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ORIGINAL ARTICLE

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The doxorubicin-cardioprotective drug dexrazoxane undergoes metabolism in the rat to its metal ion-chelating form ADR-925

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Abstract *Purpose*: Dexrazoxane 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 ADR-925. The metabolism of dexrazoxane (ICRF-187) to its one-ring open hydrolysis products and its rings-opened metal-chelating product ADR-925 was determined in a rat model in order to identify the mechanism by which dexrazoxane acts. *Methods*: A new fluorescence detection flow injection assay utilizing the metal-chelating dye calcein was developed to detect ADR-925 in blood plasma. Dexrazoxane and its one-ring open metabolites were determined by HPLC. *Results*: ADR-925 was detected within 5 min of i.v. administration of dexrazoxane to rats, suggesting that dexrazoxane is rapidly metabolized in vivo. The plasma concentrations of ADR-925 exceeded those of both one-ring open intermediates at 30 min and those of dexrazoxane by 80 min and reached a maximum at 80 min, and then slowly decreased. *Conclusions*: 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 results indicate that the one-ring open dexrazoxane intermediates are enzymatically metabolized to ADR-925 and provide a pharmacodynamic basis for the antioxidant cardioprotective activity of dexrazoxane.

Keywords Dexrazoxane · Doxorubicin · Antioxidant · Iron · ADR-925

Abbreviations *DHPase:* dihydropyrimidine amidohydrolase or dihydropyrimidinase · *HPLC:* high pressure liquid chromatography · K_f: equilibrium formation constant · *SEM:* standard error of the mean · *Tris:* Tris(hydroxymethyl)aminomethane · $t_{1/2}$: half-time · t_r: retention time

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Introduction

Dexrazoxane (ICRF-187, Zinecard, Fig. 1) is clinically used to reduce doxorubicin-induced cardiotoxicity [15, 20, 22, 30, 31]. Dexrazoxane likely acts as a cardioprotective agent by diffusing into the cell and hydrolyzing to its one-ring open intermediates *B* and *C*, and then to its fully rings-opened metal ion-chelating form ADR-925 (Fig. 1), which has a structure similar to that of EDTA. ADR-925 may be acting by chelating free iron or displacing iron bound to the iron-doxorubicin complex [1, 7, 15, 20], thus reducing iron-based oxygen radical formation. Considerable evidence has accumulated that the cardiotoxicity of doxorubicin is due to iron-based oxygen radical-induced oxidative stress on the heart muscle [27, 28] which is relatively low in antioxidant enzymes. We and others have shown that the Fe^{3+}-doxorubicin complex is reductively activated and that it redox cycles to produce the extremely damaging hydroxyl radical in a Fenton-type reaction [8, 10, 27, 28].

Our previous spectrophotometric and HPLC studies [12, 13] have shown that under physiological conditions dexrazoxane is only slowly hydrolyzed to *B* and *C* ($t_{1/2}$ 9.3 h at 37°C and pH 7.4), and the final hydrolysis product ADR-925 ($t_{1/2}$ 23 h) according to the kinetic scheme shown in Fig. 1. Given the slow rate at which hydrolysis-activation occurs in vitro it is unclear how sufficient amounts of ADR-925 could be present in heart tissue to chelate iron and prevent oxygen radical damage before dexrazoxane is eliminated (elimination $t_{1/2}$ 4.2 ± 2.9 h in humans) [24]. We have also shown [18] that dexrazoxane undergoes an enzymatic ring-opening hydrolysis in the presence of the 105,000 *g* soluble supernatant fraction of homogenates of porcine liver and kidney, but not of heart. DHPase (EC 3.5.2.2), present in the liver and kidney supernatant, has been shown to be responsible for this enzymatic hydrolysis [11, 14, 18,

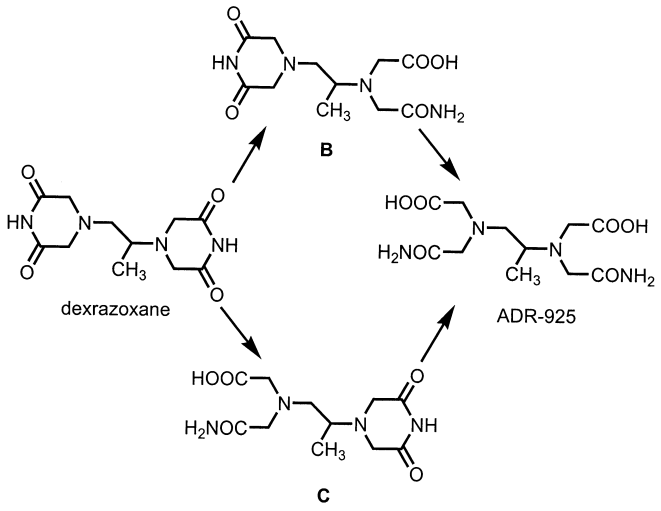

Fig. 1 Reaction scheme for the hydrolysis of dexrazoxane (ICRF-187) to intermediates **B** and **C**, and its strongly metal ion-chelating form ADR-925

19]. These studies have also shown that while dexrazoxane is a substrate for DHPase, its one-ring open hydrolysis products *B* and *C* are not.

