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Contradistinction between doxorubicin and epirubicin: in-vitro interaction with blood components

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Abstract

The molecular structure and anti-tumour activity of doxorubicin and epirubicin are similar. However, the incidence of their cardiotoxicity occurs at different cumulative dose concentrations. The purpose of this study was to investigate the in-vitro interaction of these two drugs with different blood components, namely intact erythrocytes, haemoglobin and erythrocyte ghosts. Plasma protein binding was also evaluated. The intended goal was to identify the most relevant samples among total blood, plasma or blood cells for pharmacokinetic analysis. The methodology involved the incubation of each of the blood components (the intact erythrocytes, erythrocyte ghosts, haemoglobin and plasma proteins) at physiological pH and temperature with different concentrations of each drug, followed by measurement by HPLC and fluorometry at excitation and emission wavelengths of 480 and 580 nm, respectively. The results indicated that the binding of doxorubicin and epirubicin to plasma proteins, erythrocyte ghosts and intact erythrocytes was essentially the same. However, the binding of both compounds to intact erythrocytes was significantly different from erythrocyte ghosts, which indicates that haemoglobin plays an important role in the binding to and uptake by erythrocytes. The isotherms of binding to haemoglobin revealed that the maximum binding of doxorubicin was approximately 0.42 μg mg^{-1} haemoglobin; for epirubicin this value was ten times greater than for doxorubicin. The Scatchard plot of binding of both drugs to haemoglobin exhibited two distinct binding sites for each drug. The constant of association of high affinity and low capacity binding sites was significantly greater for epirubicin, whereas the constant of association of low affinity and high capacity binding sites was significantly higher for doxorubicin. The number of high affinity binding sites per mg of haemoglobin was estimated to be 0.072 for doxorubcin and 0.030 for epirubicin. The number of low affinity binding sites was significantly greater for epirubicin (1.963) than for doxorubicin (0.305). Since the combined number of binding sites for epirubicin was more than doxorubicin, and the total uptake by erythrocytes remained the same for both drugs, it was concluded that epirubicin, being a more lipophilic compound, may diffuse more freely into the cells. Therefore, it binds more to haemoglobin, whereas doxorubicin remains more adsorbed on the surface of the cells due to its self-association property. It was concluded that the interaction of both drugs with erythrocytes, although it appears to be similar, is significantly different due to the interaction with haemoglobin. The difference in this interaction is expected to influence the disposition of both drugs in-vivo.

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Introduction

Doxorubicin is an effective agent in the treatment of breast cancer, malignant lymphomas, soft tissue sarcoma and various neoplastic diseases. This anthracycline antibiotic has a water-soluble basic amino sugar, daunosamine, that is linked

glycosidically to carbon 7 of a four-ringed water insoluble aglycone known as doxorubicinone. This aglycone is a substituted naphthacene quinone with a methoxy group at the 4-position and a hydroxyl group plus a hydroxyacetyl group at carbon 9 (Orr & Von Hoff 1994). Epirubicin, an epimer of doxorubicin, differs from doxorubicin in the 4′-OH position of the amino sugar moiety. In doxorubicin the 4′-OH group is in axial orientation whereas in epirubicin it is in equatorial orientation. In its axial configuration, the 4′-OH group is closer to the NH_2 of the amino sugar, and hence provides a higher $pK_a$and lowers lipid solubility for doxorubicin. In its equatorial orientation, the OH group, being away from the NH_2 group of daunosamine, gives epirubicin the properties of a weaker base and a more lipid soluble molecule (Bonfante et al 1979; Bonadonna et al 1980, 1993; Launchbury & Habboubi 1993). These differences in ionization and partition coefficients have been shown to affect cell penetration, binding and metabolic conjugation (DiMarco et al 1977; Launchbury & Habboubi 1993; Chassany et al 1994, 1996; Pernkopf et al 1996). Both drugs have similar cytotoxicity against a wide range of animal and human tumour cell lines (Hill & Whelan 1982; Cantoni et al 1990). The anti-tumour activity of both drugs appears to be comparable (Giuliani et al 1981; Harstrick et al 1990; Plosker & Faulds 1993). However, the incidence of their cardiotoxicity occurs at different cumulative dose concentrations. For doxorubicin the cardiotoxic dose concentration is in excess of 550 mg m^{-2} (Pratt et al 1978), whereas for epirubicin it is greater than 900 mg m^{-2} (Greg et al 1993).

