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ABSTRACT:

The enzyme kinetics of the hydrolysis of the one-ring open metabolites of the antioxidant cardioprotective agent dexrazoxane [ICRF-187; (+)-1,2-bis(3,5-dioxopiperazin-1-yl)propane] to its active metal ion binding form ADR-925 [N,N’-[(1S)-1-methyl-1,2-ethanediyl]bis[N-(2-amino-2-oxoethyl)glycine] by dihydroorotase (DHOase) has been investigated by high-performance liquid chromatography (HPLC). A spectrophotometric detection HPLC assay for dihydroorotate was also developed. Dexrazoxane is clinically used to reduce the iron-based oxygen free radical-mediated cardiotoxicity of the anticancer drug doxorubicin. DHOase was found to catalyze the ring opening of the metabolites with an apparent $K_m$ that was 11- and 27-fold greater than its natural substrate dihydroorotate. However, the apparent $K_m$ for the metabolites was 240- and 550-fold larger than for dihydroorotate. This report is the first that DHOase might be involved in the metabolism of a drug. Furosemide inhibited DHOase, but the neutral 4-chlorobenzenesulfonamide did not. Because dihydroorotate, the one-ring open metabolites, and furosemide all have a carboxylate group, it was concluded that a negative charge on the substrate strengthened binding to the positively charged active site. The presence of DHOase in the heart may explain the cardioprotective effect of dexrazoxane. Thus, dihydropyrimidinase and DHOase acting in succession on dexrazoxane and its metabolites to form ADR-925 provide a mechanism by which dexrazoxane is activated to exert its cardioprotective effects. The ADR-925 thus formed may either remove iron from the iron-doxorubicin complex, or bind free iron, thus preventing oxygen radical formation.

Dexrazoxane [ICRF-187; (+)-1,2-bis(3,5-dioxopiperazin-1-yl)propane; Zinecard; Fig. 1] is clinically used to reduce doxorubicin-induced cardiotoxicity (Hasinoff, 1998; Hasinoff et al., 1998). There is now considerable evidence to indicate that this toxicity may be due to iron-dependent oxygen free radical formation (Malisza and Hasinoff, 1995; Meyers, 1998) on the relatively unprotected cardiac muscle (Halliwell and Gutteridge, 1989). Dexrazoxane can be considered a prodrug analog of EDTA that is activated upon hydrolysis to its one-ring open intermediates (Hasinoff, 1990, 1994a,b, 1998; Hasinoff et al., 1998). Neutral dexrazoxane is permeable to cells (Dawson, 1975). ADR-925 (Fig. 1) may either remove iron from the iron-doxorubicin complex (Buss and Hasinoff, 1993) or bind free iron, thus preventing oxygen radical formation.

Our previous spectrophotometric and HPLC$^1$ studies (Hasinoff, 1994a,b) showed that under physiological conditions dexrazoxane is only slowly hydrolyzed to B and C ($t_{1/2}$ of 9.3 h at 37°C and pH 7.4) and the final hydrolysis product ADR-925 ($t_{1/2}$ of 23 h), according to the kinetic scheme shown in Fig. 1a. Given the slow rate at which 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 ($t_{1/2}$ of 4.2 ± 2.9 h in humans) (Hochster et al., 1992).

We have previously shown that B and C are rapidly formed from dexrazoxane in a primary rat hepatocyte suspension (Hasinoff et al., 1994) and in vivo using a rat model (Hasinoff and Aoyama, 1999). These results were both consistent with dexrazoxane being metabolized by dihydropyrimidine amidohydrolase EC 3.5.2.2 (DHPase). Furthermore, we have shown that pure DHPase enzymatically hydrolyzed dexrazoxane to B and C but did not enzymatically hydrolyze B and C to ADR-925 (Hasinoff et al., 1991; Hasinoff, 1993). The fact that dexrazoxane was not stoichiometrically converted into B and C in our hepatocyte suspension study (Hasinoff et al., 1994) suggested that there was a second unidentified enzyme that acted on B and C. Our previous pharmacokinetic study in the rat showed that B and C only reached relatively low steady-state levels (Hasinoff and Aoyama, 1999). Our preliminary pharmacokinetic studies in the rat (Schroeder and Hasinoff, 2001) and humans (Schroeder et al., 2002) that showed that dexrazoxane is rapidly metabolized to ADR-925 in vivo also suggested that B and C were being converted to ADR-925 enzymatically.

