg.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Treatment</th>
<th>dexrazoxane</th>
<th>dexrazoxane + 5-aminoorotic acid</th>
<th>B/C mixture</th>
<th>B/C mixture + 5-aminoorotic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ADR-925 (µmol/kg)</td>
<td>ADR-925 (µmol/kg)</td>
<td>ADR-925 (µmol/kg)</td>
<td>ADR-925 (µmol/kg)</td>
</tr>
<tr>
<td>heart</td>
<td></td>
<td>63 ± 5</td>
<td>12 ± 2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27 ± 4</td>
<td>29 ± 1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>liver</td>
<td></td>
<td>116 ± 19</td>
<td>12 ± 1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>40 ± 9</td>
<td>46 ± 4&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Significantly different than its untreated control ($p < 0.001$).

<sup>b</sup> Significantly different than its untreated control ($p = 0.005$).

<sup>c</sup> Not significantly different than its untreated control ($p = 0.7$).

<sup>d</sup> Not significantly different than its untreated control ($p = 0.5$).
Fig. 1

Dexrazoxane → DHPase → B → DHOase → ADR-925

DHPase → C → DHOase

5-aminooorotate → EDTA
Fig. 3

(a) B or C concentration (μM) vs. Time (min)

- ▲ B
- ▼ C
- ▲ B + 5-aminoorotic acid
- ▼ C + 5-aminoorotic acid

(b) ADR-925 concentration (μM) vs. Time (min)

- ◇ ADR-925
- ■ ADR925 + 5-aminoorotic acid