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# Development of the model of rat isolated perfused heart for the evaluation of anthracycline cardiotoxicity and its circumvention

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**1** In order to develop a predictive model for the preclinical evaluation of anthracycline cardiotoxicity and the means of preventing it, we have studied the functional parameters of perfused hearts isolated from rats receiving repeated doses of several anthracyclines.

**2** The anthracyclines studied were doxorubicin, epirubicin, pirarubicin and daunorubicin, and we also studied a liposomal formulation of daunorubicin (DaunoXome) and the co-administration of dexrazoxane (ICRF-187) and doxorubicin.

**3** Anthracyclines were administered i.p. at equimolar doses corresponding to 3 mg $kg^{-1}$ per injection of doxorubicin, every other day for a total of six doses. Dexrazoxane was used at the dose of 30 mg $kg^{-1}$ per injection and was administered either 30 min before or 30 min after doxorubicin. We evaluated any general toxicity towards the animals as well as alterations of left ventricular contractility and relaxation *ex vivo*.

**4** Epirubicin and daunorubicin were significantly less cardiotoxic than doxorubicin, and neither pirarubicin nor DaunoXome caused significant alterations in cardiac function. There was a direct relationship between the decrease in cardiac contractility or relaxation and anthracycline accumulation in the heart, evaluated after the same treatment schedule.

**5** Dexrazoxane induced a significant protection against doxorubicin-induced cardiac toxicity when administered 30 min before doxorubicin, whereas this protection was ineffective when administered 30 min after doxorubicin. Direct perfusion of DaunoXome in isolated hearts of untreated animals resulted in a 12-fold reduction of the accumulation of daunorubicin in heart tissue as compared to the perfusion of free daunorubicin, and did not cause alterations in cardiac function at a dosage for which free daunorubicin induced major alterations.

**6** The isolated perfused rat heart appears to be a valuable model for screening of new anthracyclines and of strategies for circumventing anthracycline cardiotoxicity.

**Keywords:** Anthracycline cardiotoxicity; rat isolated perfused heart; doxorubicin; daunorubicin; epirubicin; pirarubicin; anthracycline liposomal formulations; dexrazoxane

## Introduction

The cardiotoxicity of anthracyclines remains a clinical problem of major importance (Unverferth *et al.*, 1982). These drugs are required for the treatment of haematological malignancies and solid tumours, but their cumulative toxicity on the myocardium prevents their use at their maximum myelotoxic doses during the optimal number of courses required (Rhoden *et al.*, 1993). Paediatricians are specially aware of the possible occurrence of congestive heart failure several years after the cure of leukaemias or solid tumours in children (Steinherz *et al.*, 1992; Leandro *et al.*, 1994). Dose-reduction protocols have been proposed to avoid the risk of delayed cardiac toxicity, but this might be at the expense of the cytotoxic activity of the anthracycline (Lipshultz *et al.*, 1994). Numerous anthracyclines have been developed with the aim of obtaining potent drugs with reduced cardiac toxicity. Epirubicin (4′-epidoxorubicin) and pirarubicin (4′-O-tetrahydropyranyldoxorubicin) have been brought into routine clinical usage after it was observed that they produced less cardiotoxicity than doxorubicin and daunorubicin, the reference molecules, in both preclinical models and early clinical trials (Ganzina, 1983; Maehara *et al.*, 1989; Hérait *et al.*, 1992). In addition, several strategies for reducing cardiotoxicity of anthracyclines have been proposed, including the administration of α-tocopherol (Julicher *et al.*, 1986; Mimnaugh *et al.*, 1981), N-acetylcysteine (Villani *et al.*, 1990), glutathione (Villani *et al.*, 1990), and ICRF-187 or dexrazoxane (Herman & Ferrans, 1986; Speyer *et al.*, 1988). This last molecule has been shown to provide a prolonged protection against anthracycline cardiotoxicity in rabbits and dogs (Herman & Ferrans, 1986; Herman *et al.*, 1988) and to prevent the doxorubicin-induced decrease of the left ventricular ejection fraction in human subjects (Speyer *et al.*, 1988; 1992). However, its effects on ventricular contractility and relaxation have not yet been studied. Recently, drug encapsulation in liposomes has been proposed to reduce drug accumulation in the heart, and therefore cardiac toxicity (Gabizon, 1992). Indeed, it has been shown that DaunoXome, a liposomal formulation of daunorubicin, did not result in any cardiotoxicity in early clinical trials (Forssen & Ross, 1994).