The first three steps of de novo synthesis of pyrimidines, uracil, and thymine are carried out by the multifunctional protein called CAD...
Preparation of Purified Recombinant DHOase. Six-histidine-tagged CAD protein (DHOase) was purified from transfected hamster cells as described previously (Qiu and Davidson, 2000).

Preparation and Separation of B and C. Microgram quantities of B and C were prepared by hydrolyzing 5 mg/ml dexrazoxane with NaOH (40 μl/ml of 1 M NaOH) at 25°C for 40 min and quenching the reaction with HCl (45 μl/ml of 1 M HCl) to pH 3 as described previously (Hasinoff, 1994a). Under these conditions a mixture of dexrazoxane, B, C, and ADR-295 is produced. Dexrazoxane was efficiently removed from the reaction mixture by loading 500 μl of the mixture on a Sep-Pak Plus C18 cartridge (Waters, Mississauga, ON, Canada) and eluting with 2% (v/v) methanol at a flow rate of 1 ml/min. Although dexrazoxane was highly retained on the cartridge, B, C, and ADR-295 eluted together and were collected at elution volumes between 1.5 and 2.5 ml. HPLC analysis confirmed that dexrazoxane was not detectable in this fraction. This 1-ml fraction, pH 6, was loaded on three Sep-Pak Accell Plus QMA (Waters) ion exchange cartridges connected in series and eluted with 2% (v/v) methanol at a flow rate of 5 ml/min. Fractions containing B and C were collected at elution volumes between 3 and 4.5 ml, and those containing B and C between 5 and 9 ml. The B fraction contained less than 0.1 mol% and 0.01 mol% of C and ADR-295, respectively. The C fraction contained less than 0.1 mol% B and 0.05 mol% of C and ADR-295, respectively. These fractions were brought to pH 2 with 5 M HCl and evaporated to dryness under a stream of nitrogen, stored at −80°C, and reconstituted in water just before use. Neither of these fractions contained detectable amounts of dexrazoxane (<0.001 mol%). Typical yields of B and C were 10 and 6 μg, respectively, which represent about 1% of the starting dexrazoxane.

Kinetics of DHOase-Catalyzed Hydrolysis of B, C, and Dihydroorotate. The DHOase-catalyzed hydrolysis of B, C, and dihydroorotate was generally determined by measuring the decrease of substrate concentration as a function of time to obtain the initial velocities (v). The much lower molar absorptivity of ADR-295 (Hasinoff, 1999) compared with B and C prevented its routine use in the enzyme kinetic assays. The 60-μl reaction mixture contained Chelex-treated 10 mM Tris buffer, pH 7.4; 2 μg/ml DHOase; and B, C, or dihydroorotate at 15°C. DHOase [198 μg/ml in 30% (v/v) dimethyl sulfoxide, 5% (v/v) glycerol, 12 mM Tris buffer, pH 7.9, 0.3 M NaCl, 0.6 M imidazole, and 1 mM 1,4-diithiothreitol] was thawed for 1 min at 37°C and added to the reaction mixture to give a final DHOase concentration of 2 μg/ml. A reaction temperature of 15°C was found to greatly minimize the background hydrolysis of B and C while retaining good DHOase activity. Under these conditions, nonenzymatic hydrolysis of either B or C was not detectable at 45 min, and the substrate-concentration plots were linear to at least 1 h. When inhibitors were used either 1 mM 5-aminooxycetic acid, furosemide, or 4-chlorobenzensulfonyl- amide was incubated with DHOase in the reaction buffer for 1 min before addition of C. After incubation periods of 0, 10, 20, 30, and 45 min post-DHOase addition, 10-μl aliquots were removed and added to 25 μl of 3 mM HCl, pH 2, and stored at −80°C to stop the reaction and prevent further hydrolysis of B or C (Hasinoff, 1994a). The initial velocities for the first 10% (or less) of the reaction for the decrease of B, C, or dihydroorotate were calculated from a linear least-squares fit of five substrate concentration-time data points.

HPLC Analysis of B, C, ADR-295, and Dihydroorotate. The HPLC analysis of B and C using an ion-pair reagent with the reversed phase C18 column (detection wavelength 205 nm) has been described previously (Hasinoff, 1993, 1994a,b; Hasinoff et al., 1994; Hasinoff and Aoyama, 1999). Duplicate determinations were carried out on each sample. The calibration plots for ADR-295 (20–140 μM, n = 7), B, and C (50 μM–10 mM, n = 8) were linear (r2 = 0.995, 0.999, and 0.998, respectively). ADR-295 was determined separately (n = 2) under isocratic conditions (500 μM Na2EDTA/2 mM octanesulfonic acid, pH 3.5, 1 ml/min). After ADR-295 eluted (tR of 3.5 min) the column was washed with 500 μM Na2EDTA/methanol (20:80 v/v) for 20 min followed by reequilibration with the initial mobile phase for 25 min.

