JPP Journal of Pharmacy and Pharmacology

JPP 2001, 53: 987–997

Received May 17, 2000
Accepted October 30, 2000
ISSN 0022-3573

Contradistinction between doxorubicin and epirubicin: in-vivo metabolism, pharmacokinetics and toxicodynamics after single- and multiple-dosing in rats

Sandhya Ramanathan-Girish and Mehdi Boroujerdi

Abstract

There is compelling in-vitro evidence that the evaluation of doxorubicin or epirubicin pharmacokinetics based solely on plasma concentration may not fully elucidate the differences between the two drugs. Both compounds bind to erythrocytes and their different binding to haemoglobin may influence their disposition in the body. The purpose of the present study was to compare the pharmacokinetics and metabolism of doxorubicin and epirubicin based on the plasma concentration, amount associated with blood cells and simultaneous monitoring of biliary and urinary elimination of unchanged drug and metabolites after single- and multiple-dose injections. The level of sarcoplasmic reticulum Ca^{2+}ATPase in the heart was also measured as a biomarker of cardiotoxicity. Male Sprague-Dawley rats were treated in a parallel design with doxorubicin or epirubicin on a multiple-dosing basis (4 mg kg^{-1} per week) or as a single dose injection (20 mg kg^{-1}). Blood, urine and bile samples were collected periodically after each dose in the multiple-dosing regimen and the single dose injection, and at the end of each experiment the hearts were removed. The concentrations of each drug in plasma, blood cells, bile and urine samples were determined, and by simultaneous curve-fitting of plasma and bile data according to compartmental analysis, the pharmacokinetic parameters and constants were estimated. The concentration of drug associated with blood cells was analysed according to non-compartmental analysis. The bile and urine samples provided the in-vivo metabolic data. The level of Ca^{2+}ATPase in the heart, determined by Western blotting, was used as the toxicodynamic parameter to correlate with the kinetic data. Multiple-dosing regimens reduced the total plasma clearance and increased the area under the plasma concentration–time curve of both drugs. Also, the area under the curve of doxorubicin associated with blood cells increased with the weekly doses, and the related mean residence time (MRT) and apparent volume of distribution (Vd_{ss}) were steadily reduced. In contrast to doxorubicin, the MRT and Vd_{ss} of epirubicin increased significantly. Metabolic data indicated significant differences in the level of alcohol and aglycones metabolites. Doxorubicinol and doxorubicin aglycones were significantly greater than epirubicinol and epirubicin aglycone, whereas epirubicinol aglycone was greater than doxorubicinol aglycone. The area under the blood cells concentration–time curve correlated linearly with the changes in Ca^{2+}ATPase net intensity. The results of this study demonstrate the importance of the kinetics of epirubicin and doxorubicin associated with blood cells. Linear correlation between the reduction of net intensity of the biomarker with the area under the curve of doxorubicin associated with blood cells confirms that the differences between the two compounds are related to their interaction with blood cells. This observation together with the observed differences in metabolism may underline a significant role for blood cells in distribution and metabolism of doxorubicin and epirubicin.

Department of Pharmaceutical Sciences, Northeastern University, Boston, MA 02115, USA

Sandhya Ramanathan-Girish and Mehdi Boroujerdi

Present address: Chiron Corporation, 4560 Horton Street, M/S 4.176, Emeryville, CA 94608-2916, USA

Sandhya Ramanathan-Girish

Correspondence: M. Boroujerdi, 110 Mugar Life Sciences Building, Northeastern University, Boston, MA 02115, USA. E-mail: m.boroujerdi@neu.edu

Introduction

Doxorubicin and epirubicin have equal cytotoxicity against a wide range of animal and human tumour cell lines (Hill & Whelan 1982). Both drugs are extensively metabolized in the liver by metabolic processes of aldo-keto reduction, conjugation and microsomal cleavage reactions. The parent compounds and the metabolites are eliminated predominantly in the bile and moderately in the urine (Handa & Sao 1976; Oki et al 1977; Broggini et al 1980; Tavoloni & Guarino 1980a, b; Pan et al 1981; Bertazolli et al 1985; Camaggi et al 1986; Eksborg et al 1986; Gewirtz & Yanovich 1987; Sweatman & Israel 1987; Launchbury & Habboubi 1993; Dorr & Van Hoff 1994). Both drugs produce early transient electrocardiographic changes and a progressive cardiomyopathy. This cardiomyopathy is severe and leads to progressive congestive heart failure that is dose-dependent, cumulative and irreversible (Dorr & Von Hoff 1994). However, the incidence of cardiac damage with epirubicin is less than with doxorubicin (Bertazolli et al 1985; Launchbury & Habboubi 1993). It is interesting that structurally epirubicin is an epimer of doxorubicin and the only difference is the configuration of the 4′-OH group of the sugar moiety of the molecule. In doxorubicin the OH group is oriented in the axial configuration whereas in epirubicin it is in the equatorial configuration (Launchbury & Habboubi 1993). The risk of congestive heart failure is considerable for doxorubicin at cumulative doses greater than 550 mg m^{-2}, whereas for epirubicin the risk is approximately 30% for cumulative doses in excess of 900 mg m^{-2} (Bertazolli et al 1985; Launchbury & Habboubi 1993). The myocardial damage appears to be related to impairment of heart mitochondrial function such as disturbances in the intracellular transport of calcium (Azuma et al 1981). Doxorubicin causes mitochondria to lose their ability to sequester calcium (Olson & Mushlin 1990; Bonnadonna et al 1993; Dodd et al 1993). It depresses adenosine diphosphate stimulated respiration (Azuma et al 1981; Muhammed et al 1983) and induces alterations in membrane structure and function (Goormaghtigh et al 1982). Doxorubicin inhibits the intracellular levels of ATP and GTP of the heart and creates pathological lesions at the mitochondrial levels that are more evident than epirubicin (Alderton et al 1992). Doxorubicin, and to a lesser extent epirubicin, influence a wide variety of sub-cellular systems by direct interaction with membrane or through the production of free radicals that are formed by redox cycling of doxorubicin in the presence of iron (de Silva & Aust 1993; Pierscinski et al 1994).

It has been suggested that the disparity in toxicity between epirubicin and doxorubicin may be related to the differences in their pharmacokinetics and metabolism (Sweatman & Israel 1987; Le Bot et al 1988, 1991; Weenen et al 1984, Van der Vijgh et al 1990). Furthermore, doxorubicin is known to bind significantly to plasma membrane (Bannister et al 1983; Diociaiuti et al 1991). The present study was designed to investigate whether the difference in toxicity between doxorubicin and epirubicin correlates with their pharmacokinetic data. We compared the pharmacokinetics of doxorubicin and epirubicin after a single dose of 20 mg kg^{-1} and multiple-dose injections of 4 mg kg^{-1} per week for five weeks. The single and cumulative dose of 20 mg kg^{-1} was selected by design to be toxic. The study of their disposition was based on the time-course of concentration of either drug in plasma and their amount associated with blood cells. The comparison of their metabolism was based on the time-course of biliary and urinary elimination of unchanged drug and metabolites. To gauge the toxicity and correlate with pharmacokinetic data, sarcoplasmic reticulum (SR) Ca^{2+}ATPase concentration in the heart was measured as a biomarker of cardiotoxicity.