There are methodological problems for the early and rapid evaluation of the cardiac toxicity of new anthracyclines and of the protective approaches developed for clinical use. The best clinical predictor is the decrease of the left ventricular ejection fraction. However, this decrease is often discovered too late to avoid congestive heart failure, and excess precaution may considerably slacken the dose increase schedule. The evaluation of the risk of congestive heart failure in human subjects requires a large number of patients entering the clinical pro-

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tocols. Preclinical information on the potential cardiac toxicity of a new drug or of a new association is therefore required before the start of clinical trials. This is why we developed in a first attempt the model of the rat isolated heart, which was perfused with various concentrations of several anthracyclines, as a potential model of cardiac toxicity (Pouna *et al.*, 1995). However, despite the fact that we obtained reproducible alterations of cardiac function, which were different for the anthracyclines tested, this model could not be proposed as such because it bypasses the pharmacokinetic and metabolic steps of the drug in the body, and gave only insights on the direct action of anthracyclines on the heart muscle. It was not possible with this model to evaluate the roles of the distribution, metabolism and elimination of a given anthracycline upon its cardiac toxicity; in addition, the study of the circumvention of cardiac toxicity by drug encapsulation or by cardioprotectors could not be performed because the efficiency of these approaches could well be related to alterations in anthracycline pharmacokinetics.

We have therefore developed the isolated perfused heart preparation of the rat for evaluating the left ventricular functional parameters after treatment of the rats by various anthracyclines, eventually in association with cardiac protectors. We show in this paper that this preclinical model can be used for screening of new anthracyclines and of ways of circumventing anthracycline cardiotoxicity. The role of drug encapsulation as well as that of dexrazoxane in this purpose is exemplified in this work. Direct perfusion of anthracyclines in the isolated heart preparation of untreated rats was compared to the perfusion of hearts isolated from treated rats.

## Methods

### *Experimental animals*

*Heart perfusion from treated rats* Male Sprague-Dawley rats (10 weeks) were divided at random into several groups: (i) groups of 6–8 animals received equimolar quantities of anthracyclines i.p. every other day for 11 days (3 mg doxorubicin or epirubicin per kg body weight, 3.2 mg pirarubicin $kg^{-1}$, 2.9 mg daunorubicin or DaunoXome $kg^{-1}$); (ii) groups of 6–7 animals were treated with dexrazoxane i.p. (30 mg $kg^{-1}$), 30 min before or after doxorubicin administration (3 mg $kg^{-1}$) which was performed following the same schedule as before, (iii) a group of 8 animals received 0.9% NaCl solution (200 $\mu$l) every other day for 11 days and served as control.

The rats were weighed every two days and assessed for possible abnormalities such as ascites, diarrhoea and epistaxis. Rats were killed on the 12th day after intitial treatment, hearts were removed and perfused, cardiac functional parameters were monitored as described below and the hearts were weighed after the end of the experiments. Independent series of animals were treated as described previously in (i), except that, after removal of their hearts, a portion of about 120 mg of the left ventricle was sampled and kept frozen for evaluation of anthracycline cardiac accumulation as described below.

*Direct perfusion of isolated hearts from untreated rats* Hearts from male Sprague-Dawley untreated healthy rats (11–12 weeks) were removed and perfused according to different schedules: (i) hearts of 2 groups (6–7 animals) were perfused with Krebs-Henseleit buffer for 30 min and then with free anthracycline or liposomal daunorubicin ($10^{-6}$ M and $10^{-5}$M) for 70 min; (ii) hearts of a group of 6 animals were perfused with dexrazoxane ($10^{-4}$ M in Krebs-Henseleit buffer for 30 min, and then with doxorubicin ($10^{-5}$ M) for additional 70 min; (iii) hearts of a group of 8 animals were perfused with Krebs-Henseleit buffer for 100 min. At the end of perfusion of rat hearts with free daunorubicin or DaunoXome, left ventricle samples weighing about 120 mg were taken and intracardiac drug accumulation was estimated as described below.