An ion-pair HPLC assay was developed to determine (wavelength of 205 nm) dihydroorotate using a 10-mm μBondapak reversed phase C18 column (3.9 × 300 mm; Waters). The elution profile was 10 mM 1-octanesulfonic acid (pH 2.5, 1 ml/min) for 5 min after which the methanol concentration was linearly increased over 1 min from 0 to 40% (v/v) and maintained for 20 min. The column was reequilibrated with the initial mobile phase of 10 mM 1-octanesulfonic acid for 30 min before the next injection. Under these
which indicated that ADR-925 did not inhibit DHOase. An experiment was also done to determine whether ADR-925 metabolized the ring-opening reaction of B and C. The initial velocities were fit to Michaelis-Menten kinetic nonlinear least-squares analysis (SigmaPlot, Jandel Scientific, San Rafael, CA) of the data in the following equation:

\[ v = \frac{V_{\text{max}}[S]}{(K_m + [S])} \] (1)

The specific \( V_{\text{max}} \) and \( K_m \) values are given in Table 1.

An experiment was also done to determine whether ADR-925 inhibited DHOase. To test this, 100 \( \mu \)M ADR-925 was added to the DHOase reaction mixture before the addition of the 25 \( \mu \)M dihydroorotate. The initial velocity of the DHOase-catalyzed hydrolysis of dihydroorotate measured in the presence and absence of 100 \( \mu \)M ADR-925 was not significantly different (t test, \( p > 0.5 \), \( n = 5 \)), which indicated that ADR-925 did not inhibit DHOase. An experiment was also done to determine whether dexrazoxane was a substrate for DHOase. The hydrolysis of 500 \( \mu \)M dexrazoxane in the presence and absence of 2 \( \mu \)g/ml DHOase was followed for 2 h (15°C, pH 7.4). No statistically significant (t test, \( p > 0.1 \), \( n = 5 \)) enzymatic hydrolysis was detected above that observed for the dexrazoxane control, which indicated that neutral dexrazoxane was not a substrate for DHOase.

Experiments were also done to prove that the product of the DHOase-catalyzed hydrolysis of B and C was ADR-925. To test this B and C (1 mM) were separately incubated with 2 \( \mu \)g/ml DHOase for 2 h (15°C, pH 7.4) to obtain sufficient amounts of ADR-925 to measure. The concentrations of ADR-925 enzymatically produced as determined by HPLC at 2 h were found to be 137 and 184 \( \mu \)M, respectively, for B and C. By subtraction, the amount of ADR-925 produced was 130 and 226 \( \mu \)M, respectively, for B and C. The agreement with the directly determined values indicated that DHOase catalyzed the hydrolysis of B and C to ADR-925.

**Effect of Inhibitors on DHOase-Catalyzed Hydrolysis of C.** Given that DHOase is only one of the three activities of the multifunctional CAD enzyme, the kinetics of DHOase-catalyzed hydrolysis of C was determined in the presence of specific inhibitors of DHOase to determine whether the hydrolysis of B and C was due to the DHOase domain. 5-Aminooartic acid is an inhibitor (\( K_i \) of 6 \( \mu \)M) of mammalian DHOase-catalyzed hydrolysis of dihydroorotate, although 4-nitrobenzenesulfonamide is not (Christopherson and Jones, 1980). 4-Chlorobenzenesulfonamide and 4-nitrobenzenesulfonamide are, however, noncompetitive inhibitors (\( K_i \) of 200 and 1100 \( \mu \)M, respectively) of bacterial dihydroorotate (Pradhan and Sander, 1973). Furosemidie (Fig. 1) was included because it is a sulfonamide with a carboxylate group. As shown in Fig. 3, 1 mM 4-chlorobenzenesulfonamide did not inhibit DHOase. However, 1 mM 5-aminooartic acid and furosemidie inhibited DHOase by 91 and 80%, respectively.