The approval of dexrazoxane by the American Food and Drug Administration in May 1995 required a follow-up phase IV commitment to characterize the pharmacokinetics of the metabolites. To our knowledge there has been no published report concerning the pharmacokinetics of the metabolites other than our report on the pharmacokinetics of the conversion of dexrazoxane to *B* and *C* in the rat [16]. Because ADR-925 is likely the pharmacologically active metabolite of dexrazoxane, and because ADR-925 has not previously been determined in an in vivo model, it was decided to develop a sensitive assay to measure its presence and levels in plasma so that a greater understanding of the metabolism and cardioprotective effect of dexrazoxane could be obtained.

Materials and methods

Drugs and chemicals

Dexrazoxane hydrochloride and ADR-925 were gifts from Adria Laboratories (Columbus, Ohio). Calcein ("high purity") was from Molecular Probes (Eugene, Ore.). HPLC-grade methanol and cobalt chloride hexahydrate were from Fisher (Nepean, Canada) and the 1-heptanesulfonic acid sodium salt was from Sigma (St. Louis, Mo.).

HPLC separation of dexrazoxane, its one-ring open intermediates B and C, and ADR-925

The HPLC analysis of dexrazoxane and its one-ring open intermediates using a reversed-phase C_{18} column has been described previously [11, 12, 13, 16, 19]. The HPLC calibration plots using integrated peak areas (10 to 500 μ*M* dexrazoxane, 2 to 50 μ*M* *B* and *C*, and 20 to 100 μ*M* ADR-925 in blank rat plasma) were carried out as previously described [12, 13, 16, 19]. The calibration plots ($n = 6$–8) for dexrazoxane, *B*, *C*, and ADR-925 were linear (r^2 0.998, 0.996, 0.994, and 0.991, respectively). Absolute recoveries from spiked plasma ranged from 86% to 99% for 50 to 1000 μ*M* dexrazoxane, and from 80% to 98% for 10 to 100 μ*M* ADR-925. The limit of quantitation of dexrazoxane, *B*, and *C* in plasma was 0.5, 1, and 1 μ*M* (estimated from three times the limit of detection), respectively. Calibration plots were run before each set of samples. Between-day variation in the slopes of the calibration plots were less than 9%, 2%, and 9% for dexrazoxane, *B*, and *C*, respectively. Accuracy, which depended upon the concentration of the analyte, was estimated to be generally better than 4%.

Fluorescence flow injection analysis of ADR-925

Because of the low molar absorptivity of ADR-925 [9], a highly sensitive flow injection method of analysis for ADR-925 was developed using the fluorescent metal ion-chelating indicator calcein (which does not bind calcium or magnesium at neutral pH). Calcein fluorescence is strongly quenched by Co^{2+} with which it forms a strong 1:1 (K_f $10^{6.8}$ M^{-1}) complex [26]. The determination involved a back titration using Co^{2+}. In this assay the free Co^{2+} not bound to ADR-925 in plasma was reacted with calcein. The free calcein remaining was then determined fluorometrically by flow injection analysis. The calcein assay can be described by the following reactions with their stoichiometric coefficients:

$$1\ Co^{2+} + x\text{ADR} - 925 \rightarrow x Co^{2+} - \text{ADR} - 925 + (1-x)Co^{2+} \quad (1)$$

$$1\ \text{calcein} + (1-x)Co^{2+} \rightarrow (1-x)Co^{2+} - \text{calcein} + x\ \text{calcein} \quad (2)$$

One mol equivalent of Co^{2+} was first reacted (reaction 1) with an unknown amount, x mol, of ADR-925 (where $x < 1$). The $1-x$ mol of free Co^{2+} remaining was then reacted (reaction 2) with 1 mol of calcein to produce x mol of calcein, the fluorescence of which was measured by flow injection analysis. Thus, the unknown amount of ADR-925, x mol, was directly proportional to the fluorescence of x mol of calcein remaining in the reaction mixture after the Co^{2+} and calcein were serially added.

