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Interaction of dexrazoxane with red blood cells and hemoglobin alters pharmacokinetics of doxorubicin

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Abstract *Purpose*: The mechanism of the cardioprotective action of dexrazoxane against doxorubicin cardiotoxicity is not fully understood. It has been suggested that its hydrolysis product, ICRF-198, chelates and removes free iron and iron associated with doxorubicin-iron complex and, therefore, prevents the formation of free radical, lipid peroxidation and cardiotoxicity. Dexrazoxane is also known to inhibit topoisomerase II, to prevent the inactivation of cytochrome c oxidase by Fe^{3+}-doxorubicin and to increase the levels of transferrin receptor (trf-rec) mRNA and cellular iron uptake. This sequestration of iron and its effect on cellular iron homeostasis may also contribute to its protective effect against doxorubicin cardiotoxicity. The present project was designed to investigate the interaction of dexrazoxane with hemoglobin and red blood cells and the subsequent effect on the pharmacokinetics and toxicodynamics of doxorubicin. *Methods*: In an in vitro investigation the binding of doxorubicin (0.5–25 μg/ml) to red blood cells, erythrocyte ghosts and hemoglobin in the presence of dexrazoxane was evaluated. In an in vivo study female Sprague Dawley rats were pretreated with 100 mg/kg of dexrazoxane by intravenous injection 1 h before the injection of ^{14}C-doxorubicin (specific activity 0.4 μCi/mg, 10 mg/kg). The time-course of doxorubicin associated with blood cells and plasma was evaluated with simultaneous characterization of doxorubicin and its metabolites in the bile and urine. The serum concentration of endothelin was measured as a biomarker of cardiotoxicity in separate groups of animals. *Results*: The in vitro data indicated that dexrazoxane inhibited the binding of DOX to red blood cells in a concentration-dependent manner. At 1 μg/ml it reduced the binding of doxorubicin to red blood cells by about 30% and at 100 μg/ml by about 60%. It had no effect on the association of doxorubicin with erythrocyte ghosts. The investigation of binding of doxorubicin to hemoglobin revealed the existence of two distinct binding sites and dexrazoxane reduced the association constant of doxorubicin with the low-affinity and high-capacity class of binding sites significantly. The pharmacokinetic analysis showed that pretreatment with dexrazoxane (100 mg/kg) reduced the area under plasma concentration-time curve of doxorubicin, its mean residence time and plasma clearance significantly. Similar reductions were also shown with the pharmacokinetic analysis of doxorubicin associated with blood cells. The biliary and urinary elimination of unchanged doxorubicin increased significantly. The pretreatment reduced the serum concentration of endothelin from about 20 ng/ml to about 12 ng/ml. The per cent of this reduction was proportional to the reduction in the AUC of blood cells. *Conclusion*: The cardioprotective effect of dexrazoxane is due, in part, to its interaction with hemoglobin and red blood cells and this interaction modifies the pharmacokinetics of DOX.

Key words Doxorubicin · Dexrazoxane · Pharmacokinetics · Blood cells · Hemoglobin

Introduction

Doxorubicin (DOX) is a quinone-containing anthracycline antibiotic that in the presence of flavoenzymes is reduced to its respective semiquinone free radicals. In addition, free radicals can also be formed from the interaction of DOX with iron to form DOX-ironIII complex. This complex undergoes self-reduction to ironII, which results in the generation of a semiquinone free radical of DOX. The semiquinone reacts with oxygen to form superoxide anion radical that in the presence of iron catalyzes to hydroxyl radical. This potent free

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radical causes lipid peroxidation and DNA damage [2, 5, 8, 16]. The heart is a more susceptible organ to free radical-induced damage due to its low level of detoxifying enzymes such as superoxide dismutase [17]. Furthermore, DOX reduces the levels of the cardiac detoxifying enzymes like glutathione peroxidase and glutathione reductase [7].

Dexrazoxane (DEX), or ICRF-187, is a nonpolar cyclized derivative of ethylene diamine tetraacetic acid (EDTA). Chemically it is a bisdioxopiperazine and water-soluble enantiomer of the racemic compound razoxane, ICRF-159. The major use of DEX is to prevent anthracycline-mediated cardiotoxicity [4, 18]. It is administered in combination with cancer chemotherapeutic agents, mainly with DOX [12, 14, 18]. The mechanism of its cardioprotective action is not well understood. It has been suggested that its hydrolysis product, ICRF-198, chelates and removes free iron and iron associated with the DOX-iron complex [9, 10, 11], thus preventing the formation of free radical and subsequent lipid peroxidation. DEX is also known to inhibit topoisomerase II [13, 21], to prevent the inactivation of cytochrome c oxidase by Fe^{3+}-doxorubicin [9, 10] and to increase the levels of transferrin receptor (trf-rec) mRNA and cellular iron uptake [20]. This sequestration of iron and its effect on cellular iron homeostasis may also contribute to its protective effect against DOX cardiotoxicity.

There has been no investigation of the effect of DEX on the association of DOX with red blood cells (RBCs) and subsequent change in the pharmacokinetics of DOX. It has been shown that the pharmacokinetics of DOX remain dose-independent over a wide range of escalating doses of DEX (60–900 mg/m^2). Based on this observation it has been hypothesized that DEX has no effect on the pharmacokinetics or metabolism of DOX [12]. In the course of our investigation, we noted that the pharmacokinetics of DOX usually remain linear even at the toxic dose of 20 or 30 mg/kg [19]. It has also been suggested that DEX increases the systemic clearance of epirubicin, an epimer of DOX, and decreases its area under the curve of plasma concentration-time curve [3]. The pharmacokinetics of DEX itself have been reported to be dose-independent, and its AUC is directly proportional to dose over a wide range of dose levels, it does not bind to plasma proteins, its systemic clearance is approximately 11 ml/min per kg and its blood cell to plasma partitioning is about 0.517 [1].