**Molecular Modeling of DHOase in the DHOase Active Site.** Metabolite C was modeled into the X-ray crystal structure determined active site of Escherichia coli DHOase (Thoden et al., 2001) to determine what movement of the Arg 20 side chain would be required to fit C into the active site. The Arg 20 forms a salt bridge with the carboxylate of dihydroorotate (Thoden et al., 2001). The modeling was done with a number of distance constraints. The distances from the carbonyl carbon that undergoes hydroxide attack to the binuclear zinc centers and its bridging hydroxide were fixed at those determined in the X-ray structure of dihydroorotate bound to DHOase (Thoden et al., 2001). Also the distance between the carboxylate group of C and the guanidinium group and the two carbon atoms at each extreme of the Arg 20 side chain were also fixed to those found in the X-ray structure. The minimized structure of C in the DHOase active site is shown in Fig. 4. The distance from the carbonyl carbon in C undergoing hydroxide attack to the CZ carbon of the guanidinium group increased by 2.2 Å from 7.6 Å determined in the X-ray structure of dihydroorotate bound to DHOase (Thoden et al., 2001).

**Discussion**

This report is the first that DHOase might be involved in the metabolism of any drug. The specific \( V_{\text{max}} \) value of 0.41 \( \mu \)mol/min/mg (10 mM Tris buffer, pH 7.4, 15°C) for the hydrolysis of dihydroorotate by the recombinant 6-histidine-tagged hamster DHOase (Qiu and Davidson, 2000) used in this study is smaller than the recombinant hamster DHOase specific \( V_{\text{max}} \) values of 1.2 to 2.1 \( \mu \)mol/min/mg (50 mM HEPES buffer, pH 7.4, 37°C, 100 \( \mu \)M ZnCl₂) (Williams et al., 1995; Huang et al., 1999) and 1.2 \( \mu \)mol/min/mg (100 mM Tris buffer, pH 7.4, 37°C, 25 mM MgCl₂, 0.1 M KCl, 3.3 mM glutamine, 15 mM t-aspartate, 1.4-dithiothreitol, and 7.5% dimethyl sulfoxide) (Kelly et al., 1986). However, given the different reaction conditions (buffer, presence of

**TABLE 1**

Michaelis-Menten kinetic parameters for the DHOase-catalyzed hydrolysis of dihydroorotate and B and C, the one-ring open metabolites of dexrazoxane.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>( V_{\text{max}} ) [( \mu )mol/min/mg]</th>
<th>( K_m ) [( \mu )M]</th>
<th>( V_{\text{max}} / K_m ) [1/min/mg]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dihydroorotate</td>
<td>0.41 ± 0.07</td>
<td>20 ± 6</td>
<td>20.6</td>
</tr>
<tr>
<td>B</td>
<td>4.35 ± 0.70</td>
<td>4,800 ± 1,700</td>
<td>0.91</td>
</tr>
<tr>
<td>C</td>
<td>10.9 ± 3.8</td>
<td>11,000 ± 6,300</td>
<td>0.98</td>
</tr>
</tbody>
</table>
DHOase was used in this study, the agreement is reasonable. The structure of DHOase (Thoden et al., 2001). Only dihydroorotate, Arg 20, the binuclear zinc of (bottom). Only the heteroatoms are labeled.

The reaction was carried out in Tris buffer (10 mM, pH 7.4, 15 °C) and with DHOase in the absence of 1 mM 5-aminooarotic acid (△), furosemide (●), and 4-chlorobenzenesulfonamide (○). The results of Table 1 show that both B and C are 11- and 27-fold higher than the endogenous substrate dihydroorotate. The reason for the high \( V_{\text{max}} \) for B and C probably lies with the fact that the imide bond in B and C is much more easily hydrolyzed than is the imide bond in dihydroorotate. We previously showed that B and C underwent nonenzymatic hydrolysis (pH 7.4, 37 °C) with \( t_{1/2} \) values of 17 and 8.4 h, respectively (Hasinoff, 1994a), whereas dihydroorotate is very stable under these conditions. The \( K_m \) values for both B and C are, however, 240- and 550-fold larger than for dihydroorotate. This result suggests that neither B nor C is a good fit in the active site of DHOase. A comparison of the \( V_{\text{max}}/K_m \) values of Table 1 indicates that under nonsaturating conditions ([S] < \( K_m \)) dihydroorotate is still a 23-fold better substrate than either B or C. Given that the \( V_{\text{max}}/K_m \) values for B and C are similar, under nonsaturating conditions DHOase would act on B and C at about the same rate. The high \( K_m \) values for B and C may be partially compensated for by the high \( V_{\text{max}} \) values, thus providing a role for DHOase in the metabolism of dexrazoxane.