A stock solution of 50 μl of Co^{2+} (20 μ*M* in 50 m*M* Tris buffer, pH 5.5) was added to 10 μl plasma reconstituted to its original volume in water for 5 min to allow the reaction of ADR-925 with Co^{2+} to proceed to completion. In preliminary experiments we showed that under our assay conditions using ADR-925-spiked plasma, more than 70% of the ADR-925 reacted with Co^{2+} in 30 s and by 3 min 100% had reacted. Because ADR-925 in plasma is likely complexed with Ca^{2+} and Mg^{2+} (equilibrium formation constants K_f of $10^{6.9}$ and $10^{5.1}$ M^{-1}, respectively [25]), these results indicate that Co^{2+} rapidly displaced these metal ions from the ADR-925 in the assay mixture. Calcein (10 μl of 100 μ*M* stock solution in water) was then added, and after 30 s 920 μl Tris buffer (50 m*M*, pH 7.5) was added such that the final Co^{2+} concentration in the assay mixture was 1.0 μ*M* and the final diluted ADR-925 concentration in the assay mixture was in the working range of 0.2 to 0.9 μ*M*. If the ADR-925 concentration exceeded 0.9 μ*M*, as determined from the fluorescence signal, it was further diluted to bring it into the working range. Thus, the concentration of ADR-925 in the assay mixture was typically about 100-fold less than that in the plasma. The high sensitivity of the assay allowed possible contaminants in the plasma to be diluted to levels where they did not measurably interfere. In order to reduce interfering heavy metal contaminants the Tris buffer and calcein stock solutions were stirred with the chelating resin Chelex (Sigma, 1 g/100 ml) for 24 h prior to use and then stored over Chelex. Under these conditions the calcein stock solution was stable for a least 3 months. Care was also taken to ensure that the plasma samples only came in contact with plastic.

The sample for analysis ($n = 3$) was injected into a Rheodyne (Cotati, Calif.) injector with a 20-μl Upchurch (Oak Harbor, Wash.) PEEK sample loop using a Teflon needle (Rheodyne).

PEEK tubing (Upchurch) was used between the injector and the Shimadzu (Columbia, Md.) RF-551 fluorescence detector (λ_{ex} 496 nm, λ_{em} 517 nm). Aqueous Na_2EDTA (20 μ*M*, 1 ml/min) was used as the mobile phase to complex metal ions present in the flow system. This concentration of EDTA was shown not to affect the assay because the brief time (a few seconds) that the injected sample was in contact with EDTA was too short to allow any significant reaction to occur with the Co^{2+}-calcein complex. The calcein assay was shown to selectively detect only ADR-925 in the presence of a mixture of dexrazoxane, *B*, *C* and ADR-925. This was tested in preliminary experiments by preparing a sample that contained 100 μ*M* ADR-925, 800 μ*M* dexrazoxane, and 305 μ*M* *B* and *C*. The slopes of calibration plots ($n = 5$) using this sample and one containing 100 μ*M* ADR-925 alone were shown by *t*-test to be not significantly different ($P > 0.5$). The added Co^{2+} was shown not to detectably promote the ring opening of *B* and *C* [2] over 20 min. Plasma components were shown not to interfere with the assay as the slopes of the calibration plots in water and reconstituted plasma were not significantly different. Over the time the assay was conducted both the Co^{2+}-ADR-925 and Co^{2+}-calcein complexes were stable. Free calcein was also shown not to be able to displace ADR-925 from Co^{2+}-ADR-925 over the time the assay was conducted. Calibration plots of integrated peak areas ($n = 6$) in the range 0 to 1.0 μ*M* ADR-925 were repeated every 3 h during analysis and were found to be highly linear ($r^2 > 0.994$). Between-day variation in the slope of the calibration plots was less than 1%. The limit of quantitation of ADR-925 in plasma was estimated to be 1 μ*M*.

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 anesthetized i.p. with a combination of ketamine (90 mg/ml) and xylazine (10 mg/kg) and the left jugular vein was cannulated. The dexrazoxane hydrochloride solution (freshly prepared, 10 mg/ml in sterile saline) was administered at a dose of 40 mg/kg as an i.v. infusion (1 ml/min) into the tail vein. Blood samples (0.5 ml) were removed from the left jugular vein from a group of six rats before dexrazoxane infusion and at 5, 30, 60, 120, and 180 min after dexrazoxane infusion, and at 60, 80, 100, 120, and 140 min after dexrazoxane infusion from a second group of six rats. The blood removed was replaced with an equal volume of sterile saline. The animal protocol was approved by the University of Manitoba Animal Care Committee. The blood samples were added to heparin (10 μl of 1000 U/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 –80°C to prevent further hydrolysis [12]. 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 N_2. 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 in 10 m*M* HCl/500 μ*M* Na_2EDTA to their original volume just prior to analysis. The dexrazoxane hydrochloride that was injected was found to contain less than 0.05% *B*, 0.02% *C*, and 0.001% ADR-925 (mol%). Because only very small quantities (nanomoles) of *B* and *C* were isolatable after the HPLC separation, pharmacokinetic or plasma studies could not be carried out on these intermediates.