### *Perfusion of rat isolated hearts*

Rats were heparinised i.p. (500 iu per 100 g body weight) and anaesthetized with diethylether. The heart was quickly excised and briefly soaked in a Krebs-Henseleit solution at 4°C. Coronary perfusion was initiated through a short cannula in the aortic root and maintained at a constant pressure of $92.8 \pm 1.9$ mmHg in a nonrecirculating way by the Langendorff technique as described by Lorell *et al.* (1986). Perfusion pressure was measured by a $P_{23Db}$ transducer (Bentley Trantec) connected to the aortic infusion cannula. The heart was electrically paced at a rate of 300 beats $min^{-1}$ (5 Hz) through stimulator-activated stainless steel electrodes placed on the heart. A latex balloon attached to one end of a polyethylene catheter was placed in the left ventricle through the mitral valve. The catheter was filled with water and the other end was linked to an electronic amplifier (Thomson Medical) via a second $P_{23Db}$ transducer.

The coronary perfusion pression and the left ventricular pressure were recorded on a computer that allowed continuous monitoring of heart rate, left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), left ventricular developed pressure (LVDP) and the maximal and minimal first derivatives of LVSP as a function of time [$LV(dP/dt)_{max}$ and $LV(dP/dt)_{min}$, respectively].

The perfusate consisted of modified Krebs-Henseleit buffer, pH 7.4, containing mM: NaCl 118, KCl 4.7, $MgSO_4$ 1.2, $KH_2PO_4$ 1.2, $NaHCO_3$ 25, glucose 11, $CaCl_2$ 0.95, insulin 10 iu $l^{-1}$. It was continuously bubbled with a mixture of 95% $O_2$/5% $CO_2$ and maintained at 37°C. After 30 min stabilization with buffer, the latex balloon inserted into the left ventricle was dilated with distilled water, sufficiently to produce an LVEDP of 6 mmHg, and the functional cardiac parameters were recorded. Anthracycline solution in Krebs-Henseleit could be then perfused for 70 min.

### *Anthracycline accumulation*

The samples obtained from the hearts of rats treated with anthracyclines, or after perfusion of untreated rats, were homogenized in physiological saline (2 ml for 100 mg tissue) with a tissue homogenizer (Ultra-Turrax) and kept frozen at −80°C until extraction. Anthracyclines and metabolites were extracted from 0.5 ml aliquots of the homogenates according to the method of Baurain *et al.* (1979) after addition of 0.5 ml borate buffer (50 mM, pH 9.8), 9 ml of chloroform/methanol 4/1 (v/v) and an adequate amount of internal standard (doxorubicin in the case of pirarubicin, pirarubicin in the case of daunorubicin, and daunorubicin in the case of doxorubicin and epirubicin). After mixing and centrifuging (10 min at 3000 *g*), the solvent layer was recovered, evaporated to dryness and reconstituted in a small volume of methanol. Calibration curves were obtained after incubating heart homogenates with each anthracycline *in vitro* for 15 min at room temperature (De Jong *et al.*, 1991). For all anthracyclines, a good linearity was obtained from 0.015 to 1.5 nmol $mg^{-1}$ tissue. Chromatography was performed on a microBondapak $C_{18}$ column (Waters Associates) measuring 30 x 0.39 cm. The solvent was a mixture of ammonium formate buffer (60 mM, pH 4.0) and acetonitrile (66/34 v/v), delivered at 3 ml $min^{-1}$. Detection was achieved with a Perkin-Elmer LS1 spectrofluorometer with the excitation and emission wavelengths set at 480 nm and 592 nm respectively. Retention times and peak areas were recorded with a Perkin-Elmer LCI-100 integrator. In these conditions, the whole duration of the chromatographic analysis of each sample did not exceed 6 min.

### *Drugs*

Anthracyclines (hydrochloride forms) were obtained from Bellon Rhône-Poulenc Rorer, Neuilly-sur-Seine, France (daunorubicin, pirarubicin), from Pharmacia, Rueil-Malamaison, France (doxorubicin, epirubicin) and from Vestar,

Rungis, France (liposomal daunorubicin or DaunoXome). Dexrazoxane (ICRF-187, ADR-529) was supplied by Pharmacia, Columbus, Ohio, U.S.A. All drugs were diluted with sterile water to a concentration of 5 mg $ml^{-1}$, divided into aliquots and kept frozen until use.

### *Statistical analysis of the data*

Statistical comparisons between untreated and anthracycline-treated groups were made by Student's *t* test after ANOVA assumption of the validity of *t* test; all data are expressed as the mean value ± s.d. Statistical significance was determined as a *P* value below 0.05.