The present study was designed to investigate the effects of DEX on the interaction of DOX with RBCs and the consequent effects on the metabolism, pharmacokinetics and toxicodynamics of DOX. This study was based on the concurrent measurements of DOX in plasma and blood cells, and the amount of DOX and its metabolites in simultaneously collected bile and urine samples. Serum endothelin levels were measured as a biomarker of cardiotoxicity and used as a toxicodynamic parameter to correlate with pharmacokinetic parameters of plasma and blood cells.

Materials and methods

Materials

DEX, DOX HCl, doxorubicinol, DOX aglycone and DOXol aglycone were gifts from Pharmacia Upjohn (Albuquerque, N.M.). Radiolabeled DOX (^{14}C, specific activity 91.1 μCi/mg) was purchased from Amersham Corporation (Arlington Heights, Ill.). The radiolabeled DOX was further diluted with nonradiolabeled DOX to obtain a final specific activity of 0.4 μCi/mg. Human hemoglobin, bovine serum albumin, Trizma HCl and the total protein reagent were purchased from Sigma Chemical Co. (St. Louis, Mo.). Fresh packed human RBCs were obtained from the American Red Cross (Dedham, Mass.). Endothelin enzyme immunoassay (EIA) reagents were purchased from Peninsula Laboratories (San Carlos, Calif.).

Sodium pentobarbital (Nembutal 50 mg/ml) was purchased from Henry Schein (Port Washington, N.Y.). HPLC-grade solvents, polypropylene Eppendorf tubes, Nalgene polycarbonate ultracentrifuge tubes, borosilicate glass liquid scintillation vials and hydrogen peroxide (30%) were purchased from VWR Scientific (Boston, Mass.). Scintillation counting fluid (Scintiverse E) and all analytical grade chemicals were obtained from Fisher Scientific (Springfield, N.J.). Beckman Instruments (Fullerton, Calif.) provided BTS-450 tissue solubilizer.

Protocol of the in vitro experiment

EGs were prepared by washing the RBCs with isotonic phosphate buffer several times followed by incubation with hypotonic phosphate buffer at room temperature for 10 min. The cells were then centrifuged at 3000 rpm for 15 min and the supernatant was discarded. The procedure was repeated until a clear supernatant was obtained. The final pellet was suspended in isotonic phosphate buffer. The final yield was optimized with respect to the number of erythrocyte ghosts (EGs) per milliliter.

Erythrocytes and EGs (10 million cells per milliliter, total volume 2 ml) were incubated with different concentrations of DOX (0.5–25 μg/ml). The test groups ($n = 4$ per group) were incubated in the presence of 1 or 100 μg/ml of DEX. The control group ($n = 4$) was incubated in the absence of DEX. All samples were incubated at 37 °C for 24 h. The samples were then centrifuged at 3000 rpm for 5 min and the supernatants were analyzed by HPLC for free DOX.

The effect of DEX on the binding of DOX to hemoglobin was studied by incubation of 6% hemoglobin in normal saline (the physiological concentration of hemoglobin) with different concentrations of DOX and DEX. The sequence of addition of DEX or DOX to the incubation was considered important and evaluated according to the following protocols:

Method I DEX (100 μg/ml) and different concentrations of DOX (0 to 175 μ*M*) were added simultaneously to the solution of hemoglobin followed by incubation at 37 °C for 1 h.

Method II DEX (100 μg/ml) was added to the solution of hemoglobin first, followed by incubation at 37 °C for 1 h. DOX (0 to 175 μ*M*) was then added and the incubation continued for another hour.

Method III DOX (0 to 175 μ*M*) was added to the solution of hemoglobin first, followed by incubation at 37 °C for 1 h. DEX (100 μg/ml) was then added and the incubation continued for another hour.

Incubation of each test group ($n = 4$ per group) was accompanied by incubation of a related control group ($n = 4$ per group).

Due to the extensive binding of DOX to filters of any kind or dialysis membrane, ultracentrifugation was selected as the method of choice. The control samples and samples from methods I to III were then centrifuged at 100,000 *g* for 15 h in a Beckman ultracentrifuge (Fullerton, Calif.). The supernatants were separated and the concentration of free drug was determined by HPLC.

Protocol of the in vivo experiment

Female Sprague Dawley rats weighing 225–250 g were purchased from Taconic Farms (Germantown, N.Y.). All animals were housed in a clean environment, allowed to acclimatize for at least 1 week prior to experimentation and allowed free access to food and water. On the night before the experiment food was removed but the animals had access to water. The animals were divided into five groups (six per group). Group I (control group) received ^{14}C-DOX (specific activity 0.4 μCi/mg, 10 mg/kg) via a femoral vein. Group II (test group) received DEX pretreatment (100 mg/kg) via the tail vein 1 h before the injection of ^{14}C-DOX (specific activity 0.4 μCi/mg, 10 mg/kg) via a femoral vein. Groups I and II were used for the pharmacokinetic study. The remaining three groups (III, IV and V) were used for the measurement of endothelin levels and for the toxicodynamic study. Group III received normal saline via a femoral vein, group IV received nonradiolabeled DOX (10 mg/kg) via a femoral vein and group V received DEX pretreatment (100 mg/kg) via the tail vein 1 h before the injection of nonradiolabeled DOX (10 mg/kg) via a femoral vein.