Materials and Methods

Chemicals

Radiolabelled [^{14}C]doxorubicin with specific activity of 100 μCi mg^{-1} was purchased from Amersham Life Science, Inc. (Arlington Hights, IL). Doxorubicin HCl, epirubicin HCl and their metabolites were a gift from Upjohn-Pharmacia (Albuquerque, NM). The radiolabelled dose of doxorubicin was prepared by addition of the cold drug to the radiolabelled drug to obtain a final specific activity of 0.4 μCi mg^{-1}. Sodium pentobarbital (Nembutal, 50 mg mL^{-1}) was obtained from Henry Schein (Port Washington, NY). Monoclonal anti-SERCA2 ATPase antibody (IgG2a) was purchased from Affinity BioReagent Inc. (Golden, CO). Peroxidase-conjugated affinipure goat anti-mouse IgG was purchased from Jackson Immunoresearch Laboratories Inc. (West Grove, PA). Bovine serum albumin (BSA) blocking reagent was from Sigma Chemical Co. (St Louis, MO). Tris-glycine-SDS buffers, 7.5% pre-cast polyacrylamide gels and electroblot buffer were purchased from Owl Scientific (Woburn, MA). SDS PAGE pre-stained standards (high range) and PVDF protein-sequencing membrane were purchased from Bio-Rad Laboratories (Hercules, CA). ECL Western blotting analysis system and hyperfilm ECL were purchased

from Amersham Life Science Inc. (Arlington Heights, IL).

Animals and experimental protocol

Male Sprague-Dawley rats, 250–350 g (Taconic Farms, Germantown, NY), were randomized to either a single-dosing regimen (two groups, n = 6 per group, 20 mg kg^{-1} epirubicin or doxorubicin), or multiple-dosing regimen of once a week for five consecutive weeks (two groups, n = 30 per group, 4 mg kg^{-1} epirubicin or doxorubicin per week). Each group in the multiple-dosing regimen was divided into five subgroups (n = 6 per subgroup) and every subgroup was used to study the time-course of the drug at a given week. The weekly doses were given via the tail vein and on the day of experiment, following the bile duct cannulation, the dose was administered via the femoral vein. Blood, bile and urine samples were collected periodically for 10 h. All blood samples were centrifuged for 10 min at 3000 rev min^{-1} in heparinized tubes to separate plasma. Plasma and bile samples were frozen at −70°C in liquid nitrogen and stored at −20°C until analysis. At the end of the experiment the rats were killed and the hearts removed immediately, washed with ice-cold normal saline, blotted dry and frozen in liquid nitrogen and stored at −20°C.

Analytical methodology

Bile and urine samples were analysed by an HPLC system consisting of a solvent delivery system and autosampler with Novapak C-18 cartridge and C18 Novapak Sentry guard column (Waters Corporation, Milford, MA). The mobile phase was 0.1% v/v ammonium formate buffer–methanol (30:70%, v/v), pH 4.0. The online detectors were a Gilson fluorometer (Middleton, WI) with excitation and emission wavelength of 480 nm and 540 nm, respectively, and a radioactivity detector (Beta Flo, Packard, Meriden, CT). The guard column was replaced regularly and the retention time of the parent compound and the metabolites were determined and confirmed daily with authentic standards. Blood cells were treated with 500 μL Solvable (Beckman Instruments Inc., Fullerton, CA) and were left overnight at room temperature. The following day, samples were decolourized with hydrogen peroxide (200 μL), and glacial acetic acid (100 μL) was added to the samples to prevent quenching. The concentration of plasma and blood cells was then measured separately.

Measurement of SR Ca^{2+}ATPase concentration

The homogenates of hearts, in 10 mM Tris/maleate buffer (pH 6.8), were centrifuged at 15000 *g* for 20 min. The supernatants were centrifuged at 100000 *g* for 30 min. The pellet was then suspended in Tris/maleate buffer (0.6 M KCl) and centrifuged at 100000 *g* for 45 min. The pellet obtained after this centrifugation was re-suspended in Tris/maleate (pH 6.8) containing 0.1 M KCl (80 μL g^{-1}). The final fraction was frozen immediately in liquid nitrogen and stored at −20°C until analysis. The protein concentration in the SR fraction of all samples was determined according to the Biuret method (Gornall et al 1949). SR samples (40–80 μg) were treated with 4 μL of 1 × sample buffer (4 mL dH_2O, 800 μL glycerol, 1.6 mL 10% SDS and 1 mL 0.5 M Tris HCl, pH 6.8 for 3X) and mercaptoethanol (15–85 μL of 1X sample buffer). The samples were mixed vigorously, heated in a water bath (100°C) for 5 min and used for electrophoresis. Tris-glycine-SDS buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) was used as the running buffer. A 7.5% polyacrylamide pre-cast gel was used in conjunction with a Daiichi Mini 2-gel system for electrophoresis. After running and rinsing with water, the gel was stained with 0.25% Coomassie blue dissolved in a mixture of methanol–acetic acid–water (5:1:5) for 45 min. After de-staining and drying for 1 h at 60°C, the 110-kDa Ca^{2+} protein band was quantified using Kodak Digital Science 1D image analysis software. To prepare the samples for electroblotting, the SR vesicles were run on polyacrylamide gels under reducing conditions as described before. The gels were separated from the plates and the proteins were transferred to a PVDF membrane. Three buffers consisting of cathode, anode I and anode II were used for the transfer at 400 mA and 11 V for 1 h. Following the electro-blotting procedure, the membrane was rinsed with distilled water and immersed in 3% blocking reagent (BSA) in PBS-Tween 20 (0.3%) for 1 h at 37°C. The membrane was then washed with PBS-Tween 20 (0.05%) and incubated with the primary antibody, anti-SERCA2 ATPase antibody (1:750), for 1 h at 37°C. After washing with the primary antibody, the next incubation was carried out with the peroxidase-conjugated affinipure goat anti-mouse IgG (1:2000) at 37°C for 1 h. The ECL kit was used for detection. The hyperfilm ECL was exposed for 15 s to the detection reagent and then developed in a Kodak X-Omat M-20 processor. The band intensity was quantified using Kodak Digital Science 1D image analysis software.