## Results

### *Effect of anthracyclines on cardiac function of treated rats*

*General toxicity* In the control group, no morphological or physiological alteration was observed and rat weight was increased by 18.7 ± 2.2% during the 11 days of the study. Table 1 presents the general toxicity observed in rats treated with various anthracyclines. Doxorubicin and daunorubicin appeared as the most toxic agents, generating an important weight loss and general symptoms, such as diarrhoea, ascites and epistaxis, with a high frequency. Epirubicin and pirarubicin provided less general toxicity symptoms than doxorubicin and daunorubicin; DaunoXome had pratically no general toxicity effects except for a slight reduction in weight gain during the 11 days of the study. Association of dexrazoxane with doxorubicin provided different results according to the schedule of administration: a reduction of toxicity was evident when dexrazoxane preceded doxorubicin administration by 30 min, whereas this advantage was significantly less important when doxorubicin was injected 30 min before dexrazoxane.

*Cardiac functional parameters* In the control group, LVDP, $LV(dP/dt)_{max}$ and $LV(dP/dt)_{min}$ were respectively 89.4 ± 9.7 mmHg, 2739 ± 340 mmHg $s^{-1}$ and 1780 ± 257 mmHg $s^{-1}$. Mean heart weight in this group was 1.40 ± 0.09 g. Rats treated with doxorubicin presented important alterations of these parameters (Table 2), with especially a 33% reduction in heart contractility [$LV(dP/dt)_{max}$] and a 29% reduction in heart relaxation [$LV(dP/dt)_{min}$]. Epirubicin and daunorubicin treatments induced less alterations in these parameters, since heart contractility was reduced by 18% by both drugs, and relaxation was not significantly altered by epirubicin. Pirarubicin appeared to be devoid of cardiotoxicity at the dose used, since heart contractility and relaxation were not significantly different from control. When compared to daunorubicin, DaunoXome appeared not to be cardiotoxic. Dexrazoxane

**Table 1** General toxicity of anthracyclines in rats

| | *Body weight variation (%)* | *Diarrhoea* | *Ascites* | *Epistaxis* | *Early death* |
|---|---|---|---|---|---|
| Control | +18.7 ± 2.2 | 0/8 | 0/8 | 0/8 | 0/8 |
| Doxorubicin (6 × 3 mg $kg^{-1}$) | −23.0 ± 6.0*** | 4/8 | 3/8 | 8/8 | 1/8 |
| Epirubicin (6 × 3 mg $kg^{-1}$) | −8.3 ± 2.3***††† | 0/7 | 0/7 | 7/7 | 0/7 |
| Pirarubicin (6 × 3.2 mg $kg^{-1}$) | −2.8 ± 1.1***††† | 0/7 | 0/7 | 0/7 | 0/7 |
| Daunorubicin (6 × 2.9 mg $kg^{-1}$) | −20.0 ± 3.9*** | 4/6 | 2/6 | 5/6 | 1/6 |
| DaunoXome (× 2.9 mg $kg^{-1}$) | +7.1 ± 3.5***‡‡‡ | 0/8 | 0/8 | 0/8 | 0/8 |
| Dexrazoxane (6 × 30 mg $kg^{-1}$) *then* doxorubicin (6 × 3 mg $kg^{-1}$) | −14.8 ± 5.6***† | 0/6 | 0/6 | 1/6 | 1/6 |
| Doxorubicin (6 × 3 mg $kg^{-1}$) *then* dexrazoxane (6 × 30 mg $kg^{-1}$) | −17.0 ± 4.7*** | 0/7 | 0/7 | 7/7 | 2/7 |

Rats were treated with different anthracyclines at equimolar doses, every other day for 11 days. Control rats were treated with 0.9% NaCl. The rats were weighed and assessed for eventual abnormalities such as ascites, diarrhoea and epistaxis as described in Methods. Dexrazoxane was administered 30 min before or after doxorubicin dose. Data are expressed as mean ± s.d. Significant differences as compared to control values have been indicated (***$P<0.001$), as well as significant differences for dexrazoxane plus doxorubicin as compared to doxorubicin alone (†$P<0.05$; †††$P<0.001$), and significant differences for DaunoXome as compared to free daunorubicin (‡‡‡$P<0.001$).