Groups I and II were anesthetized by intraperitoneal sodium pentobarbital (Nembutal, 45 mg/kg) and their bile duct and tail vein were cannulated. The animals were maintained under constant anesthesia throughout the 10-h experiment. The cumulative dose of sodium pentobarbital did not exceed 50 mg per animal. Blood samples (about 200 μl) were collected in heparinized Eppendorf tubes at 5, 15, 30 and 45 min and then every hour for 10 h. Bile samples were collected periodically. Urine was collected during and at the end of the experiment by puncturing the bladder. The blood samples were centrifuged at 2000 rpm for 15 min and the blood cells were separated from the plasma. The plasma, bile and urine samples were frozen in liquid nitrogen immediately after collection and stored at −20 °C until further analysis. The blood cells were stored at 4 °C.

Groups III, IV and V were anesthetized and only blood samples were collected (250 μl) without heparin. The blood samples were centrifuged at 2000 rpm for 15 min. The serum portions were collected, frozen in liquid nitrogen and stored at −20 °C.

Analytical methodology

The samples of blood cells, normalized with respect to volume, were first treated with tissue solubilizer at room temperature for 24 h. The samples were then decolorized with hydrogen peroxide (300 μl) and glacial acetic acid (100 μl) was added to prevent quenching. After the addition of liquid scintillation counting fluid, the samples were left overnight in the refrigerator. A liquid scintillation counter (Wallac, Gaithersburg, Md.) was used to measure the total radioactivity of all biological samples of groups I and II. Bile and urine were then analyzed by HPLC to quantify unchanged DOX and its major metabolites. The HPLC system consisted of a Waters solvent delivery system, Novapak C-18 cartridge, C-18 Novapak Sentry guard column (Waters Corporation, Milford, Mass.) and a Hitachi AS-2000 autosampler (Danbury, Ct.). The mobile phase was 0.1% v/v ammonium formate buffer/methanol (30:70% v/v), pH 4.0. The detections were carried out using a Gilson Fluorometer (Middleton, Wis.) with excitation and emission wavelengths of 480 nm and 540 nm, respectively, and an online radioactivity detector (Beta Flo, Packard, Meriden, Ct.). The guard column was replaced regularly and the retention times of DOX and each metabolite were determined and confirmed daily with authentic standards.

The serum samples of groups III, IV and V were analyzed by an enzyme immunoassay procedure for levels of the peptide endothelin. All the required reagents were purchased from Peninsula Laboratories (San Carlos, Calif.). A microtiter plate reader (Bio-Tek Instruments, Winooski, Vt.) was used for reading the ELISA plates for measurement of the peptide concentrations in serum. Briefly, the procedure involved placing the antipeptide antibody, the standard serum samples or serum samples from groups III, IV or V and biotinylated peptide in a coated 96-well plate. The biotinylated peptide competes with the standard peptide or the peptide in the sample for binding sites on the antibody. The mixture was incubated overnight at room temperature. The wells were then washed five times to remove the excess unbound biotinylated peptide. A solution of streptavidin-horseradish peroxidase conjugate was added to the wells and the plates were incubated at room temperature for 60 min to allow the conjugate to bind to the immobilized primary antibody-biotinylated peptide complex. After washing the excess conjugate, a solution of 3,3′,5,5′-tetramethyl benzidine dihydrochloride was added at room temperature and 50 min later the formation of colored complex was stopped by 2 *N* HCl. The intensity of the color was measured at 450 nm with the microtiter plate reader. The concentration of the peptide was determined from the inverse relationship between the concentration of the peptide and the intensity of the colored samples.

Data analysis

We evaluated the in vitro interaction of DOX with RBCs and EGs according to the Freundlich isotherm. This isotherm formulates the relationship between the amount of DOX associated with RBC or EG with the equilibrium concentration of free DOX as follows:

$$\frac{x}{m} = K(C_{\mathrm{eq}})^n \tag{1}$$

where x/m is the amount of DOX associated per unit mass of cells, K is an estimate of the association capacity, C_{eq} is the equilibrium concentration of DOX and n is the intensity of the association.

We assessed the interaction of DOX with hemoglobin by a Scatchard plot and the Hill equation. Since the preliminary evaluation of the data indicated that multiple classes of binding sites might exist for DOX on hemoglobin, the following version of the Scatchard plot was used:

$$r = \frac{n_1 k_1 [D_{\mathrm{f}}]}{1 + k_1 [D_{\mathrm{f}}]} + \frac{n_2 k_2 [D_{\mathrm{f}}]}{1 + k_2 [D_{\mathrm{f}}]} + \cdots \tag{2}$$

where r is the concentration of DOX (micrograms per milliliter) bound per milligram of hemoglobin, n is the number of binding sites, k is the association constant and $[D_{\mathrm{f}}]$ is the free concentration of DOX.

The Hill equation was selected to determine the presence of cooperativity in the binding of DOX and DEX to hemoglobin. The equation is:

$$\log\left(\frac{r}{r_{\max} - r}\right) = n \ \log[C] - n \ \log \ K \tag{3}$$

where r has the same definition as above, $r_{\max}$ is the maximum binding capacity, C is the equilibrium concentration, K is the dissociation constant and n indicates the nature of the binding.