Results

The results shown in Fig. 2 for dexrazoxane, *B*, and *C* are nearly identical to those we previously obtained for rats likewise dosed at 40 mg/kg of dexrazoxane hydrochloride [16]. The intermediates *B* and *C* appeared in the plasma very quickly after dexrazoxane administration and then after a rapid decrease over 30 min changed very little. It can also be seen from the data in Fig. 2 that the concentration of *B* was always larger than that of *C* (by a nearly constant 4.0 ± 0.3-fold, range 3.1–5.1) as we have previously observed [16]. ADR-925 also appeared in the plasma very quickly, increased with time, and by 30 min exceeded the concentration of *B* and *C*. It peaked at about 80 min, and exceeded the concentration of dexrazoxane at that time, and then slowly decreased with time to 180 min. The sum of the concentrations of dexrazoxane and its three metabolites (Fig. 2) decreased rapidly over the first 60 min and then slowly decreased with time.

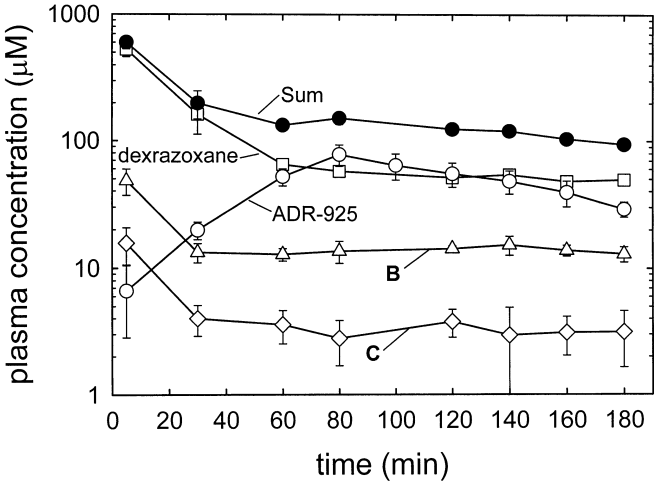

Fig. 2 Average rat plasma concentrations of dexrazoxane (*open squares*), *B* (*open triangles*), *C* (*open diamonds*), ADR-925 (*open circles*), and *Sum* (the sum of the concentrations of dexrazoxane, *B*, *C* and ADR-925) (*closed circles*) after i.v. dosing with 40 mg/kg of dexrazoxane hydrochloride. *Error bars* represent the SEMs from determinations on 6–12 rats, except for the 120-min and 140-min data which are from 3 rats. Where the error bars are not shown they are smaller than the symbols

Because the calcein assay measures only free ADR-925, experiments were done to determine the relative amount of free ADR-925 in plasma. In preliminary experiments it was shown that stirring Chelex with preformed Fe^{3+}-ADR-925 at pH 5.5 resulted in complete displacement of Fe^{3+} from its complex with ADR-925 within 20 min. The amount of free ADR-925 in plasma samples collected at 5, 60, and 180 min time did not show a measurable increase in ADR-925 levels after Chelex treatment, which indicated that the relative amount of Fe^{3+}-ADR-925 in plasma was too small to measure.

In order to test the possibility that the calcein assay might detect other dexrazoxane-derived chelating metabolites, an experiment was carried out on a single rat in which a single large blood sample, sufficient to measure the ADR-925 level both by HPLC and by the calcein assay, was taken at 120 min after dexrazoxane infusion. The plasma concentration of ADR-925 was measured to be 52 and 49 μ*M* using the HPLC and

calcein assays, respectively. The close agreement of these two values indicates that there were no other calcein assay-detectable dexrazoxane-derived chelating metabolites formed in vivo.