**Table 2** Cardiac functional parameters in rats treated with anthracyclines

| | *LVDP* (mmHg) | *LV* $(dP/dt)_{max}$ (mmHg $s^{-1}$) | *LV* $(dP/dt)_{min}$ (mmHg $s^{-1}$) | *Heart weight* (mg) |
|---|---|---|---|---|
| Control | 89.4 ± 9.7 | 2739 ± 340 | −1780 ± 257 | 1337 ± 87 |
| Doxorubicin (6 × 3 mg $kg^{-1}$) | 67.8 ± 13* | 1836 ± 451** | −1264 ± 287* | 1010 ± 107*** |
| Epirubicin (6 × 3 mg $kg^{-1}$) | 79.3 ± 15 | 2246 ± 361* | −1504 ± 304 | 1064 ± 101*** |
| Pirarubicin (6 × 3.2 mg $kg^{-1}$) | 84.3 ± 5.3 | 2426 ± 204††† | −1683 ± 61† | 1114 ± 125** |
| Daunorubicin (6 × 2.9 mg $kg^{-1}$) | 81.4 ± 7.0* | 2222 ± 227* | −1408 ± 174* | 1057 ± 72*** |
| DaunoXome (6 × 2.9 mg $kg^{-1}$) | 91.5 ± 6.2 | 2754 ± 230 | −1635 ± 230‡ | 1209 ± 56**‡ |
| Dexrazoxane (6 × 30 mg $kg^{-1}$) *then* doxorubicin (6 × 3 mg $kg^{-1}$) | 88.0 ± 8.8† | 2419 ± 216*† | −1700 ± 196 | 1116 ± 73** |
| Doxorubicin (6 × 3 mg $kg^{-1}$) *then* dexrazoxane (6 × 30 mg $kg^{-1}$) | 70.8 ± 7.4 | 1924 ± 270* | −1166 ± 99.7* | 1064 ± 55*** |

Rats were treated with different anthracyclines at equimolar doses, every other day for 11 days. Control rats were treated with 0.9% NaCl. Dexrazoxane was administered 30 min before or after doxorubicin dose. On the 12th day, rats were killed for cardiac functional study as described in Methods. After 30 min stabilization with buffer, the latex balloon inserted into the left ventricle was dilated with distilled water, sufficiently to produce an LVEDP of 6 mmHg and the functional cardiac parameters were recorded. Data are expressed as mean ± s.d. Significant differences as compared to controls have been indicated (*$P<0.05$; **$P<0.01$; ***$P<0.001$) as well as significant differences as compared to doxorubicin (†$P<0.05$; †††$P<0.001$) and to daunorubicin (‡$P<0.05$; ‡‡$P<0.01$).

treatment provided significant protection of the heart when administered before doxorubicin, whereas it had only a minor effect on doxorubicin-induced alterations of cardiac parameters when administered 30 min after doxorubicin.

*Cardiac accumulation of anthracyclines* The accumulation of various anthracyclines in the left ventricle of rats treated for 11 days with 6 doses of anthracyclines are presented in Table 3, together with the changes in heart contractility and relaxation obtained after the same treatments. It clearly appears that there is a direct relationship between intra cardiac drug accumulation and both contractility and relaxation of the heart muscle. It should be noted that only daunorubicinol was found in the heart tissue after daunorubicin or DaunoXome treatment, and that only traces of pirarubicin were found in the heart after pirarubicin treatment.

### *Effect of direct perfusion of anthracyclines in the rat isolated heart preparation*

*Cardiac functional parameters* Direct perfusion of doxorubicin, epirubicin and pirarubicin has already been studied and published (Pouna *et al.*, 1995). We wished to evaluate left ventricular dysfunction with this approach and we attempted to correlate it to drug accumulation. Comparison of free daunorubicin to DaunoXome is presented in Table 3. When both formulations were perfused at a concentration of $10^{-5}$ M, no alteration of cardiac function was seen after 70 min perfusion of DaunoXome, whereas a significant alteration of cardiac function was observed after 70 min perfusion of free drug.

With this protocol of direct perfusion, we also evaluated the potential effect of dexrazoxane on doxorubicin-induced ventricular dysfunction. The perfusion of $10^{-4}$ M dexrazoxane for 30 min prior to doxorubicin perfusion at a dose of $10^{-5}$ M did not modify the effect of doxorubicin on left ventricular contractility and relaxation (data not shown).

*Cardiac accumulation of daunorubicin* Daunorubicin accumulation in the heart muscle was dependent on the concentration of free drug present in the perfusate (Table 4): when the concentration was increased from $10^{-6}$ to $10^{-5}$ M, the accumulation increased 11 fold. At the only concentration of DaunoXome studied ($10^{-5}$ M), cardiac accumulation was of the same order of magnitude as that obtained with $10^{-6}$ M free daunorubicin (Table 4). Daunorubicinol was found in heart extracts after free daunorubicin perfusion (at $10^{-6}$ M and $10^{-5}$ M), and represented 13–15% of unchanged drug (data not shown). In contrast, no daunorubicinol was found in the heart after DaunoXome perfusion.