The in vivo data related to plasma concentration and amount associated with blood cells were analyzed by noncompartmental analysis using the following equations:

$$\mathrm{Cl} = \frac{\mathrm{Dose}}{\mathrm{AUC}} \tag{4}$$

$$\mathrm{MRT} = \frac{\mathrm{AUMC}}{\mathrm{AUC}} \tag{5}$$

$$\mathrm{Vd_{ss}} = \mathrm{MRT} \times \mathrm{Cl} \tag{6}$$

where Cl is the clearance term, AUC is the area under the concentration-time curve, MRT is the mean residence time, AUMC is the area under the first moment curve and $\mathrm{Vd_{ss}}$ is the volume of distribution.

The analysis of the biliary data was achieved by simultaneous curve fitting of plasma data and biliary elimination of unchanged DOX. After several trials of different models and using the appropriate curve-fitting criteria, the following two-compartment equations were chosen to predict the lines and the related biliary rate constants:

$$\text{Plasma: } C_p = Ae^{-\alpha t} + Be^{-\beta t} \quad (7)$$

$$\text{Bile: } A_B = k_b\left(\frac{A\beta + B\alpha}{\alpha\beta}\right) - k_b\left(\frac{Ae^{-\alpha t}}{\alpha} + \frac{Be^{-\beta t}}{\beta}\right) \quad (8)$$

where C_p is the plasma concentration, A_B is the cumulative amount eliminated at time t, A and B are the coefficients of the exponential terms of plasma data, α and β are the hybrid rate constants of disposition and k_b is the first-order rate constant for biliary excretion.

At time $t = \infty$,

$$A_B^{\infty} = k_b\left(\frac{A\beta + B\alpha}{\alpha\beta}\right) \quad (9)$$

where A_B^{∞} corresponds to the total amount that ultimately will be eliminated in the bile, i.e. the plateau level of the cumulative curve of biliary elimination.

The change in endothelin concentration, as the toxicodynamic parameter, exhibited a threshold and therefore the following E_{max} model was used to define the sigmoidal curve:

$$E = \frac{E_{max} \times T^{\gamma}}{ET_{50}^{\gamma} + T^{\gamma}} \quad (10)$$

where E is the serum endothelin level, E_{max} is the maximum serum endothelin level, T is the time, ET_{50} is the time required to reach 50% of maximum endothelin levels and γ is the degree of sigmoidicity [6].

Statistical comparisons were carried out using the two-tailed paired and unpaired Student's t-test as needed. WinNonlin (Scientific Consulting, Cary, N.C.) was used for pharmacokinetic analysis and EXCEL (version 5.0, Microsoft Corporation, Redmond, Wash.) was used for statistical analysis.

Results

In vitro data

The effect of DEX on the interaction of DOX with RBCs is shown in Figs. 1 and 2. The comparison of the related constants of association is presented in Table 1. The data indicate that the addition of DEX at a concentration of 1 μg/ml reduced the association of DOX with RBCs by about 30% and at 100 μg/ml by about 50–60%. The data also reveal that regardless of the mechanism of association of DOX with EGs, either by encapsulation or adsorption on the membrane, DEX at a concentration of either 1 or 100 μg/ml had no effect on the overall uptake of DOX with EGs. The results of in vitro investigation demonstrated the importance of hemoglobin in the interaction of DOX or DEX with RBCs.

Isotherms of the binding of DOX to hemoglobin are presented in Fig. 3. The most significant reduction in the binding was achieved when DEX was added to the solution of hemoglobin 1 h before DOX (method II). The inhibition was also significant when both drugs were added to the solution of hemoglobin simultaneously (method I). The addition of DEX 1 h after incubation of hemoglobin with DOX had no effect on the binding – the data was essentially the same as that from the control and no significant change was observed. The analysis of the data by the Scatchard plot revealed the existence of two distinct binding sites for DOX on

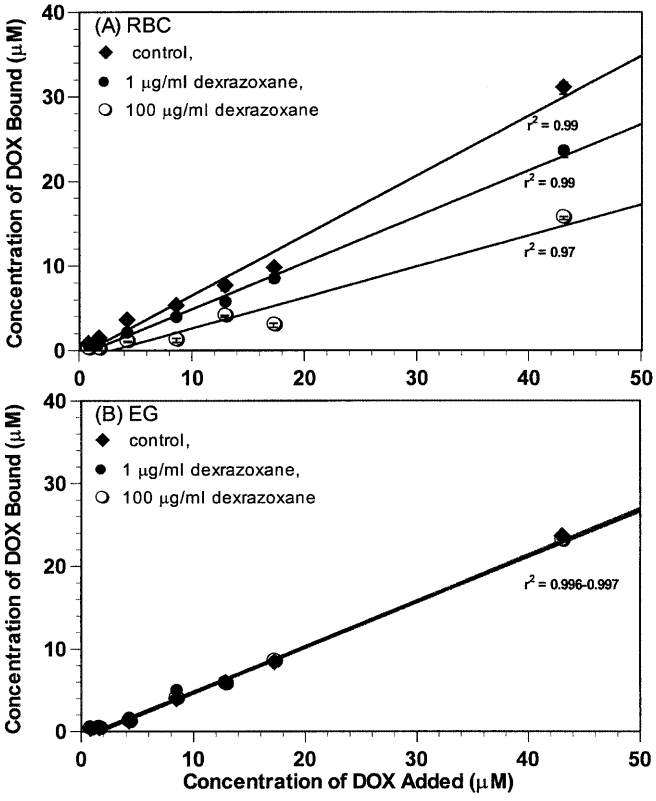

Fig. 1A,B Comparison of the effect of DEX on the association of DOX with RBCs and EGs. Isotherm **A** indicates that the effect of DEX is concentration-dependent whereas isotherm **B** shows its lack of effect on EG. The data presented are means ± SD ($n = 4$)

