Influence of Verapamil and Digoxin on the *in Vitro* Binding of Doxorubicin to the Rat Heart

Loreto ÁLVAREZ-CEDRÓN, Francisco G. LÓPEZ, and José M. LANAÓ*

Department of Pharmacy and Pharmaceutical Technology, University of Salamanca, Av. Campo Charro, 37007-Salamanca, Spain. Received October 31, 1997; accepted May 15, 1998

A study has been carried out to characterize the binding of doxorubicin to heart homogenates and subcellular fractions. The technique chosen to perform the study was equilibrium dialysis and the levels of anthracycline in the samples obtained from the dialysis were assessed using HPLC. Doxorubicin has high affinity to heart homogenates and subcellular fractions such as nuclear, mitochondrial and microsomal. The binding was saturable and dose-dependent. Doxorubicin binding is decreased in the presence of digoxin and especially verapamil.

Key words doxorubicin; heart; subcellular fraction; binding; verapamil; digoxin

Doxorubicin (DXR) is an antineoplastic agent used in the treatment of some acute leukaemias, lymphomas and certain solid tumours. It is often used in multiple therapy regimens in combination with other cytostatic agents.^{1–3)} Despite its efficacy, its dose-dependent cardiotoxicity is a strong side effect which limits its use. Electrocardiographic alterations, cardiomyopathy and congestive heart insufficiency make up the most common clinical complications and this is accompanied by risks of acute and/or chronic toxicity that may become irreversible and eventually lethal for the patient.^{4–6)}

Several biochemical hypotheses have been advanced to account for the mechanism through which these side effects occur: inhibition of DNA synthesis,^{7,8)} the formation of free radicals and superoxides,^{8–10)} the appearance of active metabolites such as doxorubicinol,^{4,8)} modifications in calcium homeostasis,^{9,11,12)} and the release of vasoactive substances.⁴⁾ Thus, the use of protective agents such as antioxidants,^{13,14)} chelating agents¹⁵⁾ and calcium antagonists^{9,16,17)} has been proposed to ameliorate the side effects associated with this compound. Parallel to its biochemical basis, the toxicity of these drugs may be related to their broad distribution, uptake and accumulation in the heart.^{1,18)} In the light of this, it seems justifiable to use protective drugs that have a high affinity to heart tissue such as verapamil (VPM)^{10,17)} and digitalics, like digoxin (DGX),^{19–21)} because they could reduce the binding to heart which is involved with the cardiotoxicity. The aim of the present work was to conduct an *in vitro* study of DXR binding to heart homogenates and isolated subcellular fractions and to assess the possible effect of DGX and VPM on the binding of DXR to heart tissue and subcellular fractions.

MATERIALS AND METHODS

Tissue Homogenates Homogenates were prepared from Wistar rat (mean weight 260 ± 10 g) hearts at a concentration of 50 mg/ml in phosphate buffer (1/15 mol/l, pH 7.4) to study the binding of DXR to heart tissue. These homogenates were used in the different binding experiments.

Subcellular Fractions Using the differential centrifugation technique at 4 °C, nuclear (10 min, 3000 rpm), mitochondrial (30 min, 12000 rpm) and microsomal (30 min, 22500 rpm) subcellular fractions were obtained from heart tissue homogenates (10% w/v) prepared in 0.25 mol/l su-

crose.²²⁾

Equilibrium Dialysis The *in vitro* binding studies of DXR to heart homogenate and to different subcellular fractions were carried out using the equilibrium dialysis technique.^{22–24)} For dialysis, 1 ml of homogenate (from tissue or subcellular fractions) at wet tissue weight concentration of 50 mg/ml was placed in one compartment of the dialysis cell against 1 ml of a solution of DXR of known concentration prepared in phosphate buffer. The time of dialysis required to reach distribution equilibrium was 4 h. The process was carried out at physiological temperature (37 °C).

Previous studies had shown that DXR is not adsorbed onto the dialysis membrane (m.w.c. 10000/i.d. 0.63 mm). Neither was any significant volume shift across the membrane observed. Using this technique, three types of experiments were performed.

1. The study of DXR binding to heart homogenates: 1a. In the equilibrium situation, for an unbound concentration range between 50 and 4000 ng/ml. 1b. As a function of time, determining unbound concentrations at 20, 25, 30, 60, 120, 180, 240 min. Initial DXR concentration was 1000 ng/ml. The experiment was repeated five times.

2. The study of DXR binding to heart subcellular fractions (nuclear, mitochondrial and microsomal) in the equilibrium situation for an unbound concentration range between 50 and 500 ng/ml.