## Discussion

Cardiac toxicity of anthracyclines remains a major problem which has been increasing with the use of these anticancer agents in curable haematological malignancies, especially in paediatrics. However, the occurrence of congestive heart failure is a rare event below a threshold cumulative dose, and this threshold is difficult to assess during the development of a new anthracycline in humans subjected to clinical trials (Hérait *et al.*, 1992). The measure of left ventricular ejection fraction is a clinical evaluation of myocardial contractility and is routinely used to monitor anthracycline therapy in human subjects (Basser & Green, 1993), whereas endomyocardial biopsy remains far from routine clinical use. Numerous models have been developed for the understanding of anthracycline cardiotoxicity, using myocardial cells (Jiang *et al.*, 1994), papillary muscles (Lee *et al.*, 1991), microsomes (Vile & Winterbourn, 1989; Ondrias *et al.*, 1990), mitochondria (Solem & Wallace, 1993), isolated atria (Monti *et al.*, 1986; Temma *et al.*, 1993) or isolated hearts directly perfused with anthracyclines (Pelikan *et al.*, 1986; Rabkin, 1983; Del Tacca *et al.*, 1987; Pouna *et al.*, 1995). The interest of these models is to identify the possible targets of anthracyclines in the heart and to understand the

**Table 3** Relationship between cardiac dysfunction and anthracycline accumulation in the heart after anthracycline treatment

| | *Myocardial content* (nmol $g^{-1}$ tissue) | *Heart contractility* (% change) | *Heart relaxation* (% change) |
|---|---|---|---|
| Control | 0 | – | – |
| Doxorubicin | 8.0 ± 4.0 | 33 | 29 |
| Epirubicin | 2.5 ± 1.0 | 18 | 16 |
| Pirarubicin | not detectable | 11 | 5 |
| Daunorubicin | 2.7 ± 0.8* | 19 | 21 |
| DaunoXome | 1.3 ± 0.3* | 0 | 8 |

*Present as daunorubicinol exclusively.
Rats were treated with different anthracyclines at equimolar doses, every other day for 11 days. On the 13th day, rats were killed and a sample of the left ventricle was taken and frozen. Anthracycline content was extracted and estimated as described in Methods.

**Table 4** Relationship between cardiac dysfunction and anthracycline accumulation in the heart after 70 min perfusion

| *Drug* | $10^{-6}$ M | | $10^{-5}$ M | |
|---|---|---|---|---|
| | *Drug accumulation* (nmol $g^{-1}$ tissue) | *LV* $(dP/dt)_{max}$ *at 70 min* (% of initial value) | *Drug accumulation* (nmol $g^{-1}$ tissue) | *LV* $(dP/dt)_{max}$ *at 70 min* (% of initial value) |
| Daunorubicin | 78 ± 10 | 101.5 ± 6.8 | 850 ± 50 | 42.6 ± 6.4*** |
| DaunoXome | ND | ND | 63 ± 8$^{\ddagger\ddagger\ddagger}$ | 100.0 ± 5.5 |

ND: not determined. At the end of heart perfusion with free daunorubicin or DaunoXome, a sample of myocardium was taken from the left ventricle to determine drug accumulation as described in Methods. In the control group, the LV $(dP/dt)_{max}$ decreased to 97.8 ± 6.8% of initial value. Values are means ± s.d. of data obtained from at least six independent experiments. We have indicated a value significantly different from that of the control (***) or from that observed with daunorubicin ($^{\ddagger\ddagger\ddagger}$), both at the $P<0.001$ level.

mechanisms of cardiotoxicity. However, they are of little interest for the prediction of heart function impairment induced by anthracyclines. High drug concentrations are often required for the production of significant effects on most of these models (Lee *et al.*, 1991; Temma *et al.*, 1993; Solem & Wallace, 1993; Jiang *et al.*, 1994), whereas 10 to 100 fold less drug concentrations are sufficient to obtain alterations of cardiac function in animals and man. As a consequence, it is not possible to relate the observations made on these experimental models to the *in vivo* situation and to use them as predictors of the clinical toxicity.