hemoglobin. These binding sites were identified as class 1 (high affinity and low capacity) and class 2 (low affinity and high capacity). The related data are presented in Fig. 4. The addition of DEX according to method II reduced the association constant of DOX for class 2 binding sites significantly (Table 2). The product of the number of binding sites, n, and the association constant, k, known as the binding capacity, was also less for class 2 binding sites using method II. In addition, the analysis of the data by the Hill plot gave the slopes 0.99 for the control, 1.02 for method I, 0.933 for method II and 0.984 for method III. The closeness of these values to 1 indicates that both compounds bind to the same classes of binding sites (Fig. 4).

In vivo data

The results of noncompartmental analysis of plasma concentration of DOX are presented in Table 3. The pretreatment of animals with DEX lowered the plasma concentration of DOX and decreased the plasma AUC, MRT and Vd_{ss}, but the plasma clearance increased significantly. The results of noncompartmental analysis of DOX associated with blood cells are presented in Table 4. The significant reduction in AUC, MRT and Vd_{ss} in pretreated animals was consistent with the

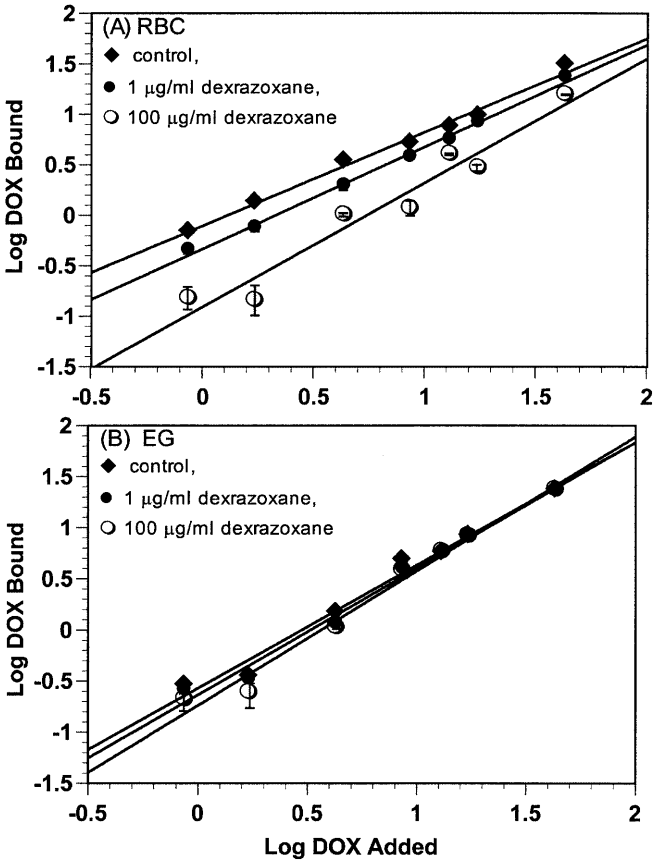

Fig. 2 Freundlich isotherms of the effect of DEX on the association of DOX with RBCs and EGs. This comparison further supports the observation that DEX has no effect on EGs ($n = 4$)

reduction in these parameters in plasma, but contrary to plasma data the clearance of DOX associated with blood cells was decreased significantly.

The total radioactivity (i.e., unchanged DOX plus its metabolites) recovered in the bile of the control group during the experiment accounted for 30.82 ± 4.32% of the dose with an overall biliary rate constant, k_b, of 4.86 h^{-1}. This amount included approximately 11.45 ± 1.81% unchanged DOX (k_b 1.92 h^{-1}), 2.75 ± 0.25% doxorubicinol (k_b 0.48 h^{-1}), 1.37 ± 0.42% DOX aglycone (k_b 0.24 h^{-1}) and 1.28 ± 0.11% doxorubicinol aglycone (k_b 0.24 h^{-1}). The remaining radioactivity was assumed to be the unidentified metabolites including the conjugates. The effect of coadministration of DEX on

Table 1 Effect of DEX on the constant of association of DOX (at concentrations in the range 0–45 μ*M*) with human erythrocytes and erythrocyte ghosts (EGs). The values presented are means ± SD ($n = 4$)

Incubation	$K_{erythrocytes}$	$K_{erythrocyte\ ghosts}$
DOX (control)	0.71 ± 0.03	0.55 ± 0.02
DOX and DEX (1 μg/ml)	0.55 ± 0.01*	0.56 ± 0.04
DOX and DEX (100 μg/ml)	0.36 ± 0.01*	0.55 ± 0.06