Data analysis

WinNonlin (version 1.1; SCI Software, Apex, CA) was used for pharmacokinetic data analysis. The pharmacokinetic parameters of plasma were determined by simultaneous curve-fitting of plasma and biliary data. The

Table 1 Parameters and constants of simultaneous curve-fitting of plasma concentrations and biliary elimination of doxorubicin according to the equations of two-compartment open model using WinNonlin.

Parameter/ constant	Week 1 (4 mg kg^{-1})	Week 2 (4 mg kg^{-1})	Week 3 (4 mg kg^{-1})	Week 4 (4 mg kg^{-1})	Week 5 (4 mg kg^{-1})	Acute dose (20 mg kg^{-1})
A (mg L^{-1})	2.35 ± 0.13	3.34 ± 0.26	2.92 ± 0.23	6.20 ± 0.31	6.83 ± 0.55	10.71 ± 0.42**
B (mg L^{-1})	0.25 ± 0.04	0.42 ± 0.03	0.46 ± 0.02	1.03 ± 0.07	1.33 ± 0.27	2.31 ± 0.20**
α (h^{-1})	5.17 ± 0.54	9.17 ± 0.90	9.67 ± 0.96	7.27 ± 0.60	4.93 ± 0.84	4.45 ± 0.42
β (h^{-1})*	0.12 ± 0.06	0.12 ± 0.02	0.16 ± 0.18	0.23 ± 0.24	0.25 ± 0.06	0.13 ± 0.02
k_b(h^{-1})	0.18 ± 0.01	0.15 ± 0.01	0.14 ± 0.01	0.15 ± 0.06	0.13 ± 0.02	0.07 ± 0.01**
AUC (mg h L^{-1})*	2.54 ± 0.36	3.86 ± 0.28	3.17 ± 0.15	5.33 ± 0.35	6.70 ± 1.20	20.17 ± 1.68**
CL (L h^{-1} kg^{-1}) *	1.60 ± 0.23	1.04 ± 0.70	1.26 ± 0.06	0.75 ± 0.05	0.61 ± 0.12	0.99 ± 0.11

A and B are the coefficients of the exponential terms of the concentration in plasma; α and β are the hybrid rate constants of disposition; and k_b is the first-order rate constant for biliary excretion. Data are presented as mean ± s.d., n = 6. *$P < 0.05$ multiple-dosing parameters. **$P < 0.05$ single-dosing parameters.

Table 2 Parameters and constants of simultaneous curve-fitting of plasma concentrations and biliary excretion of epirubicin according to the equations of two-compartment open model using WinNonlin.

Parameter/ constant	Week 1 (4 mg kg^{-1})	Week 2 (4 mg kg^{-1})	Week 3 (4 mg kg^{-1})	Week 4 (4 mg kg^{-1})	Week 5 (4 mg kg^{-1})	Acute dose (20 mg kg^{-1})
A (mg L^{-1})	2.42 ± 1.8	3.08 ± 0.83	4.99 ± 1.63	1.93 ± 0.28	3.37 ± 0.87	79.46 ± 15.21**
B (mg L^{-1})	0.15 ± 0.05	0.15 ± 0.08	0.25 ± 0.20	0.06 ± 0.02	0.07 ± 0.01	1.07 ± 0.38**
α (h^{-1})	22.52 ± 10.0	12.19 ± 3.2	10.25 ± 3.6	4.20 ± 1.14	4.27 ± 1.44	17.09 ± 2.22
β (h^{-1})*	0.34 ± 0.06	0.26 ± 0.12	0.13 ± 0.18	0.04 ± 0.02	0.01 ± 0.03	0.06 ± 0.06**
k_b(h^{-1})	0.97 ± 0.24	0.97 ± 0.18	0.49 ± 0.18	0.74 ± 0.18	0.30 ± 0.20	0.56 ± 0.06
AUC (mg h L^{-1})*	0.55 ± 0.23	0.83 ± 0.37	2.41 ± 1.70	2.04 ± 0.51	7.98 ± 1.60	2.48 ± 7.22**
CL (L h^{-1} kg^{-1})*	8.81 ± 3.65	6.05 ± 2.73	3.26 ± 2.2	2.08 ± 0.52	0.52 ± 0.10	0.89 ± 0.34**

A and B are the coefficients of the exponential terms of the concentration in plasma; α and β are the hybrid rate constants of disposition; and k_b is the first-order rate constant for biliary excretion. Data are presented as mean ± s.d., n = 6. *$P < 0.05$ multiple-dosing parameters. **$P < 0.05$ single-dosing parameters.

selection of the most appropriate pharmacokinetic model was achieved by evaluating different compartmental models and the use of Akaike and Schwarz criteria (Ludden et al 1994). The following are the general equations for the two-compartment open model used for the simultaneous curve-fitting.

$$\text{Plasma: } C_t = Ae^{-\alpha t} + Be^{-\beta t} \quad (1)$$

$$\text{Bile: } A_B = k_b((C\beta + D\alpha)/\alpha\beta) - k_b((Ce^{-\alpha t}/\alpha) + (De^{-\beta t}/\beta)) \quad (2)$$

Where, C_t is the plasma concentration at time t; A and B are the coefficients of the exponential terms of the concentration in plasma; C and D are the coefficients of the exponential terms associated with biliary elimination; α and β are the hybrid rate constants of disposition; A_B is the cumulative amount in the bile; and k_b is the first-order rate constant for biliary excretion.

As t approaches ∞ equation 2 becomes equation 3:

$$A_B{}^{\infty} = k_b((A\beta + B\alpha)/\alpha\beta) \quad (3)$$

Where $A_B{}^{\infty}$ corresponds to the total amount of drug/metabolite that ultimately will be eliminated in the bile.

The time-course of drug associated with blood cells was analysed by non-compartmental analysis with the use of the following general equations:

$$CL = \text{Dose}/AUC \quad (4)$$

$$MRT = AUMC/AUC \quad (5)$$

$$Vd_{ss} = (MRT)(CL) \quad (6)$$

Where CL is clearance; AUC represents the area under the curve; MRT is the mean residence time; AUMC is

the area under the first moment curve; and Vd_{ss} is the volume of distribution.

Relevant pharmacokinetic parameters of plasma and blood cells were correlated with the net intensity of the SR Ca^{2+} pump protein. In addition to the linear correlation of the parameters, the application of the following E_{max} model was also evaluated.

$$E = (E_{max} \times P)/(P + EC50) \quad (7)$$

In this model, E is the percentage reduction in the net intensity of the protein; E_{max} is the maximum percentage of reduction of protein; P is the pharmacokinetic parameter such as AUC or maximum concentration; and EC50 is the magnitude of P that may result in 50% reduction of E_{max}.