3. The study of DXR binding in the presence of DGX and VPM. The homogenates were incubated with high concentration solutions of DGX (100 ng/ml) and VPM (1000 µg/ml) during a period of 4 h. This methodology guaranteed an accumulation of these drugs in heart tissue. This *in vitro* situation could be comparable with an *in vivo* chronic therapy with both drugs. 3a. To heart homogenates in the equilibrium situation for an unbound concentration range between 100 and 1500 ng/ml; and as a function of time determining unbound concentration range at 20, 25, 30, 60, 120, 180, 240 min. Initial DXR concentration was 1000 ng/ml. The experiment was repeated five times. 3b. To subcellular fractions in the equilibrium situation using an unbound concentration range of 50 to 500 ng/ml.

Analytical Technique An HPLC technique was used to determine unbound DXR concentrations in the different samples, employing a reverse phase column (nucleosil C-18, 10 µm, 25 × 0.4 cm), a mobile phase (70% methanol–30%

* To whom correspondence should be addressed.

H₃PO₄ 0.01 mol/l, pH=3.01±0.04) with a flow rate of 2 ml/min. The pump was equipped with a fluorescence detector ($\lambda_{\min}$ 469/ $\lambda_{\max}$ 555). Samples were quantified immediately after dialysis and were not treated prior to determination.^{25–27} The amount of DXR bound to heart was calculated by the difference between the initial and unbound concentrations, normalizing the amount of DXR bound per gram of tissue or subcellular fraction. The partition coefficient (PC) was determined as the ratio between the bound and unbound concentration in the equilibrium situation. Statistical analysis of the technique showed good linearity and reproducibility, the sensitivity limit being 50 ng/ml. The interday coefficient of variation of the analytical technique was less than 12%.

Kinetic Analysis For the *in vitro* studies on DXR binding to heart homogenates and subcellular fractions, a non-linear model that describes specific and saturable binding was used:

$$C_b = \sum_{i=1}^m \frac{C_{\max} \cdot C_u}{K_{di} + C_u}$$

where C_b (ng/g) is bound concentration; C_u (ng/ml) is the unbound concentration; $C_{\max}$ (ng/g) is the maximum saturation concentration; K_{di} (ng/ml) is the dissociation constant and m is the number of binding sites for each class, i .²⁸⁾

For the *in vitro* studies of the binding of DXR to heart as a function of time a first-order uptake model was used:

$$C_b = C_b^{\max}(1 - e^{-Kt})$$

where C_b is the bound concentration at time t ; $C_b^{\max}$ is the maximum bound concentration and K , is the first-order binding constant.

The experimental results were fitted to the different binding models proposed using a non-linear regression program. The statistical criteria for studying the goodness of fit were the parameter estimation error, Akaike's information criterion (AIC) and residuals analysis.²⁹⁾

Statistical Analysis Statistical comparison of the PC and the binding constants of DXR to heart tissue in the different experimental situations was performed using parametric statistics after confirming distribution normality.²⁹⁾

RESULTS

Binding to Heart Homogenates DXR is characterized by its strong affinity for heart tissue homogenates at therapeutic concentrations. For an unbound concentration in the equilibrium situation of about 100 ng/ml (99.49±5.48), the mean PC of DXR was $4.32 \times 10^2 \pm 0.21 \times 10^2$. The binding of DXR to heart homogenates also proved to be a concentration-dependent non-linear process, the PC gradually decreasing parallel to the increase in the unbound concentration (C_u) of drug, which was characterized by the following potential equation:

$$PC = 31.90 \times 10^2 \cdot C_u^{-0.46}$$

Figure 1 shows the potential relationship between PC and unbound concentration of DXR in equilibrium situation. Using goodness of fit statistical criteria obtained by non-linear regression, the non-linear binding model with only one type of affinity proved to be the best model to describe the binding of DXR to heart tissue homogenates. The equation

Fig. 1. Potential Relationship between PC vs. Unbound Concentration of DXR in Equilibrium Situation

fitted to describe the non-linear process was the following:

$$C_b = \frac{20.89 \times 10^4 \pm 15.94 \times 10^3 \cdot C_u}{54.17 \times 10^3 \pm 10.19 \times 10^3 + C_u}$$

where C_b is the bound concentration, and C_u is the unbound concentration.

The binding of DXR to heart homogenates as a function of time was characterised by a first-order process, as may be seen in Fig. 2. The estimated mean first-order binding constant had a value of $3.10 \times 10^{-2} \pm 1.73 \times 10^{-2} \text{ min}^{-1}$ and the binding half life was $28.20 \pm 15.01 \text{ min}$. Accordingly, 95% of the equilibrium of the distribution process is reached in about 2 h and 99% in 3 h. DXR also showed strong affinity for the different subcellular fractions studied. Figure 3 shows the PC of DXR and its statistical comparison in heart homogenate and the nuclear, mitochondrial and microsomal fractions for unbound DXR concentrations in the equilibrium situation ranging between 100 and 200 ng/ml.