The X-ray structure of homodimeric bacterial E. coli DHOase with dihydroorotate bound has recently been determined (Thoden et al., 2001). Each subunit contains a binuclear zinc center, and dihydroorotate is bound to one subunit with the negatively charged carboxylate group of dihydroorotate, forming a salt bridge with the positively charged guanidinium group of Arg 20. Assuming mammalian DHOase has a similar structure, the positively charged active site is the reason that B and C with their negatively charged carboxylate groups are substrates for DHOase, but neutral dexrazoxane is not. A mechanism is proposed in which a bridging hydroxide group attacks the re-face of dihydroorotate (Thoden et al., 2001). Given the structural similarity of dihydroorotate to B and C, a similar mechanism is likely. Although the sequence identity homology of the hamster DHOase domain of CAD with that of E. coli DHOase is only 20%, there are clusters of highly conserved amino acids (Simmer et al., 1990). The Arg 20 is, however, conserved in human DHOase (Thoden et al., 2001). The presence of the guanidinium salt bridge had been predicted previously based on the homology of DHOase with carbonic anhydrase II (Williams et al., 1995).

The results of the molecular modeling shown in Fig. 4 indicated that C can be accommodated in the DHOase active site with a movement of the Arg 20 side chain of 2.2 Å. Although this distance is not large, this and other interactions in the binding pocket are, nonetheless, sufficient to increase the \( K_m \) value for C to 11,000 μM from 20 μM for dihydroorotate (Table 1). It should be noted the modeling was done with bacterial DHOase and may not be valid for the DHOase domain of the CAD enzyme.

The ability of furosemide, but not 4-chlorobenzenesulfonamide, to inhibit DHOase is probably due to the presence of a negatively charged carboxylate group on furosemide (Fig. 1) allowing for stronger binding to the positively charged active site of DHOase (Williams et al., 1995; Thoden et al., 2001). Furosemide is a widely used diuretic in the treatment of congestive heart failure. Given that only micromolar peak plasma levels of furosemide are achieved after a typical 40-mg dose (Straughn et al., 1986), it is unlikely that furosemide inhibition of DHOase has any significant pharmacological effects in vivo.

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). The level of DHOase activity in heart homogenate is 23% of that found in the liver (Kennedy, 1974). The presence of DHOase in the heart suggests that B and C may be enzymatically hydrolyzed by DHOase to ADR-925 in the heart tissue. Hydrolysis would likely be occurring in other tissues and in the blood.

![Fig. 3. Initial velocity plots for the DHOase-catalyzed hydrolysis of metabolite C without DHOase (●) and with DHOase in the absence (○) and the presence of 1 mM 5-aminooarotic acid (△), furosemide (●), and 4-chlorobenzenesulfonamide (○).](image)

![Fig. 4. Ball and stick structure of dihydroorotate in the active site of DHOase (top).](image)

The structure was obtained from the X-ray crystal determination of E. coli DHOase (Thoden et al., 2001). Only dihydroorotate, Arg 20, the binuclear zinc atoms, and their bridging hydroxide are shown. The carboxylate group of dihydroorotate forms a salt bridge with the guanidinium group of Arg 20. Ball and stick structure of C in the active site of DHOase obtained by molecular modeling (bottom). Only the heteroatoms are labeled.
as well. The presence of DHOase in the heart, in particular, may explain the cardioprotective effect of the iron-doxorubicin complex (Buss and Hasinoff, 1993), or bind free iron, thus preventing oxygen radical formation.

The rapid appearance of ADR-925 in our preliminary pharmacokinetic studies in the rat (Schroeder and Hasinoff, 2001) and humans (Schroeder et al., 2002) is consistent with a DHOase-catalyzed conversion of B and C to ADR-925. We previously showed that B and C were present at relatively low steady-state levels in a rat pharmacokinetic study (Hasinoff and Aoyama, 1999). This result is consistent with the subsequent metabolism of B and C to ADR-925 by DHOase. We also previously showed that the zinc hydrolase DHPase, which is present in the liver and kidneys, but not in the heart (Hasinoff et al., 1991), converted dexrazoxane to B and C, but did not convert these intermediates to ADR-925 (Hasinoff, 1993). In preliminary studies we also showed that B and C were permeable enough to be taken up by attached neonatal rat myocytes and dequench an intracellularly trapped iron-calcein complex (Hasinoff et al., 2002). Thus, DHPase and DHOase acting in succession on the parent drug and its metabolites B and C provide a mechanism by which dexrazoxane may exert its cardioprotective effects.

References