Results

Multiple-dose study

The calculated parameters and constants of plasma concentration are presented in Tables 1 and 2. The similarities between the two compounds were the gradual increase in AUC and decline in total body CL over

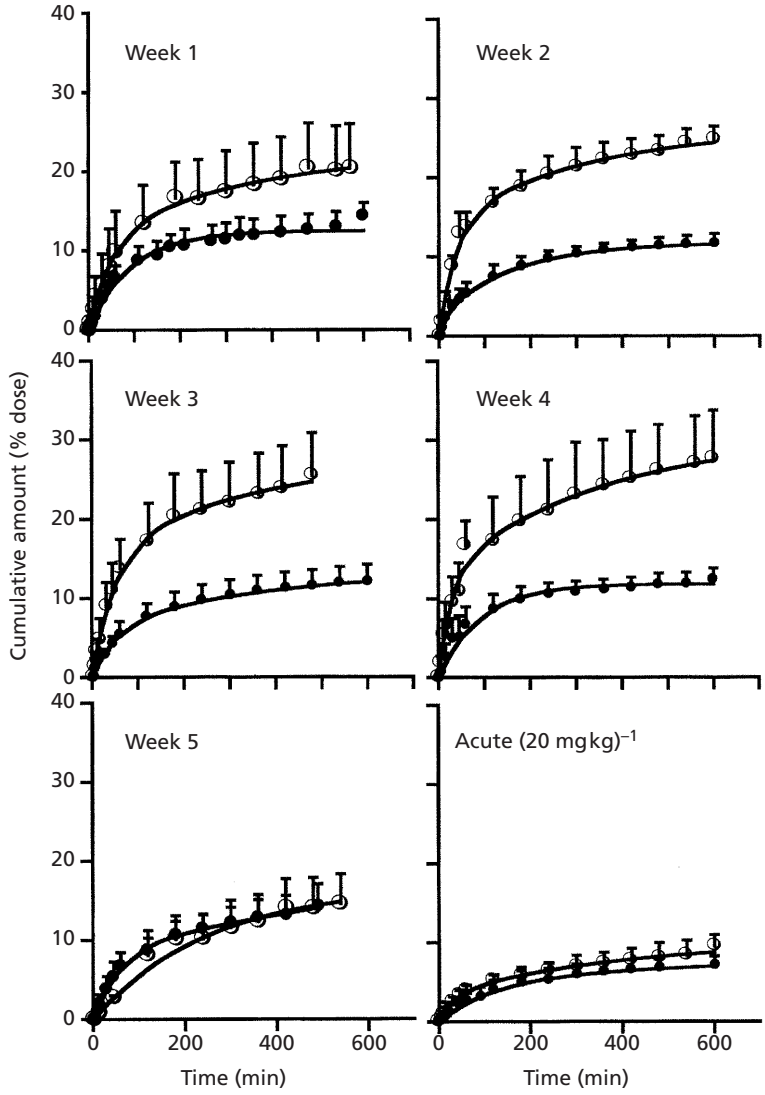

Figure 1 Cumulative amount of unchanged doxorubicin (●) and epirubicin (○) eliminated in the bile after multiple-dosing (4 mg kg^{-1} per week) and single-dosing (20 mg kg^{-1}) regimens. Each point represents the mean ± s.d. of the observed data points from six rats. The solid lines represent the predicted values and are generated by the equation: $A_B = k_b((A\beta + B\alpha)/\alpha\beta) - k_b((A/\alpha)(\exp\text{-}\alpha t) + (B/\beta)(\exp\text{-}\beta t))$.

Table 3 Parameters of non-compartmental analysis of doxorubicin associated with blood cells after single- or multiple-dosing regimen.

Parameter/ constant	Week 1 (4 mg kg^{-1})	Week 2 (4 mg kg^{-1})	Week 3 (4 mg kg^{-1})	Week 4 (4 mg kg^{-1})	Week 5 (4 mg kg^{-1})	Acute dose (20 mg kg^{-1})
$AUC_{0-\infty}$ (mg h L^{-1})*	4.81 ± 0.60	6.17 ± 0.51	7.54 ± 0.61	7.85 ± 0.60	8.61 ± 0.66	30.92 ± 2.85**
$MRT_{0-\infty}$ (h)*	14.07 ± 1.75	10.70 ± 0.88	16.27 ± 1.32	9.79 ± 0.75	8.77 ± 0.67	11.29 ± 2.10
Vd_{ss} (L)*	3.85 ± 0.48	1.88 ± 0.15	2.10 ± 0.17	1.20 ± 0.10	0.99 ± 0.07	2.25 ± 0.62
$CL_{blood\ cells}$ (L h kg^{-1})	0.84 ± 0.10	0.65 ± 0.05	0.54 ± 0.04	0.51 ± 0.04	0.46 ± 0.03	0.65 ± 0.08

Data are presented as mean ± s.d., n = 6 per week and acute dose. *$P < 0.05$ multiple-dosing parameters. **$P < 0.05$ single-dosing parameters.

Table 4 Parameters of non-compartmental analysis of epirubicin associated with blood cells after single- or multiple-dosing regimen.

Parameter/ constant	Week 1 (4 mg kg^{-1})	Week 2 (4 mg kg^{-1})	Week 3 (4 mg kg^{-1})	Week 4 (4 mg kg^{-1})	Week 5 (4 mg kg^{-1})	Acute dose (20 mg kg^{-1})
$AUC_{0-\infty}$ (mg h L^{-1})*	3.37 ± 0.28	1.42 ± 0.21	9.41 ± 3.18	3.73 ± 1.01	8.62 ± 1.18	51.55 ± 4.32**
$MRT_{0-\infty}$ (h)*	6.72 ± 0.57	2.19 ± 0.32	31.34 ± 10.6	8.18 ± 2.21	37.90 ± 5.2	1.75 ± 0.43**
Vd_{ss} (L)*	2.18 ± 0.18	1.72 ± 0.25	3.20 ± 1.08	2.12 ± 0.57	4.26 ± 0.60	0.17 ± 0.68**
$CL_{blood\ cells}$ (L h^{-1} kg^{-1})	1.19 ± 0.11	2.87 ± 0.43	0.48 ± 0.16	1.15 ± 0.32	0.47 ± 0.07	0.39 ± 0.06**

Data are presented as mean ± s.d., n = 6. *$P < 0.05$ multiple-dosing parameters. **$P < 0.05$ single-dosing parameters.

the course of the multiple-dosing study. The differences were the reduction in the hybrid rate constant of disposition of epirubicin over the course of treatment, and the elevation in the biliary rate constant which was greater than for doxorubicin. The change in the biliary rate constant indicates that the overall biliary elimination of unchanged epirubicin is greater than unchanged doxorubicin. However, since the reduction of this rate constant between the first and the last weeks of the multiple-dosing was significant for epirubicin ($P < 0.05$), it was concluded that the multiple-dosing regimen of the toxic dose influenced the biliary elimination of unchanged epirubicin. The weekly comparisons of the cumulative amount of unchanged drug eliminated in the bile for both drugs are presented in Figure 1. The observed reduction in the cumulative amount of unchanged epirubicin in bile was consistent with the gradual increase in AUC over the course of study. This reduction, from 20.36 ± 5.60 (% dose) in the first week to 14.60 ± 3.75 (% dose) in the fifth week, was statistically significant ($P < 0.05$). This observation again indicated that the biliary elimination of unchanged epirubicin was reduced due to the multiple-dosing regimen. The cumulative amount of doxorubicin in bile remained unchanged. There were some small fluctuations but no significant changes between the levels of doxorubicin in the first (14.32 ± 1.70 % dose) and fifth weeks (14.25 ± 2.82 % dose).