Both drugs undergo similar phase 1 metabolism by the cytosolic aldo-keto reductases and NADPH cytochrome P450 reductases (Gewirtz & Yanovich 1987; Behnia & Boroujerdi 1999). The cardiotoxicity of both drugs is known to be initiated by free radicals (Rajagopalan et al 1988; Olson & Mushlin 1990). This hypothesis has been further supported by the protective role of antioxidants (Doroshow et al 1981; Vora & Boroujerdi 1996). The formation of oxygen radicals from heart muscle or its sub-cellular organelles has also been documented (Piccinini et al 1990; Ogura et al 1991). The role of iron in the formation of the free radicals is of critical importance (Myers et al 1982; Gutteridge et al 1984). It is the doxorubicin–iron (III) complex that undergoes self-reduction to an iron (II) complex and forms a semiquinone free radical, which reduces oxygen or hydrogen peroxide to yield the hydroxyl free radical (Muindi et al 1984). Therefore, the important biological element that facilitates the development of the cardiotoxicity is the availability of iron. The total iron content in the body of normal adults is approximately 3–5 g. The portion associated with myoglobin in the heart is estimated to be 0.5 g or less. The total iron associated with haemoglobin is 1.5–3 g, more than 50% of the total iron content. The remaining iron is bound to transferrin or stored in ferritin (Smith & Reynard 1992). The role that haemoglobin may play in disposition of epirubicin and doxorubicin or their interaction at the cellular level in-vitro is not fully understood. The purpose of this study was to investigate the in-vitro interaction of these two drugs with different blood components, namely intact erythrocytes, haemoglobin and erythrocyte ghosts. Plasma protein binding was also evaluated. The intended goal was to identify the most relevant samples among total blood, plasma or blood cells for pharmacokinetic analysis.

Materials and Methods

Chemicals and biological samples

Human erythrocytes and plasma were obtained from the American Red Cross (Dedham, MA). Bovine serum albumin (fatty acid- and globulin-free lyophilized powder), total protein reagent (to determine total protein content of the samples) and haemoglobin (ferric) were purchased from Sigma Chemical Co. (St Louis, MO). All HPLC solvents and analytical grade chemicals were purchased from Fisher Scientific (Springfield, NJ).

Analytical methodology

The HPLC system consisted of a solvent delivery system (Waters, Milford, MA), an autosampler (Hitachi AS-2000; Danbury, CT) and a fluorescence detector (Gilson, Middleton, MI). The column was a Novapak C18 cartridge (Waters), the mobile phase was methanol–ammonium formate buffer (0.1%, v/v, pH 4.0), 70:30 (v/v), the flow rate was 2 mL min^{-1}, and detection was carried out at excitation and emission wavelengths of 480 and 580 nm, respectively.

Binding of doxorubicin and epirubicin to intact erythrocytes

The number of erythrocytes was determined by a Neubaeur haemocytometer. Approximately 20×10^6 cells were suspended in isotonic phosphate buffer (pH 7.4) and incubated at 37°C for 24 h with different concentrations of doxorubicin or epirubicin (1 mL; $n = 6$). The stability of the drugs during the incubation was monitored by control samples containing only drug

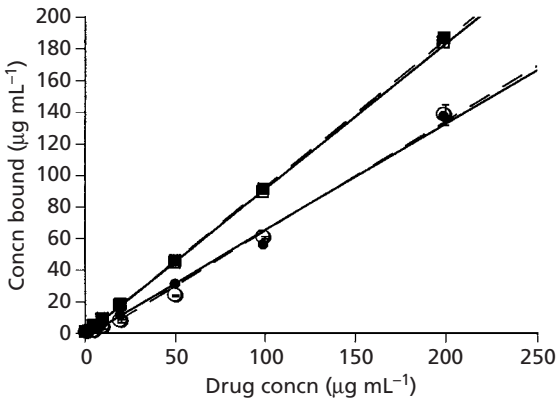

Figure 1 Isotherms of the interaction of doxorubicin and epirubicin with erythrocytes (●, ○, respectively) and erythrocyte ghosts (■, □, respectively). Data are shown as mean ± s.d., n = 6.

solutions. The final concentration of each drug after addition to cell suspensions (10^6 cells mL^{-1}) was 0.5–100 μg mL^{-1}. After the incubation, the samples were centrifuged at 7500 *g* for 15 min and the free drug was measured by a fluorometer (Perkin Elmer, Norwalk, CT) at excitation and emission wavelengths of 480 and 580 nm, respectively.