The results thus point to the existence of a very significant reduction in the PC of DXR to subcellular fractions as compared with the situation found for heart homogenates. However, no differences in the binding of DXR to the different subcellular fractions themselves were apparent. Parallel to the situation with heart homogenates, DXR binding to the different subcellular fractions was a concentration-dependent non-linear process accompanied by a progressive decrease in the PC as the unbound concentration of drug increased. This process was characterised by the following potential equations:

$$\begin{aligned} \text{nuclear fraction:} & \quad PC = 2.71 \times 10^2 \cdot C_u^{-1.03} \\ \text{mitochondrial fraction:} & \quad PC = 3.19 \times 10^2 \cdot C_u^{-1.06} \\ \text{microsomal fraction:} & \quad PC = 1.66 \times 10^2 \cdot C_u^{-0.96} \end{aligned}$$

In the presence of DGX and VPM the PC of DXR in heart tissue homogenates, for high and low unbound drug concentrations was significantly reduced, as seen in Figs. 4 and 5.

Table 1 shows a comparison of the mean PC of DXR and its statistical significance between control heart tissue and heart tissue incubated with DGX and VPM. Figure 6 shows a

Fig. 2. Mean Curve ($\pm$ S.D.) of DXR Bound Concentration to Heart vs. Time

Fig. 3. Study of the PC of DXR in Subcellular Fractions

Unbound concentrations from 100 to 200 ng/ml.

Fig. 5. Study of the PC of DXR to Heart

Control, DGX and VPM groups. Concentrations unbound from 1100 to 1600 ng/ml.

Fig. 4. Study of the PC of DXR to Heart

Control, DGX and VPM groups. Unbound concentrations from 150 to 200 ng/ml.

comparison of the mean DXR uptake curves as a function of time by tissue homogenates previously treated with DGX and VPM and the control group. The mean binding constant values for the control group, the DGX-treated group and the VPM-treated group were $3.1 \times 10^{-2} \pm 1.73 \times 10^{-2}$, $2.65 \times 10^{-2} \pm 9.26 \times 10^{-3}$ and $9.83 \times 10^{-3} \pm 6.01 \times 10^{-3} \text{ min}^{-1}$ respectively, with a statistically significant reduction ($p < 0.05$) in the DXR-heart binding constant in the presence of VPM. In the presence of VPM the time necessary for distribution equilibrium to be reached increased, being around 5 h for 95% and 8 h for 99% of the distribution process, respectively. Tables 2 shows a statistical comparison of the results obtained following incubation of subcellular fraction homogenates with DGX and VPM. As seen, differences exist in binding at the nuclear and the mitochondrial but not at the microsomal level. Both for the nuclear and mitochondrial fractions there are differences in PC in the presence of DGX and VPM for a concentration range between 250 and

Table 1. Statistical Comparison of PC ($\pm$ S.D.) in Heart Homogenates

Unbound concentration range (ng/ml)	PC			C/D		C/V		D/V	
	C	D	V	P	Significance	P	Significance	P	Significance
150—200	2.38 $\pm$ 0.42	1.07 $\pm$ 0.01	0.77 $\pm$ 0.03	1.59 $\times 10^{-3}$	S	1.51 $\times 10^{-4}$	S	6.46 $\times 10^{-3}$	S
250—350	1.76 $\pm$ 0.01	0.50 $\pm$ 0.01	0.46 $\pm$ 0.03	<10 $^{-6}$	S	<10 $^{-6}$	S	0.43	NS
750—1100	1.89 $\pm$ 0.05	1.00 $\pm$ 0.03	0.88 $\pm$ 0.03	1.23 $\times 10^{-4}$	S	1.80 $\times 10^{-4}$	S	0.37	NS
1100—1600	1.18 $\pm$ 0.03	0.47 $\pm$ 0.01	0.42 $\pm$ 0.01	<10 $^{-6}$	S	<10 $^{-6}$	S	0.31	NS

Control (C), DGX (D) and VPM (V) groups.