The non-compartmental analysis of doxorubicin and epirubicin associated with blood cells is presented in Tables 3 and 4. For doxorubicin, the AUC increased with the weekly doses and the MRT and Vd_{ss} reduced continually. The increase in AUC and reduction in MRT and Vd_{ss} of doxorubicin between the first and last week were statistically significant ($P < 0.05$). In contrast, the MRT and Vd_{ss} of epirubicin increased significantly over the course of the multiple-dosing study and the weekly changes in AUC and MRT of blood cells were consistent with the changes in AUC and the disposition rate constant of plasma data. The increase in MRT corresponds to a longer association of the drug with blood cells, and, therefore, a smaller rate constant of release from blood cells. This slower release would affect the overall disposition rate constant calculated from plasma data. Accordingly, the clearance of blood cells, that should not be confused with the clearance of plasma, declined significantly for both drugs, particularly between the first and last week of multiple dosing. The variability of the clearance between the weeks was more pronounced for epirubicin than doxorubicin.

The metabolism study indicated that in bile, the level of the alcohol metabolite doxorubicinol was substan-

Table 5 Total amount of biliary elimination of doxorubicin or epirubicin and their metabolites (% dose) in 10 h after a single dose of 20 mg kg^{-1} or each weekly dose of 4 mg kg^{-1} in the multiple-dosing regimen.

Compound	Week 1 (4 mg kg^{-1})	Week 2 (4 mg kg^{-1})	Week 3 (4 mg kg^{-1})	Week 4 (4 mg kg^{-1})	Week 5 (4 mg kg^{-1})	Single dose (20 mg kg^{-1})
Doxorubicin	14.32±1.70	11.74±1.15	11.98±2.27	12.30±1.54	14.24±2.82	7.00±1.24
Epirubicin	20.36±5.59*	24.76±1.66*	25.44±5.39*	27.63±6.16*	14.58±3.75	9.52±1.43*
Doxorubicinol	1.82±0.47	1.47±0.32	1.81±0.52	1.57±0.32	1.70±0.24	1.85±0.04
Epirubicinol	0.33±0.17*	0.33±0.04*	0.27±0.23*	0.27±0.07	0.08±0.05*	0.47±0.25
Doxorubicin aglycone	1.67±0.43	1.33±0.26	1.52±0.57	1.27±0.22	1.46±0.32	1.13±0.15
Epirubicin aglycone	0.36±0.18*	0.16±0.07*	0.36±0.23*	0.51±0.25*	0.33±0.39*	0.22±0.07*
Doxorubicinol aglycone	1.95±0.33	1.40±0.55	1.53±0.31	1.27±0.31	1.42±0.25	1.13±0.39
Epirubicinol aglycone	0.43±0.38*	4.25±0.85*	2.63±0.98	3.36±1.06*	1.46±1.69	1.97±0.77*

Data are presented as mean±s.d., n = 6. *$P < 0.05$ significantly different compared with doxorubicin.

tially higher than epirubicinol, and doxorubicin aglycone was significantly higher than epirubicin aglycone. However, epirubicinol aglycone was higher than doxorubicinol aglycone. The lower level of epirubicinol is in agreement with the increase in the level of epirubicinol aglycone. Comparison of the total amount of unchanged drug and metabolites for each compound is presented in Table 5. The cumulative amount of unchanged drug and the metabolites eliminated in the urine revealed that approximately 3–5% of epirubicin was eliminated unchanged in the urine and no significant changes occurred between the weeks. The total amount of doxorubicin eliminated unchanged after the first week was approximately 6.17±1.7% of the first dose, and this amount continually reduced over the course of the study. Aglycones of doxorubicin and epirubicin and aglycones of doxorubicinol and epirubicinol were detected in negligible quantities in a few samples during the initial hours. The level of doxorubicinol and epirubicinol in the urine samples was 2–3% of the dose and declined significantly over the course of study.

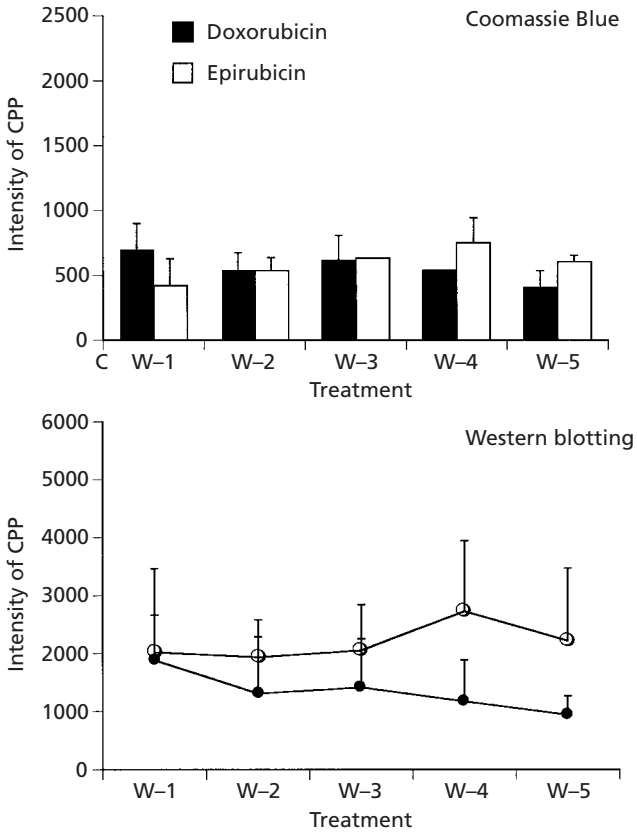

Figure 2 Comparison of the changes in the net intensity of the sarcoplasmic reticulum Ca^{2+}ATPase protein (CPP) after multiple-dosing with doxorubicin (●) or epirubicin (○) for five weeks.