Binding of doxorubicin and epirubicin to erythrocyte ghosts

This experiment was carried out to determine the magnitude of binding of both drugs to erythrocytes without haemoglobin. Erythrocytes were washed with an equal volume of hypotonic phosphate buffer (0.9 g sodium phosphate monobasic anhydrous and 0.7077 g sodium phosphate dibasic in 1 L double-distilled water). After each wash, the cells were centrifuged at 7500 *g* for 15 min. The procedure was repeated several times until the pellet and supernatant became almost colourless. Similar concentration ranges of doxorubicin or epirubicin were incubated in the presence of erythrocyte ghosts (10^6 mL^{-1}). The incubation conditions and fluorometric determination were the same as described for erythrocytes. The stability of each drug was also monitored with each incubation.

Binding of doxorubicin and epirubicin to haemoglobin

Haemoglobin solution with a physiological concentration of 6% (w/v) was incubated with different concentrations of doxorubicin or epirubicin (0.2–100 μg mL^{-1}, n = 4 per concentration) at 37°C for 90 min followed by ultracentrifugation at 100000 *g* for 15 h. We selected ultracentrifugation over other separation techniques, such as dialysis or filtration, because of the extensive binding of doxorubicin to filters and dialysis membranes or probes. The conventional preventive measures such as storage of filters, membranes or microdialysis probes with solutions of albumin or Tween-20 did not reduce the binding of doxorubicin significantly. The 15-h ultracentrifugation provided a clear supernatant containing the free drug concentration. The supernatants were further analysed by HPLC.

Binding of doxorubicin and epirubicin to plasma proteins

Human plasma (3 mL) was incubated with doxorubicin or epirubicin solution (1 mL) to give a concentration range of 0.25–50 μg mL^{-1}. Incubation was carried out in quadruplicate at 37°C for 90 min followed by ultracentrifugation at 100000 *g* for 15 h. The supernatants were analysed for free drug by the fluorometric assay as described for the erythrocyte binding study. The con-

Table 1 Interaction of doxorubicin and epirubicin with erythrocytes, erythrocyte ghosts and plasma proteins according to the Freundlich isotherm.

Sample	Adsorption intensity		Adsorbtion capacity	
	Doxorubicin	Epirubicin	Doxorubicin[a]	Epirubicin[a]
Erythrocytes	0.96	1.23	2.73*	0.75*
Erythrocyte ghosts	1.12	1.06	14.26	12.74
Plasma proteins	0.96	0.99	0.03	0.03

[a]μg of drug bound per mg of sample. *$P < 0.05$.

centration of proteins in plasma was determined by the Biuret method (Gornall et al 1949).

Results

The isotherms of doxorubicin and epirubicin binding to erythrocytes and erythrocyte ghosts are presented in Figure 1. The regression analysis of the data yielded a zero y-intercept and slopes of 0.65–0.66 for erythrocytes and 0.91–0.93 for erythrocyte ghosts. The slopes represent the association constant of the drugs. The data were very reproducible and the standard deviation of the six measurements per data point was not significant. The coefficient of determination of all lines (r^2) was 0.99. There was no significant difference between the two drugs in their interaction with erythrocytes or erythrocyte ghosts. The interaction of both drugs with erythrocytes was significantly different from erythrocyte ghosts. The isotherms of binding of doxorubicin and epirubicin to plasma proteins indicated no significant difference between their binding to plasma proteins. The overall association constants of doxorubicin and epirubicin with plasma proteins, determined by the regression analysis, were 0.70 and 0.66, respectively ($r^2 = 0.99$).

We further analysed the data according to the Freundlich isotherm using the following equation:

$$\text{Log r} = \text{nLog C} + \text{Log K}$$

where r is the amount of drug bound per mg of protein or per unit mass of erythrocyte or erythrocyte ghosts. The independent variable is the logarithm of the unbound concentration of drug (Log C). The slope, n, is the intensity of interaction and the inverse logarithm of the y-intercept, and K is the binding capacity. A comparison of the two parameters in the interaction, namely the intensity and capacity of the interaction are presented in Table 1. The difference between the two compounds in their interaction with erythrocytes was statistically significant.