Discussion

ADR-925, the rings-opened hydrolysis product of dexrazoxane, is a strong chelator of Fe^{2+} ($K_f 10^{10.0}$ M^{-1}) [25] and of Fe^{3+} ($K_f 10^{18.2}$ M^{-1}) [4]. We have previously shown that ADR-925 is able to quickly ($t_{1/2}$ 1.7 min) and completely remove Fe^{3+} from its complex with doxorubicin [1, 7, 15, 20] and thus prevents iron-based doxorubicin-mediated free-radical oxidative stress on the heart muscle [6, 27, 28]. The free iron or non-transferrin-bound iron concentration is normally very low (<0.4 μM) in plasma [5]. The ADR-925 plasma levels that we measured, even 5 min after dexrazoxane infusion, greatly exceeded this value and thus all of the plasma free iron would be complexed to ADR-925. This small amount would not be detectable in the presence of free ADR-925 at levels that we typically measured. Thus, the amount, if any, of iron transported from the tissue to the plasma due to dexrazoxane treatment was too small to determine.

The peak plasma concentration of dexrazoxane of 530 ± 70 μM shown in Fig. 2 is comparable to that seen in humans of 340 ± 80 μM at a dose of 600 mg/m^2 [24]. The rapid appearance of ADR-925 in plasma suggests that either *B* or *C*, or both, were rapidly metabolized to ADR-925. The relative lack of a change in the level of ADR-925 after 80 min suggests that this metabolite was in a steady state.

We have previously shown that rat plasma [17] does not significantly promote the hydrolysis of dexrazoxane over 2.5 h. We have also previously shown that DHPase, which is present in the liver and the kidney, can efficiently hydrolyze dexrazoxane [11, 14, 17, 18, 19] 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 the one-ring open intermediates *B* and *C* into ADR-925 [18]. Thus, the rapid appearance of ADR-925 in the plasma (Fig. 2) suggests that there is another unknown enzyme that metabolically converts *B* and *C* into ADR-925.

In a study [29] utilizing racemic ^{14}C-razoxane at 120 mg/kg i.v. in the rat it has been found that intact razoxane accounts for 46% of the total radioactivity at 2 h in the plasma, which suggests that a rapid metabolism of razoxane has occurred. For comparison, the results in Fig. 2 show that dexrazoxane accounted for 41% of the total of the concentrations of dexrazoxane and its three metabolites. The good agreement between these two values strongly suggests that we have accounted for all of the major circulating metabolites of dexrazoxane.

Dexrazoxane undergoes a slow base-catalyzed hydrolysis (Fig. 1) in vitro to *B* and *C* ($t_{1/2}$ 9.3 h) and then to ADR-925 ($t_{1/2}$ 23 h) under physiological conditions (37°C and pH 7.4) [12, 13]. Given the slow rate of the in vitro hydrolysis of dexrazoxane under physiological conditions, little of the *B*, *C*, or ADR-925 seen in vivo could have been formed from base-catalyzed hydrolysis [12, 13], and therefore must have resulted from rapid enzymatic metabolism. Previous animal studies have shown that the timing of dexrazoxane dosing is critical for it to exert its protective effects [21, 23]. Maximal protective effects are seen when dexrazoxane is given between 3 h before and 3 h after daunorubicin treatment of Syrian golden hamsters [23]. Likewise, doxorubicin-induced cardiomyopathy is less severe in beagle dogs receiving dexrazoxane simultaneously than those receiving dexrazoxane 2 h after doxorubicin [21]. These results were the basis for the recommendation that in a clinical setting after completing the infusion of dexrazoxane, and prior to a total elapsed time of 30 min (from the beginning of the dexrazoxane infusion), the intravenous injection of doxorubicin should be given. The results of this study showing that ADR-925 rapidly appeared in the plasma after dexrazoxane administration, now provide a pharmacodynamic basis for this dosing schedule.

This study did not directly address how dexrazoxane exerts its cardioprotective effects in the heart. We have previously shown that while rat primary hepatocytes [19] and supernatants of liver and kidney homogenates do hydrolyze dexrazoxane, that of heart does not [18]. The fact that dexrazoxane is not metabolized in plasma [17] indicates that dexrazoxane is metabolized extravascularly. Dexrazoxane, being a small neutral molecule and able to permeate cells [3], should easily be taken up by tissues. The rapid appearance of *B* and *C* in the plasma (Fig. 2) indicates that these charged metabolites were rapidly released from tissue into the blood. Thus, it follows that even if dexrazoxane is not metabolized in the heart, the metabolites may be taken up by heart tissue and chelate free iron and thus prevent the formation of the Fe^{3+}-doxorubicin complex. In summary, the rapid appearance of ADR-925 in the plasma (Fig. 2) indicated that *B* and *C* were metabolized to ADR-925. The enzyme or enzymes, and their site, that are responsible for the conversion of *B* and *C* into ADR-925 are unknown. We are continuing our studies to identify these enzymes so that the metabolic activation of dexrazoxane can be fully described.

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