Single-dose study

The pharmacokinetic parameters and constants of the single-dose study were determined by simultaneous curve-fitting of plasma concentration and the biliary elimination of unchanged drug (Tables 1 and 2). Apart from the expected changes in the dose-dependent parameters, such as AUC, A and B, the most notable differences were the reduction in the biliary rate constant of doxorubicin and reduction in the disposition rate constant of epirubicin. Considering the overall gradual reduction in plasma clearance in multiple dosing, the single dose of 20 mg kg^{-1} reduced the clearance of epirubicin more significantly than doxorubicin. The AUC of epirubicin associated with blood cells was much higher than doxorubicin whereas MRT and Vd_{ss} were significantly lower than in the multiple-dosing study.

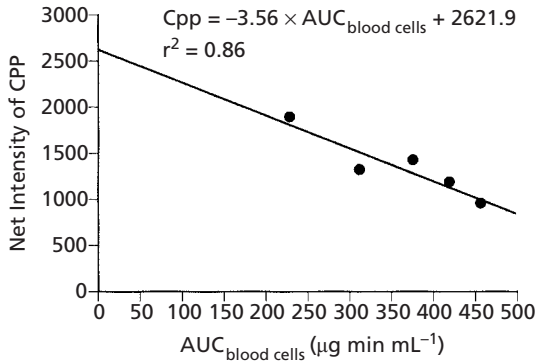

Figure 3 Linear correlation of area under the blood cells concentration–time curve ($AUC_{blood\ cells}$) with sarcoplasmic reticulum Ca^{2+}ATPase protein (CPP) after multiple-dosing with doxorubicin.

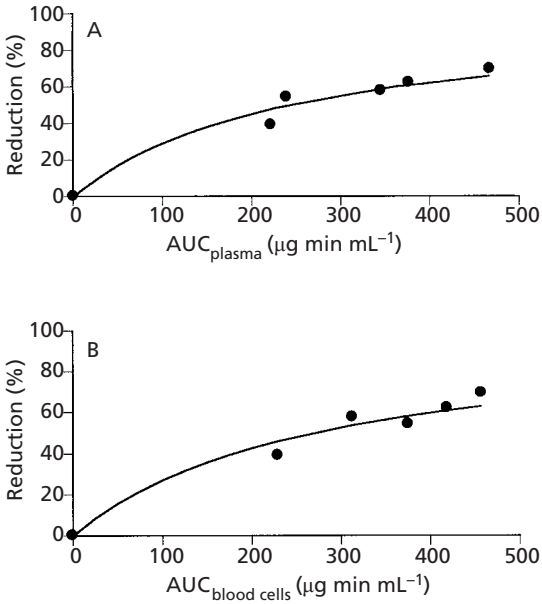

Figure 4 Correlation of area under the plasma concentration–time curve (AUC_{plasma}) (A) or area under blood cells concentration–time curve ($AUC_{blood\ cells}$) (B) with sarcoplasmic reticulum Ca^{2+}ATPase protein after multiple dosing with doxorubicin according to the E_{max} equation: $E = (E_{max} \times P)/(P + EC50)$.

The data are presented in Tables 3 and 4. The biliary elimination of parent compound and the metabolites are presented in Table 5.

SR Ca^{2+}ATPase protein

The amount of cardiac damage after multiple-dosing regimens was estimated by changes in the SR Ca^{2+} pump protein. The net intensity of the protein was measured by Western blotting in the SR fraction of the heart. The comparison was made with the SR fraction of the heart of saline-treated rats. The net intensity of the 110-kDa protein band after Coomassie blue staining and Western blotting is shown in Figure 2. The intensity of the band after the repeated dosing with doxorubicin was found to decrease with respect to the control and the first dose. The repeated dosing of epirubicin increased the net intensity of the band compared with the control. We correlated the calculated pharmacokinetic parameters of doxorubicin and epirubicin with the net intensity of the protein. Among all parameters and constants, only the AUC of doxorubicin associated with blood cells correlated linearly with a coefficient of determination of $r^2 = 0.86$ (Figure 3). No correlation was observed between the net intensity of the protein and any of the parameters for epirubicin. For doxorubicin, an E_{max} pharmacodynamic model was then selected to relate the percentage reduction in the weekly net intensity of the protein to AUC_{plasma} or $AUC_{blood\ cells}$ (Figure 4). According to this analysis the AUC_{plasma} or $AUC_{blood\ cells}$ that corresponded to a 50% reduction of the protein was 4.08 ± 2.50 mg h L^{-1} and 4.50 ± 3.14 mg h L^{-1}, respectively. According to the simple linear correlation this value was predicted as 6.13 mg h L^{-1} for blood cells.

Discussion

We used a uniform experimental protocol to evaluate the pharmacokinetic, metabolism and toxicodynamic differences between epirubicin and doxorubicin after single- and multiple-cardiotoxic doses. The concentrated single-dose (20 mg kg^{-1}), that is approximately equivalent to 900 mg m^{-2}, is a cardiotoxic dose for both epirubicin and doxorubicin and the cumulative dose of the multiple-dosing regimen is also in the toxic range. Therefore, the results of this study deal with the major differences between these two very similar molecules at a toxic dose level.

The evaluation of the plasma and bile data, both observed and calculated values, proved that the biliary elimination of unchanged epirubicin is greater than doxorubicin. The difference in the predicted values of the biliary rate constants after either multiple-dosing regimens or a single dose is in full agreement with this observation. Hence epirubicin leaves the body faster, and the lower AUC in the first- to fourth-week confirms this observation. There was, however, an abrupt change in the fifth week of epirubicin dosing. The AUC increased significantly and the elimination parameters

declined substantially. We evaluated the time-course of bile flow rate during the multiple-dosing experiments to ensure that the reduction in biliary elimination of epirubicin in the fifth week was not related to a change in bile flow rate. The bile flow rate remained mainly at plateau during the course of study. Therefore it was concluded that the bile flow could not be the reason for the abrupt changes associated with the fifth dose of epirubicin. The interesting observation was then the association of both drugs with blood cells. This association was extensive for both drugs, it increased consistently during the multiple-dosing of doxorubicin, but was irregular for epirubicin. The amount of epirubicin associated with blood cells increased significantly after the fifth dose.

Thus, the blood cells undoubtedly play a significant role in the overall disposition of both drugs. The AUC of the amount associated with blood cells reflects the magnitude of the association of these drugs with blood cells. Therefore, it seems reasonable to advocate that the pharmacokinetic analysis of these two drugs should be based on measurement of total blood concentration and not just plasma. We defined the clearance of the blood cells as a proportionality constant that when multiplied by the concentration of drug associated with blood cells, provides the rate of release of drug from blood cells. The clearance of doxorubicin associated with blood cells was lower than epirubicin and was steadily reduced during the multiple-dosing study, suggesting a stronger interaction between doxorubicin and blood cells. We believe that the continual increase in AUC of doxorubicin associated with blood cells and the stability of the interaction are related to the self-association of the molecule and its binding to haemoglobin. The MRT of a drug associated with blood cells is also indicative of the strength of its interaction with blood cells. MRT can be interpreted as the time required for 63.3% of the drug to be released from blood cells. A longer MRT would indicate a longer association of drug with blood cells. The MRT of the single-dose study of doxorubicin was estimated as 11.29 ± 2.10 h, whereas for epirubicin it was less than 2 h. It is very likely that the interaction of doxorubicin with blood cells, in particular red blood cells, may contribute to the formation of the reactive metabolite. The in-vivo interaction of the metabolites of these compounds with blood cells is not known at this time. The decline in Vd_{ss} of doxorubicin in the multiple-dosing study was also in agreement with the reduction in clearance and MRT. For epirubicin, this parameter was inversely proportional to the clearance.