The binding of doxorubicin to haemoglobin was significantly different from epirubicin. The isotherms of binding of both drugs were non-linear and hyperbolic (Figure 2A). The data were analysed according to the Michaelis-Menten equation using PCNONLIN (Scientific Consulting Inc., Lexington, KY). The predicted maximum binding capacity for doxorubicin was 20.12 μg mL^{-1}, corresponding to 0.42 μg drug per mg haemoglobin. For epirubicin the value was 192.17 μg mL^{-1}, corresponding to 4.0 μg drug per mg protein. The required concentration for 50% saturation of the binding sites was 19.31 μg mL^{-1} for doxorubicin and 221.23 μg mL^{-1} for epirubicin. The Scatchard plot revealed two distinct classes of binding sites for each drug per mg haemoglobin (Figure 2B; Table 2). The constant of association of epirubicin with the class 1 binding sites (K_1) was significantly greater than that of doxorubicin, whereas the constant of association of doxorubicin with class 2 binding sites (K_2) was significantly greater than that of epirubicin. The number of class 1 binding sites

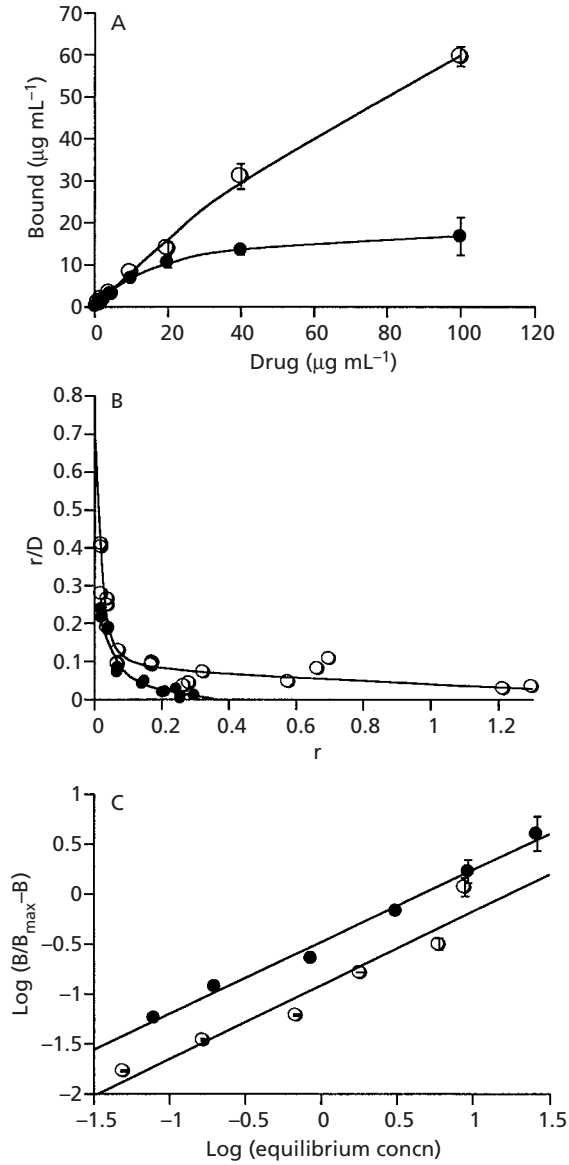

Figure 2 Comparison of the binding of doxorubicin (●) and epirubicin (○) to haemoglobin: isotherm (A), Scatchard (B) and Hill (C) plots. The solid lines represent the predicted values by the Michaelis-Menten, Scatchard and Hill equations, respectively. Data are shown as mean ± s.d., n = 4.

Table 2 Binding parameters of doxorubicin and epirubicin to haemoglobin, estimated by the Scatchard plot[a].

Drug	High affinity low capacity sites		Low affinity high capacity sites	
	n_1 (sites mg^{-1})	K_1(mL μg^{-1})	n_2 (sites mg^{-1})	K_2 (mL μg^{-1})
Doxorubicin*	0.072	3.09	0.305	0.096
Epirubicin*	0.030	21.29	1.963	0.039

[a] $r = n_1K_1[D]/(1+K_1D) + n_2K_2[D]/(1+K_2D)$, where r is the concentration of bound drug per concentration of haemoglobin, D is the concentration of unbound drug, n_1 and n_2 are the high affinity and low affinity binding sites per mg of haemoglobin, respectively, and K_1 and K_2 are the corresponding association constants. *$P < 0.005$.

per mg haemoglobin (n_1) for doxorubicin was approximately twice that of epirubicin. The number of class 2 binding sites (n_2) was significantly greater for epirubicin. We also evaluated the data according to the following version of the Hill plot:

$$\text{Log}\,(B/(B_{max}-B)) = n_H\,\text{Log}\,(C_{eq}) - n_H\,\text{Log}\,K_D$$

where B is drug bound to haemoglobin, B_{max} is the maximum binding capacity estimated from the Michaelis-Menten equation, C_{eq} is the equilibrium concentration, K_D is the dissociation constant and n_H is the slope. A value greater than 1 for the slope would have indicated a positive correlation. However, the estimated values for doxorubicin and epirubicin were 0.72 and 0.74, respectively, an indication of non-cooperative binding (Figure 2C).