For all these reasons, pharmacologically relevant models are required for the preclinical evaluation of anthracycline cardiotoxicity. However, in the laboratory animal, overall survival or morphological cardiac alterations after long-term treatment have been used rather than physiological alterations. This is the reason why we tried to study the functional alterations occurring in the myocardium of rats treated with anthracyclines. From these animals, the hearts could be isolated and perfused with a buffered solution and its functional ability could be studied. This model is simple and reproducible enough for rapid screening and quantitative evaluation of the actual harmful effect of anthracyclines on the heart, which is the decrease of the contractility of the left ventricle. The model of the rat isolated perfused heart is able to provide information about the cardiac toxicity of a molecule within two weeks. Early alterations of ventricular contractility and relaxation can be directly and easily quantified. Using the isolated perfused rat heart model, De Wildt *et al.* (1985) observed no alteration in myocardial contraction after 24 days of treatment with doxorubicin (cumulative dose: 7 mg $kg^{-1}$), whereas they observed a disturbance in the contraction when the cumulative dose reached 11 mg $kg^{-1}$ and 52 days of treatment. In our study, the cumulative dose was higher (18 mg $kg^{-1}$) and administered over a shorter period of time, and led to a significant decrease of cardiac contractility and relaxation. We did not explore in this study the electrophysiological properties and the spontaneous heart rate; when alterations in these parameters are produced by anthracyclines, they are generally transient and reversible and are therefore not indicative of functional alterations in the myocardium. It has been suggested that the mechanism of chronic cumulative cardiac toxicity of doxorubicin could be different from that of acute cardiotoxicity (Monti *et al.*, 1986). However, it seems reasonable, following Legha *et al.* (1982), to consider chronic cardiac toxicity as a consequence of repeated acute lesions occurring after each administration. These authors have especially shown that endomyocardial biopsies obtained early after doxorubicin treatment were predictive of the late lesions observed after chronic administration.

We observed in this study that epirubicin, pirarubicin and daunorubicin were less cardiotoxic than doxorubicin, the reference anthracycline. In other studies, the effects of epirubicin were identical (Vile & Winterbourn, 1989; Hirano *et al.*, 1994) or less pronounced (Llesuy *et al.*, 1985) than those of doxorubicin. Similarly, pirarubicin has been shown to be less cardiotoxic than doxorubicin in two studies (Temma *et al.*, 1993; Hirano *et al.*, 1994) and more cardiotoxic in a third one (Del Tacca *et al.*, 1987). Discrepancies were also found for daunorubicin, which was shown either less cardiotoxic (De Jong *et al.*, 1993) or more cardiotoxic (Vile & Winterbourn, 1989) than doxorubicin. In previous work, we had studied direct perfusion of several anthracyclines in the isolated heart preparation from untreated rats (Pouna *et al.*, 1995). This approach provided information on the possible mechanisms of cardiac toxicity, but could not take into account drug distribution and metabolism in the intact animal, which appear as a determinant factor of cardiac toxicity. This is exemplified by the fact that epirubicin, when perfused directly in the rat isolated heart, appeared more cardiotoxic than doxorubicin, whereas it appeared less cardiotoxic than doxorubicin when injected into the animal prior to heart removal and study. We suggested in our previous study that epirubicin should be less cardiotoxic than doxorubicin in clinics because of pharmacokinetic reasons, and we confirm this hypothesis here. Indeed, the accumulation of epirubicin in the heart of animals is at least 3 fold lower than that of doxorubicin administered at the same dose in the same conditions. This difference is higher than the difference observed by van der Vijgh *et al.* (1990) comparing the AUCs (0–48 h) of doxorubicin and epirubicin in mice hearts. Pirarubicin appeared much less cardiotoxic than doxorubicin both in treated and intact animals; its accumulation after direct heart perfusion was much higher than that of doxorubicin (Pouna *et al.*, 1995) and we therefore attributed its reduced cardiac toxicity to pharmacodynamic reasons related to differences in the mechanism of action of this compound as compared to doxorubicin. However, we now show that, in the intact animal, repeated treatment leads to barely detectable levels of drug accumulation in the heart, which could also well explain its reduced cardiac toxicity. Such a rapid disappearance of pirarubicin from the heart has also been observed in mice (Iguchi *et al.*, 1985).