The results of the in-vivo metabolism study demonstrated that the metabolism of doxorubicin is significantly different from epirubicin. Doxorubicinol and epirubicinol are known to be more cardiotoxic than the parent compounds. The levels of doxorubicinol after the single dose of 20 mg kg^{-1} or the multiple-dosing regimen were significantly greater than epirubicinol ($P < 0.05$). However, the levels of epirubicinol in the urine and aglycones of epirubicin and epirubicinol in the bile were greater than doxorubicinol, doxorubicin aglycone and doxorubicinol aglycone. The total amount of epirubicinol and epirubicinol aglycone was approximately equal to the total amount of doxorubicinol and doxorubicinol aglycone. Therefore, we concluded that the interaction of both molecules with aldo-keto reductases was similar. However, the interaction of epirubicinol with NADPH-dependent cytochrome P450 reductase to form epirubicinol aglycone was more favourable than doxorubicinol. Furthermore, since the formation of doxorubicin aglycone was greater than epirubicin aglycone, it was concluded that the interaction of doxorubicin with the enzyme NADPH-dependent cytochrome P450 was more favourable than epirubicin. Therefore, it would be reasonable to assume that the combination of hydroxyl group of epirubicinol of carbon 13 and the equatorial configuration of the 4′-OH of the sugar moiety are needed for the interaction of epirubicinol with NADPH-dependent cytochrome P450 to be more favourable than doxorubicinol.

The overall urinary elimination of the parent compounds and their metabolites did not add up to a significant portion of the dose. There are, however, a few differences that are important to note. For example, the urinary elimination of doxorubicin and doxorubicinol decreased regularly with each dose of the multiple-dosing regimens. This can be attributed to the nephrotoxicity of these two compounds. Also, since the elimination of epirubicin did not reduce significantly, we concluded that epirubicinol and epirubicin might not be as nephrotoxic as doxorubicin and its alcohol metabolite. The polar metabolites of both compounds, such as glucuronide and sulphate conjugates, were not a significant portion of total urinary elimination of the two compounds in our animal model.

We selected SR Ca^{2+}ATPase protein as the cardiotoxicity biomarker. It has been shown that the cardiotoxicity of doxorubicin may be due to early damage to the DNA, mediated by hydroxyl free radicals of the alcohol metabolite. This damage to DNA may ultimately result in a decreased synthesis of proteins that are critical for the proper functioning of the myocardium (Crooke et al 1983; Mimnaugh et al 1983; Muindi et al 1984; Sinha et al 1984; Monti et al 1995; Di Liegro et al 1996). The reduction in the net intensity of the 110-kDa

SR Ca^{2+} protein band after repeated dosing with doxorubicin may be explained on the basis of the effect of free radical formation and doxorubicinol and the related decrease in protein synthesis. The decrease in the amount of the Ca^{2+} protein would result ultimately in calcium overload and hence myocyte damage. It was interesting that the reduction of Ca^{2+} protein in rats treated with epirubicin was not as significant as doxorubicin. The linear correlation of the reduction of the net intensity of Ca^{2+} protein with AUC of doxorubicin associated with blood cells further confirms that the drug associated with blood cells may play a significant role in cardiotoxicity of doxorubicin.

In summary, the reason that epirubicin is less cardiotoxic than doxorubicin is that the interaction of epirubicinol with NADPH-dependent cytochrome P450 reductase seems to be more favourable than doxorubicinol. Therefore, the concentration of doxorubicinol would have a longer half-life than epirubicinol and the concentration of epirubicinol aglycone would always be greater than doxorubicinol aglycone. This by itself would make doxorubicin a more toxic compound than epirubicin. Furthermore, the amount of doxorubicin associated with blood cells was significantly greater than epirubicin and correlated well with Ca^{2+} protein. This linear correlation raises the question as to whether blood cells, particularly red blood cells, are involved in the formation of free radicals. Finally, it is the conclusion of this study that the pharmacokinetic analysis of doxorubicin and epirubicin should be based on the total concentration of blood and not just plasma.

References

Alderton, P. M., Gross, J., Green, M. D. (1992) Comparative study of doxorubicin, mitoxantrone and epirubicin in combination with ICRF-187 (ADR-529) in a chronic cardiotoxicity animal model. *Cancer Res.* **52**: 194–201

Azuma, J., Sperelakis, N., Hasegawa, H., Tanimoto, T., Vogel, S., Ogura, K., Awata, N., Sawamura, A., Harada, H., Ishiyama, T., Morita, Y., Yamamura, Y. (1981) Adriamycin cardiotoxicity: possible pathogenic mechanisms. *J. Mol. Cell. Cardiol.* **13**: 381–397

Bannister, J. V., Thornalley, P. J. (1983) The production of hydroxyl radicals by adriamycin in red blood cells. *FEBS Lett.* **157**: 170–172

Bertazolli, C., Rovero, C., Ballerini, L., Lux, B., Balconi, F. (1985) Experimental systemic toxicology of 4′-epidoxorubicin, a new less cardiotoxic anthracycline antitumor agent. *Toxicol. Appl. Pharmacol.* **79**: 412–422

Bonadonna, G., Santora, A., Gianni, L., Bonfante, V. (1993) Drugs ten years later: epirubicin. *Ann. Oncol.* **4**: 359–369

Broggini, H., Colombo, T., Martini, A., Donelli, M. G. (1980) Studies on the comparative distribution and biliary excretion of doxorubicin and 4′-epidoxorubicin in mice and rats. *Cancer Treat. Rep.* **64**: 897–904

Camaggi, C. M., Strocchi, E., Comparsi, R., Testoni, F., Pannuti, F. (1986) Biliary excretion and pharmacokinetics of 4′-epidoxorubicin (epirubicin) in advanced cancer patients. *Cancer Chemother. Pharmacol.* **18**: 47–50

Crooke, S. T., Mirabelli, C. K., Huang, C. H., Mong, S., Wong, A. (1983) Sequence and topology: specific attack on DNA and chromatin. In: Chen, Y. C. (ed.) *Development of Target-oriented Anticancer Drugs*. Raven Press, New York, p. 207

de Silva, D. M., Aust, S. D. (1993) Ferritin and ceruloplasmin in oxidative damage: review and recent findings. *Can. J. Physiol. Pharmacol.* **71**: 715–720