Table 2. Statistical Comparison of PC ($\pm$ S.D.) in Nuclear, Mitochondrial and Microsomal Subcellular Heart Fractions

Unbound concentration range (ng/ml)	Subcellular fraction	PC			C/D		C/V		D/V	
		C	D	V	P	Significance	P	Significance	P	Significance
50—100	Nuclear	3.43 $\pm$ 1.33	2.40 $\pm$ 0.36	3.11 $\pm$ 0.89	2.57 $\times 10^{-2}$	S	0.9	NS	0.87	NS
	Mitochondrial	3.48 $\pm$ 0.76	3.05 $\pm$ 0.20	2.36 $\pm$ 0.90	0.37	NS	3.22 $\times 10^{-2}$	S	0.23	NS
	Microsomal	2.32 $\pm$ 0.23	2.59 $\pm$ 0.89	2.47 $\pm$ 1.48	0.68	NS	0.85	NS	0.90	NS
250—300	Nuclear	1.10 $\pm$ 0.01	0.28 $\pm$ 0.01	0.28 $\pm$ 0.01	2.74 $\times 10^{-5}$	S	<10 $^{-6}$	S	0.98	NS
	Mitochondrial	1.14 $\pm$ 0.11	0.68 $\pm$ 0.02	0.65 $\pm$ 0.04	3.46 $\times 10^{-4}$	S	1.07 $\times 10^{-2}$	S	0.69	NS
	Microsomal	0.41 $\pm$ 0.01	0.46 $\pm$ 0.01	0.61 $\pm$ 0.01	0.58	NS	6.11 $\times 10^{-2}$	NS	0.13	NS

Control (C), DGX (D) and VPM (V) groups.

Fig. 6. DXR Bound Concentration to Heart vs. Time
Control, DGX and VPM groups.

350 ng/ml; in both unbound concentration ranges, the effects of VPM at mitochondrial level and of DGX at the nuclear level are remarkable. No significant differences were seen in any case on comparing the variations in partition due to the presence of DGX and VPM.

DISCUSSION

The use of DXR is limited principally by dose-dependent cardiotoxicity. Several clinical studies have suggested a possible role of digitals preparations, especially DGX, in preventing chronic DXR cardiotoxicity^{20,21} presumably through competitive inhibition of myocardial uptake, however others have disputed these findings.¹⁹ Therefore numerous reports suggest that VPM and other calcium antagonists are effective

in limiting the general toxicity and the functional cardiotoxicity of DXR.^{4,10,17}

The present results point to the strong affinity of DXR to heart tissue homogenates *in vitro* and confirm the great affinity for heart tissue observed *in vivo* by other authors.^{10,18} This strong affinity is the pharmacokinetic basis of DXR-elicited cardiotoxicity, regardless of the biochemical mechanism(s) proposed at the cellular level to justify the drug's toxicity. Additionally, the fact that DXR binding to heart homogenates is a specific and saturable concentration-dependent process suggests that its distribution in heart tissue *in vivo* would be a dose-dependent process; hitherto, this has not been reported in the literature. Unfortunately, the loss of integrity of the tissue studied during the homogenisation step precludes extrapolation of the results to *in vivo* studies.

DXR binding to subcellular fractions is high and specific. However, for the same experimental conditions, binding in heart homogenates is higher than in any of the individual subcellular fractions and there are no differences in binding between the different subcellular fractions. The specific nature of DXR binding to the different subcellular fractions assayed here could account for the non-linear process observed in the binding of the drug to heart homogenates. The present results suggest that the strong affinity of DXR for heart tissue and the specific nature of the binding of the drug could account for its cardiotoxicity. Although the mechanism through which DXR causes alterations in the structure and function of the myocardium remains to be fully elucidated, the hypotheses so far advanced such as free radical and superoxide formation,^{9,10,13} doxorubicinol metabolite^{4,8} and especially, modifications of mitochondrial membrane functions^{4,7} point to mechanisms related to the penetration and uptake of DXR in myocardium cells.

Our results show a decrease in the PC of DXR in heart homogenates and in subcellular fractions, in the presence of DGX, and especially VPM. Moreover, we observed a re-

duced DXR myocardial uptake constant in those homogenates previously treated with VPM. In the case of VPM, this protective effect has been reported previously for low doses,¹⁰⁾ and the explanation for it was based on the possible inhibition of the intracellular calcium overload induced by DXR.⁴⁾ The present results, without challenging any of the hypotheses concerning the biochemical mechanisms involved in protection against DXR cardiotoxicity, suggest the existence of significant modifications due to VPM in the process of DXR binding to heart tissue that could at least partially account for the protective effect attributed to VPM.

Nevertheless, according to the studies of Cordon-Cardo,³⁰⁾ P-glycoprotein expression was detected in heart muscle fiber. Based on this reasoning we can explain a displacement DXR binding to the P-glycoprotein in preparations pretreated with VPM or DGX. On the other hand, we should not forget that *in vitro* experimental assays that use tissue homogenates do not reproduce, in fact, the real transport mechanisms and binding models. Therefore, these *in vitro* modifications induced by DGX and VPM on the myocardial accumulation should be confirmed with an *in vivo* experimental study. According to some authors,^{10,31)} animals treated with these drugs, especially VPM, showed changes in DXR distribution. Finally, a possible explanation of our findings is that VPM and DGX may alter the kinetics of DXR in the heart and, perhaps, in other organs as well.

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