*$P < 0.05$ with respect to control

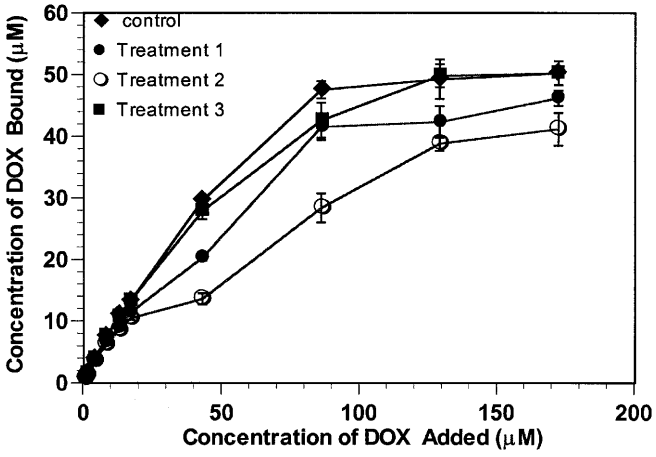

Fig. 3 Effect of DEX on the binding of DOX to hemoglobin. The incubations were carried out with different sequences of addition DOX and DEX to 6% hemoglobin solution at 37 °C. The control contained only DOX. In method I DOX and DEX were added simultaneously, in method II DEX was added 1 h before DOX, and in method III DOX was added 1 h before DEX. The data presented are means ± SD ($n = 4$)

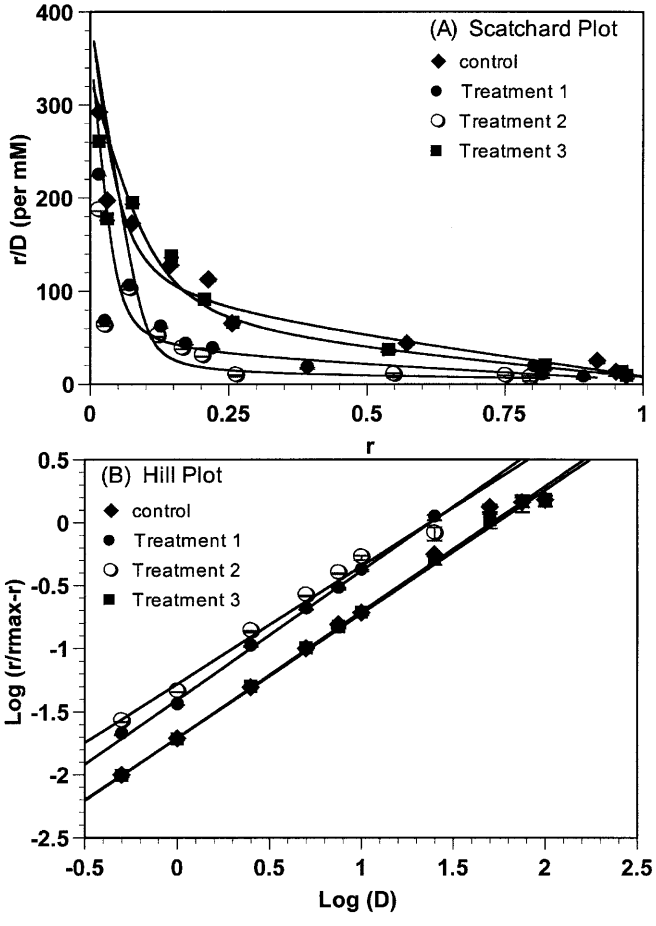

Fig. 4 Scatchard and Hill plots of the effect of DEX on the binding of DOX to hemoglobin. The control contained only DOX. In method I DOX and DEX were added simultaneously, in method II DEX was added 1 h before DOX, and in method III DOX was added 1 h before DEX. The data presented are means ± SD ($n = 4$)

Table 2 Effect of DEX (100 μg/ml) on the parameters of the Scatchard plot of binding of DOX (0–175 μ*M*) to hemoglobin when it was added to the incubation simultaneously with DOX (method I), 1 h before DOX (method II) or 1 h after DOX (method III). The values presented are means ± SD ($n = 4$) (n_1, k_1 number of binding sites and association constant for class 1 binding sites, respectively; n_2, k_2 number of binding sites and association constant for class 2 binding sites)

Incubation	n_1	$k_1(\mathrm{m}M^{-1}) \times 10^{-3}$	n_2	$k_2\ (\mathrm{m}M^{-1}) \times 10^{-1}$
DOX (control)	0.05 ± 0.001	6.39 ± 0.27	1.05 ± 0.06	8.00 ± 0.21
DOX and DEX (I)	0.04 ± 0.001	13.78 ± 0.52	1.08 ± 0.004	3.19 ± 0.52
DOX and DEX (II)	0.03 ± 0.002*	9.91 ± 0.18*	1.55 ± 0.023	0.70 ± 0.12*
DOX and DEX (III)	0.05 ± 0.001	8.33 ± 0.25	1.03 ± 0.04	4.99 ± 0.87*

*$P < 0.05$ with respect to control

Table 3 Effect of coadministration of DEX on the pharmacokinetic parameters and constants of plasma concentration of DOX using noncompartmental analysis. Data were calculated by WinNonlin and are presented as means ± SD ($n = 6$). (*AUC* area under plasma concentration-time curve, *MRT* mean residence time, V_{ss} volume of distribution)

Treatment	AUC (mg · h/l per kg)	MRT (h)	V_{ss} (l)	Clearance (ml/min)
DOX (10 mg/kg)	30.04 ± 2.25	11.40 ± 1.33	3.80 ± 0.42	5.50 ± 0.81
DOX (10 mg/kg) and DEX (100 mg/kg)	20.78 ± 1.57*	4.77 ± 0.88*	2.28 ± 0.04	8.00 ± 1.64*