Di Liegro, I., Castelli, A., Matzanke, B. F., Bill, E., Trautwein, A. X. (1996) The interaction of Fe(III), adriamycin and daunorubicin with nucleotides and DNA and their effects on cell growth of fibroblasts (NIH-3T3). *Biometals* **9**: 121–130

Diociaiuti, M., Molinari, A., Calcabrini, A., Arancia, G. (1991) Electron energy loss spectroscopy analysis of adriamycin plasma membrane interaction. *J. Microsc.* **164**: 95–106

Dodd, D. A., Atkinson, J. B., Olson, R. D., Buck, S., Cusack, B. J., Fleischer, S., Boucek, R. J. (1993) Doxorubicin cardiomyopathy is associated with a decrease in calcium release channel of the sarcoplasmic reticulum in a chronic rabbit model. *J. Clin. Invest.* **91**: 1697–1705

Dorr, R. T., Von Hoff, D. D. (1994) *Cancer Chemotherapy Handbook*. Appleton and Lange, Norwalk, CT, p. 395

Eksborg, S., Stendahl, U., Lonroth, U. (1986) Comparative pharmacokinetic study of adriamycin and 4′-epiadriamycin after their simultaneous intravenous administration. *Eur. J. Clin. Pharmacol.* **30**: 629–631

Gewirtz, D. A., Yanovich, S. (1987) Metabolism of adriamycin in hepatocytes isolated from the rat and the rabbit. *Biochem. Pharmacol.* **36**: 1793–1798

Goormaghtigh, E., Vandenbranden, M., Ruysschaert, J. M., De Kruijff, B. (1982) Adriamycin inhibits the formation of non-bilayer lipid structures in cardiolipin containing model membrane. *Biochim. Biophys. Acta* **685**: 137–143

Gornall, A. G., Bardawill, C. J., David, M. M. (1949) Determination of serum proteins by means of the Biuret reagent. *J. Biol. Chem.* **177**: 751–766

Handa, K., Sao, S. (1976) Stimulation of microsomal NADPH oxidation by quinone group-containing anticancer chemicals. *Gann* **67**: 523–528

Hill, B. T., Whelan, R. D. M. (1982) A comparison of the lethal and kinetic effects of doxorubicin and 4′-epidoxorubicin in vitro. *Tumori* **68**: 29–37

Launchbury, A. P., Habboubi, N. (1993) Epirubicin and doxorubicin: a comparison of their characteristics, therapeutic activity and toxicity. *Cancer Treat. Rev.* **19**: 197–228

Le Bot, M., Begue, J. M., Kernaleguen, D., Robert, J., Ratanasavanh, D., Airiau, J., Riche, C., Guillouzo, A. (1988) Different cytotoxicity and metabolism of doxorubicin, daunorubicin, epirubicin, esorubicin and idarubicinin cultured human and rat hepatocytes. *Biochem. Pharmacol.* **37**: 3877–3887

Le Bot, M., Glaise, D., Kernaleguen, D. (1991) Metabolism of doxorubicin, daunorubicin and epirubicin in human and rat hepatoma cells. *Pharmacol. Res.* **24**: 243–252

Ludden, T. M., Beal, S. L., Sheiner, L. B. (1994) Comparison of the Akaike Information Criterion, the Schwarz criterion and the F-Test as guides to model selection. *J. Pharmacokinet. Biopharm.* **22**: 431–445

Mimnaugh, E. G., Gram, E. G., Trush, M. A. (1983) Stimulation of mouse heart and liver microsomal lipid peroxidation by anthracycline anticancer drugs: characterization and effects of reactive oxygen scavengers. *J. Pharmacol. Exp. Ther.* **226**: 806–816

Monti, E., Prosperi, E., Supino, R., Bottiroli, G. (1995) Free radical-dependent DNA lesions are involved in the delayed cardiotoxicity by adriamycin in the rat. *Anticancer Res.* **15**: 193–197

Muhammed, H., Ramasarma, T., Kurup, C. K. R. (1983) Inhibition of mitochondrial oxidative phosphorylation by adriamycin. *Biochim. Biophys. Acta* **722**: 43–50

Muindi, J. R., Sinha, B. K., Gianni, L., Myers, C. E. (1984) Hydroxyl radical production and DNA damage induced by anthracycline-iron-complex. *FEBS Lett.* **172**: 226–230

Oki, T., Komiyama, T., Tone, H., Inui, T., Takeuchi, T., Umezawa, H. (1977) Reductive cleavage of anthracycline glycosides by microsomal NADPH-cytochrome c reductase. *J. Antibiot.* (*Tokyo*) **30**: 613–615

Olson, R. D., Mushlin, P. S. (1990) Doxorubicin cardiotoxicity: analysis of prevailing hypothesis. *FASEB J.* **4**: 3076–3086

Pan, S. S., Pedersen, L., Bachur, N. R. (1981) Comparative flavoprotein catalysis of anthracycline antibiotics. Reductive cleavage and oxygen consumption. *Mol. Pharmacol.* **19**: 184–186

Pierscinski, G., Drzewoski, J., Nowak, D. (1994) The influence of doxorubicin and 4′-epidoxorubicin on lipid peroxidation in mouse heart, lungs and liver. Part II. *Pol. J. Pharmacol.* **46**: 55–59

Sinha, B. K., Trush, M. A., Kennedy, K. A., Mimnaugh, E. G. (1984) Enzymatic activation and binding of adriamycin to nuclear DNA. *Cancer Res.* **44**: 2892–2896

Sweatman, T. W., Israel, M. (1987) Comparative metabolism and elimination of adriamycin and 4′-epiadriamycin in the rat. *Cancer Chemother. Pharmacol.* **19**: 201–206

Tavoloni, N., Guarino, M. (1980a) Biliary and urinary excretion of adriamycin on anesthetized rats. *Pharmacology* **20**: 256–267

Tavoloni, N., Guarino, M. (1980b) Disposition and metabolism of adriamycin in the rat. *Pharmacology* **21**: 244–255

Van der Vijgh, W. J. F., Maessen, P. A., Pinedo, H. M. (1990) Comparative metabolism and pharmacokinetics of doxorubicin and 4′-epidoxorubicin in plasma, heart and tumor of tumor-bearing mice. *Cancer Chemother. Pharmacol.* **26**: 9–12

Weenen, H., van Maanen, J. M., de Planque, M. M., McVie, J. G., Pinedo, H. M. (1984) Metabolism of 4′-modified analogs of doxorubicin-unique glucuronidation pathway for 4′-epidoxorubicin. *Eur. J. Clin. Oncol.* **20**: 919–926