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 N,N’-[1S]-1-methyl-1,2-ethanediyl[bis(N-[2-amino-2-oxoethyl])glycine (ADR-925). Dexrazoxane undergoes an initial metabolism to its two one-ring open intermediates N-[2-amino-2-oxoethyl]-N’-[1S]-2-(3,5-dioxo-1-piperazinyl)-1-methyl[glycine (B) and N-[2-amino-2-oxoethyl]-N’-[2S]-2-(3,5-dioxo-1-piperazinyl)propyl]glycine (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 dihydroorotidase and the second by dihydroorotate (DHOase), but not vice versa. To determine whether DHOase was important in the metabolism of dexrazoxane, its metabolite and that of B and C to ADR-925 were 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 significantly 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 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, although 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). To determine the importance of DHOase-mediated metabolism of dexrazoxane, the metabolism of dexrazoxane and B and C was studied in rats that had been pretreated with the potent DHOase inhibitor 5-aminoorotic acid (Christopherson and Jones, 1980).

Materials and Methods

Materials. Dexrazoxane hydrochloride and ADR-925 were gifts from Adria Laboratories (Columbus, OH) and were used as supplied. High-pressure liquid chromatography (HPLC)-grade methanol was from Fisher (Nepean, ON, Canada). Catalase (C-30), Tris, Chelex resin, 1-heptanesulfonic acid, and 1-oc-tanesulfonic acid were from Sigma (St. Louis, MO). Calcine was from Invitrogen (Carlsbad, CA). Pharmacokinetic analyses were carried out using WinNonlin 4.0 (Pharsight, Mountain View, CA) using an empirical, noncompartmental method for an i.v. bolus infusion or a one-compartment method as appropriate. The effect of DHOase inhibition on pharmacokinetic parameters was studied in rats that had been pretreated with the potent DHOase inhibitor 5-aminoorotic acid (Christopherson and Jones, 1980).

Analysis of B, C, and ADR-925. The HPLC analysis of dexrazoxane, B, C, and ADR-925 was performed using a reversed-phase C18 column (detection wavelength 205 nm) that had been described (Schroeder et al., 2002, 2003; Schroeder and Hasinoff, 2005). ADR-925 was determined on a BMG (Durham, NC) Fluostar Galaxy 96-well fluorescence plate reader (λex 485 nm, λem 520 nm) using the calcine 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 before 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, Basel, Switzerland) for 5 min. The homogenate was centrifuged at 0°C for 2 h at 18,000g 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- to 3-day-old Sprague-Dawley rats as we have described (Hasinoff et al., 2003a, 2007; Schroeder et al., 2005). Briefly, minced ventricles were serially digested with collagenase and trypsin in phosphate-buffered saline/1% (w/v) glucose at 37°C in the presence of DNase and preplated for 1 h in large Petri dishes to deplete fibroblasts that 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 0 in 24-well plastic culture dishes (5 × 10^4 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 2. 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 days after treatment, and the samples were frozen at −80°C and analyzed within 1 week. After the last supernatant sample was taken, the myocytes were lysed with 250 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 (Sunnyvale, 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

Effect of Pretreating Rats with 5-Aminoorotic Acid before 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 Fig. 2, A and B. 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 noncompartmental area-under-the-curve (AUC_{5–120}) analysis of the concentration of dexrazoxane or ADR-925 from the first to the last time point (5–120 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 before 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 with rats treated with dexrazoxane alone (15,100 ± 970 μM · min compared with 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 concentration were obtained directly from the observed data at 5 min.

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 decreased. 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 with 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 nonmetabolic 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). Because of 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 before 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 with rats treated with dexrazoxane alone (940 ± 240 μM · min compared with 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.

Effect of Pretreatment with 5-Aminoorotic Acid before 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. 3, A and B. 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_{max} for B (Fig. 3A) for rats pretreated with 5-aminoorotic acid before treatment with a B/C mixture rats was not significantly different (p = 0.9) compared with rats treated with a B/C mixture alone (427 ± 111 μM compared with 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 treated with 5-aminoorotic acid before treatment with a B/C mixture was not significantly different (p = 0.6) compared with rats treated with the B/C mixture alone (5.8 ± 2.9 compared with 4.8 ± 1.0 min, respectively). The fact that the pharmacokinetic parameters C_{max} and the half-life for elimination of B and C were unchanged when rats were pretreated with 5-aminoorotic acid before treatment with the B/C mixture compared with 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_{max} value
was not the result of 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 6.5 ± 1.4 can be compared with 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 before treatment with a B/C mixture was significantly decreased (p = 0.018) compared with rats treated with a B/C mixture alone (49 ± 2 μM compared with 137 ± 38 μM, respectively). Whereas the half-life for ADR-925 for rats pretreated with 5-aminoorotic acid before treatment with a B/C mixture rats did increase, the increase was not quite significant (p = 0.053) compared with rats treated with a B/C mixture alone (25 ± 9 min compared with 9 ± 4 min, respectively). Also, the plasma level of ADR-925 in either treatment group was not significantly different at 1.5 h (p = 0.4).

Effect on ADR-925 Tissue Levels of Pretreatment with the DHOase Inhibitor 5-Aminooorotic Acid before Treatment with Dexrazoxane or the B/C Mixture. ADR-925 levels in the 18,000g supernatant fraction of heart and liver tissue homogenates of rats treated 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 = 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 with total 14C-labeled dexrazoxane levels determined at 5 min of 320 and 640 μmol/kg in heart and liver, respectively, for mice administered 100 mg/kg dexrazoxane (Mhatre et al., 1982).

Effect of 5-Aminooorotic 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 whether inhibition of DHOase reduced the ability of dexrazoxane to protect myocytes from doxorubicin-induced damage. As shown in Fig. 4, A and B, 5, 20, and 100 μM dexrazoxane significantly reduced LDH release in a concentration-dependent manner from myocytes (p = 0.021, 0.004, and <0.001 at 24 h, and p = 0.008, 0.003, and <0.001 at 72 h, respectively) from doxorubicin-induced LDH release. Doxorubicin significantly increased LDH release compared with untreated control myocytes (p < 0.001) at both times. Pretreatment of myocytes with 5-aminoorotic acid compared with 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

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, 1999a,b; 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 an 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_{0-120} values and plasma levels (Fig. 2B) in rats pretreated with 5-aminoorotic acid before dexrazoxane treatment, relative to rats not treated, indicated that DHOase played a major role in the overall metabolism of dexrazoxane to ADR-925.
The rapid appearance of ADR-925 is likely because of a combination of DHOase-mediated enzymatic hydrolysis in tissue, chemical instability, and Ca²⁺- and Mg²⁺-promoted conversion to ADR-925 by these metal ions present in 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 max is most likely caused by the chemical instability of B and C in plasma. Blood and plasma stability experiments showed that whereas dexrazoxane is stable (t 1/2 > 300 min), C is not (t 1/2 50 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 is 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.

**Effect on ADR-925 Tissue Levels of Pretreatment with the DHOase Inhibitor 5-Aminoorotic Acid before Treatment with Dexrazoxane or the B/C Mixture.** Because the liver contains both DHase 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 DHase) (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. Neutral dexrazoxane also may have been distributed into the tissues more completely and quickly than the anionic and presumably less permeable B.


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