*$P < 0.05$

Table 4 Effect of coadministration of DEX on the pharmacokinetic parameters and constants of DOX associated with blood cells using noncompartmental analysis. Data were calculated by WinNonlin and are presented as means ± SD ($n = 6$). (*AUC* area under plasma concentration-time curve, *MRT* mean residence time, V_{ss} volume of distribution)

Treatment	AUC (mg · h/l per kg)	MRT (h)	V_{ss} (l)	Clearance (ml/min)
DOX (10 mg/kg)	63.41 ± 5.61	5.39 ± 0.98	1.08 ± 0.02	3.40 ± 0.22
DOX (10 mg/kg) and DEX (100 mg/kg)	49.33 ± 2.47*	3.28 ± 0.14*	0.68 ± 0.01	1.90 ± 0.18*

*$P < 0.05$

the metabolic profile of DOX is presented in Fig. 5. The total radioactivity recovered in the bile of DEX-treated animals was 34.67 ± 2.72% of the dose of DOX with an overall biliary rate constant of 6.9 h^{-1}. The unchanged DOX accounted for 13.38 ± 2.10% of the dose (k_b 2.64 h^{-1}), and doxorubicinol was 2.18 ± 0.52% (k_b 0.42 h^{-1}), DOX aglycone was 1.69 ± 0.12% (k_b 0.36 h^{-1}) and doxorubicinol aglycone was 1.86 ± 0.08% (k_b 0.36 h^{-1}). The increase in the amount of unchanged DOX in the bile of the DEX-treated animals and the corresponding biliary rate constant is consistent with the decrease in the AUC of the plasma and blood cell data.

The total radioactivity recovered in the urine of the control group was 7.80 ± 0.46% of the dose whereas in DEX-pretreated animals it increased slightly to 8.03 ± 0.41% of the dose. The significant difference was the excretion of unchanged DOX that was increased from 4.77 ± 0.48% in control animals to 5.67 ± 0.19% of the dose in DEX-pretreated animals.

The endothelin levels of groups III, IV and V were measured according to a method described previously. There were some fluctuations, and we decided to use the average of the baseline levels of group III (about 2.00 ng/ml) as the basal concentration. The serum levels of the treated groups were then normalized with respect to the basal concentration. The levels of group IV after receiving DOX increased to a maximum concentration of about 20 ng/ml 6 h after the injection. DEX pretreatment reduced this level to about 12 ng/ml. The calculated parameters are presented in Table 5.

Discussion

Based on the analysis of in vitro data, DEX interacted with the content of RBCs and not the membrane. This emphasizes the role that hemoglobin plays in the interaction of DEX with RBCs. The affinity of DEX for the hemoglobin of RBCs inhibited the binding of DOX to RBCs. This inhibition was concentration dependent and higher concentrations of DEX lessened the association of DOX with RBCs. From the effects of DEX on the binding of DOX to hemoglobin we conclude that both DOX and DEX might interact with the same binding sites on hemoglobin. However, neither compound is able to replace the other. The analysis of the data by the Scatchard plot provided information related to two distinct classes of binding sites. Whether either of the two binding sites is the iron moiety of the hemoglobin remains to be determined.

The pharmacokinetic data revealed that DOX is distributed and eliminated from blood differently in DEX-pretreated animals. The change can be attributed mainly to the reduction in binding of DOX to RBCs and free iron. The increase in biliary excretion of unchanged