In order to validate the model, we used two approaches which had been sugggested to reduce anthracycline cardiac toxicity. The first one, which consists in drug encapsulation in liposomes or nanospheres, was developed with the aim of preferential targetting to the tumour rather than to the heart (Gabizon, 1992). Indeed, it clearly appeared from our results that daunorubicin encapsulation in liposomes was followed by a complete disappearance of cardiac toxicity. This could be attributed to a dramatic reduction of drug accumulation in the left ventricle, which could be shown both after treatment of rats with the two formulations of daunorubicin, and after direct perfusion of the hearts of untreated rats. Liposomal daunorubicin is used successfully at present for the treatment of Kaposi's sarcoma (Presant *et al.*, 1993) and has been administered in patients without clinical cardiac toxicity, at cumulative doses far beyond the usual threshold admitted for daunorubicin. It can be hypothesized that the differential uptake of daunorubicin between tumour cells and myocytes is responsible for the improvement of the therapeutic index allowed by liposomal encapsulation. In human subjects, DaunoXome produced daunorubicin plasma concentrations which were 35 fold higher than those obtained after the administration of the same dose of free daunorubicin (Forssen & Ross, 1994). In rat plasma, the ratio of the areas under the curve (AUC) DaunoXome/free daunorubicin exceeded 200, but daunorubicin accumulation in normal tissues, especially the heart, was lower when the drug was injected as the liposomal form rather than as free drug (Forssen & Ross, 1994).

A second approach consisted in the use of a molecule which has been developed for preventing anthracycline cardiotoxicity. Dexrazoxane has been proposed long ago as a cardioprotector which could allow the use of doxorubicin beyond its cumulative threshold (Herman *et al.*, 1988; Bu'Lock *et al.*, 1993). In mice, dexrazoxane does not appear to modify doxorubicin antitumour effect (Verhoef *et al.*, 1988). This molecule provides a prolonged protection against anthracycline cardiotoxicity, and not only a delay of the appearance of cardiac alterations (Herman & Ferrans, 1986). The mechanism by which dexrazoxane protects the heart against anthracycline toxicity is not well understood but is thought to involve metal chelation (Hasinoff & Kala, 1993). Indeed, dexrazoxane administered systemically is taken up intracellularly and hydrolysed to its diacid, diamide metabolite, ICRF-198 (Hasinoff *et al.*, 1990), which is a strong ion metal chelator, with a structure similar to EDTA (Thomas *et al.*, 1993). Iron and copper are especially thought to facilitate doxorubicin-dependent free radical production in heart tissue (Hasinoff *et al.*, 1989). It has, therefore, been suggested that dexrazoxane exerts its protective effect by preventing doxorubicin-metal interaction (Shipp *et al.*, 1993).

Our study is the first to describe the protective effects of dexrazoxane, directly on cardiac contractility and relaxation. We have shown that the administration of dexrazoxane, at a dose which presents no toxicity (Leismann *et al.*, 1981), and

before the administration of doxorubicin, was able to restore the level of the LVDP to the values observed in doxorubicin-naive rats. Similarly, ventricular relaxation presented no alteration when dexrazoxane was associated with doxorubicin; only contractility remained marginally affected by doxorubicin treatment when the drug was associated with dexrazoxane. However, these advantages were lost when doxorubicin administration preceded that of dexrazoxane. It had already been shown by Herman & Ferrans (1993) that protection was significantly better in dogs receiving dexrazoxane and doxorubicin simultaneously than in those given dexrazoxane 2 h after doxorubicin. The importance of the schedule of administration should be kept in mind during the clinical evaluation of this association.

Doxorubicin cardiotoxicity in this study was evaluated by alteration of several parameters and was associated to a decrease in heart weight. This decrease can be explained by the inhibiting effect of doxorubicin on protein synthesis in the heart muscle (Lewis *et al.*, 1983), especially the cytoskeletal-contractile system (Rabkin & Sunga, 1987; Ito *et al.*, 1990). The addition of dexrazoxane to doxorubicin results in the restoration of most functional parameters to their control value, but does not restore the heart weight, which cannot be, therefore, used as a valuable index of cardiac toxicity since it does not reflect the cardiac function. It should be mentioned that association of dexrazoxane to doxorubicin treatment also reduced the general toxicity of the anticancer drug (body weight loss, diarrhoea and ascites). We have observed that the effects of dexrazoxane could only be evidenced when whole animals are treated with the drug, and not in the *ex vivo* model. This is obviously in relation to the fact that dexrazoxane only acts through its metabolite, ICRF-198 (Voest *et al.*, 1994), and that the metabolic capacities of the heart are probably insufficient for a quantitative production of ICRF-198 from dexrazoxane (Hasinoff *et al.*, 1990).

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