Discussion

Based on the data presented here, it seems unlikely that protein binding by itself plays a significant role in the differences between the cardiotoxicity of doxorubicin and epirubicin. The corresponding association constants were essentially the same. The interaction of both drugs with intact erythrocytes emphasized the importance of erythrocytes in the disposition of both drugs. Comparison of the association constants of erythrocytes with those of erythrocyte ghosts revealed a higher degree of association for both drugs with erythrocyte ghosts. This was expected since the procedure utilized in this study for preparation of the erythrocyte ghosts yielded intact ghosts with preserved membrane (Al-Achi & Boroujerdi 1990a, b). Therefore, the association of both compounds with erythrocyte ghosts was due to a large extent to the encapsulation phenomenon. There was no significant difference between the association constants of epirubicin and doxorubicin in their interaction with erythrocyte ghosts. We concluded that the extent of uptake by erythrocyte ghosts, whether related to binding to membrane or encapsulation, or both, was the same for both drugs. Comparison of the data from erythrocyte ghosts with intact erythrocytes also indicated that both drugs permeate through the membrane of intact erythrocytes and a significant portion of either drug, not necessarily equal portions, remains inside and potentially bound to haemoglobin. Therefore, the lower uptake by intact erythrocytes with respect to erythrocyte ghosts can be attributed to the limit that haemoglobin imposes on the presence of drug inside the erythrocytes, either by its physical presence or limited binding sites. We used the Freundlich isotherm to estimate the two parameters of this association, namely the intensity (slope), and capacity (y-intercept) of the interaction. According to this analysis, there was a significant difference between the two compounds in the intensity and capacity of their interaction with erythrocytes. The higher intensity of epirubicin is consistent with its higher lipid solubility. The higher capacity of doxorubicin is consistent with the self-association property on the surface of the cells (Dalmark & Storm 1981). Both parameters were higher for doxorubicin in the interaction with erythrocyte ghosts.

The isotherms of binding of epirubicin and doxorubicin to haemoglobin indicated that this protein has limited capacity for doxorubicin and the maximum binding for this compound does not exceed 0.42 μg drug per mg haemoglobin. The predicted maximum binding of epirubicin was ten times greater than for doxorubicin. The Scatchard plots clearly revealed two classes of binding sites, class 1 (high affinity and low capacity) and class 2 (low affinity and high capacity) binding sites. For doxorubicin the number of class 1 binding sites was significantly greater than epirubicin, but doxorubicin

had a weaker affinity for this class of binding sites. Epirubicin had a higher affinity for class 1 binding sites. Since a smaller number of class 1 binding sites are available for this drug, they can become saturated much faster for epirubicin than doxorubicin. The number of class 2 binding sites was significantly greater for epirubicin, but doxorubicin had a greater affinity for this class of binding site. Hence, the class 2 binding sites were saturable for doxorubicin. These results indicate that the binding of doxorubicin to class 2 binding sites was more favourable than its binding to class 1, whereas binding of epirubicin was more favourable to class 1 binding sites. This conclusion is consistent with the Hill plot data analysis which releaved the interaction of both compounds with haemoglobin to be non-cooperative. We also concluded that since epirubicin is more lipophilic and its combined number of binding sites (class 1 and 2) on haemoglobin are greater than for doxorubicin, its interaction with erythrocytes is mainly with haemoglobin. Doxorubicin, because of its self-association property, may remain significantly adsorbed on the surface of the erythrocytes.

The nature of the class 1 and 2 binding sites on haemoglobin is not known at this time. It is possible that the iron moiety may be a part of class 1 binding sites, or that non-specific binding sites are part of class 2 binding sites. The data presented here suggest that the nature of the interaction of doxorubicin with intact erythrocytes is different from that of epirubicin. Haemoglobin plays a significant role in this interaction and may influence the disposition of both drugs in-vivo differently.

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