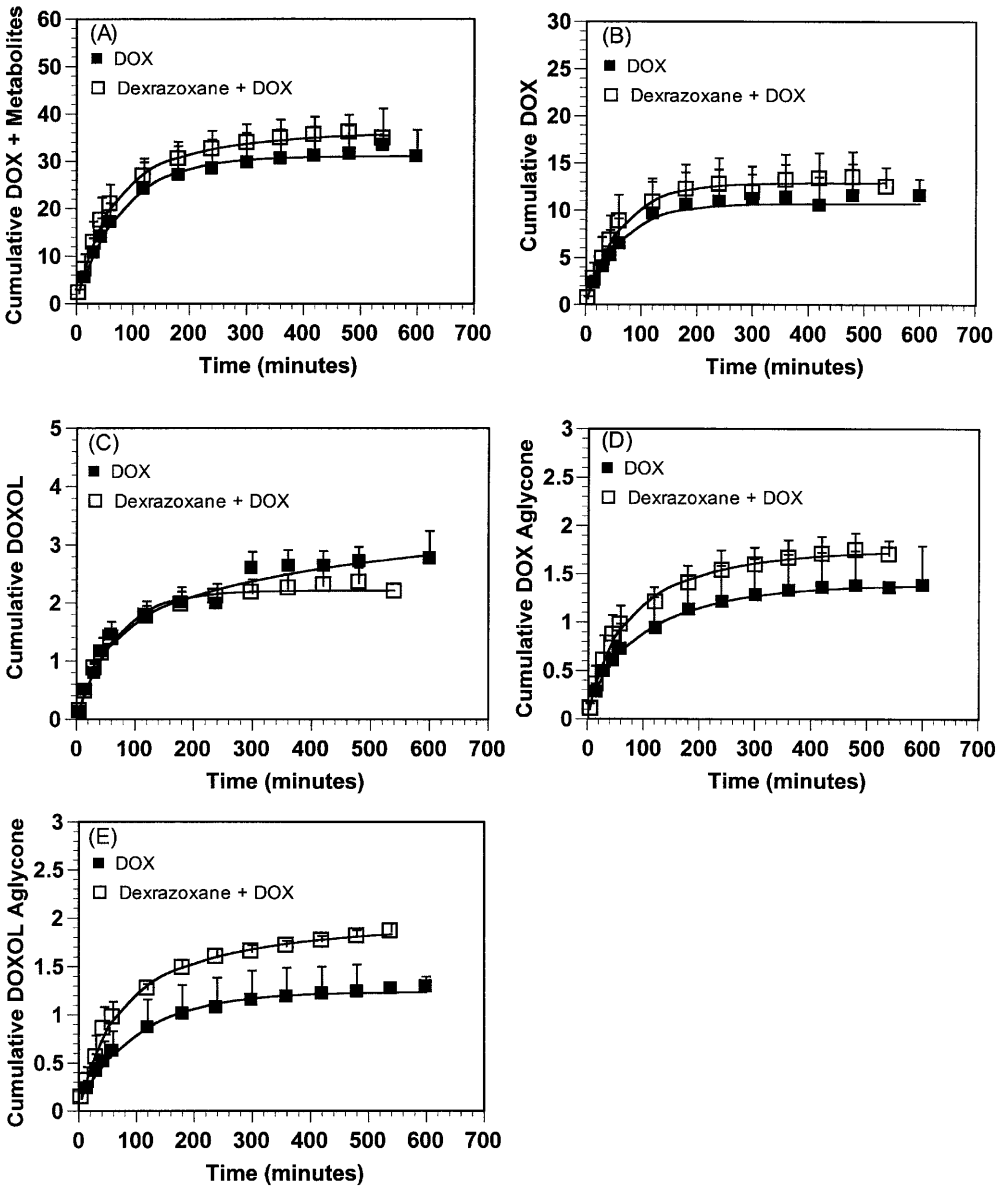

Fig. 5 Effect of DEX on the biliary elimination of DOX and its metabolites. The data presented are means ± SD ($n = 6$)

DOX is an indication of higher availability of unchanged drug in the systemic circulation. The lower MRT suggested that the DEX pretreatment not only increased the amount of DOX eliminated unchanged, but also shortened the biological half-life of DOX. The difference between the clearance of DOX calculated from the plasma and blood cell data was also significant. DEX administration increased the plasma clearance but reduced the blood cell clearance. This reduction in blood cell clearance of DOX suggests that DEX impeded the rate of release of DOX from blood cells into the plasma. The minor changes observed in the metabolism of DOX were mainly related to the increase in the concentration of the parent compound. The effects of DEX on NADPH cytochrome P450 reductase and the formation of the aglycones remain to be investigated.

Table 5 Toxicodynamic parameters from the fitting of the E_{max} model to serum endothelin concentrations following the injection of DOX or DEX followed by DOX. Values are means ± SD ($n = 6$)

Treatment	E_{max} (ng/ml)	ET_{50} (h)	γ
DOX	23.45 ± 6.63	3.81 ± 1.45	2.22 ± 0.15
DEX + DOX	12.12 ± 1.21*	4.85 ± 0.51	6.22 ± 2.91

*$P < 0.05$

To determine the pharmacokinetic-toxicodynamic relationships a biomarker of cardiotoxicity was required. We selected the endothelin level for this purpose. Endothelin is an endogenously occurring peptide found in the body. It is a 21 amino acid peptide produced in the endothelial cells. There are three pharmacologically distinct endothelins (endothelin-1, -2 and -3). Endothelin-1 has been implicated in the pathogenesis of various cardiovascular diseases. It has also been implicated in various coronary syndromes such as unstable angina

and acute myocardial infarction. The levels of endothelin-1 of patients with congestive heart failure have been shown to be increased fourfold [15]. The serum levels of endothelin-1 of groups IV and V were increased to a maximum concentration after a lag-time of 6 h. This lag-time or threshold provided a sigmoidal time course for the marker and necessitated the application of an E_{max} sigmoidal model as described in Data analysis.

The coadministration of DEX with DOX lowered the maximum level of endothelin from about 20 ng/ml (group IV) to about 12 ng/ml (group V). Based on the observed data, the E_{max} model predicted that the maximum endothelin level would be 23.45 ± 6.63 ng/ml and 12.12 ± 1.21 ng/ml for groups IV and V, respectively. The times required to reach 50% of the maximum levels were estimated as 3.81 ± 1.45 h for group IV and 4.85 ± 0.51 h for group V. To determine whether the parameters of the E_{max} model were consistent with the changes in the pharmacokinetic parameters of plasma or blood cells, various correlative analyses were attempted. Among the pharmacokinetic parameters and constants, only the ratio of the maximum endothelin levels of group V to those of group IV corresponded to the ratio of the total amount of DOX associated with blood cells of group II to the total amount of group I, that is:

$$\left[\text{endothelin } C_{max}\right]_{\text{group V}} / \left[\text{endothelin } C_{max}\right]_{\text{group IV}} = \left[(\text{AUC} \times \text{Cl})_{\text{blood cells}}\right]_{\text{group II}} / \left[(\text{AUC} \times \text{Cl})_{\text{blood cells}}\right]_{\text{group I}}$$

Such a relationship could not be established with the calculated data from plasma concentration of DOX. The above correlation may well be an indication of the significant role that blood cells play in the cardiotoxicity of DOX.

There remains a need for further investigation to improve our understanding of the role that RBCs, and particularly hemoglobin, play in the disposition and cardiotoxicity of DOX.

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