In vitro and *in vivo* Binding of Epirubicin to Red Blood Cells and Human Plasma Proteins

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In this study, the *in vitro* interaction of epirubicin (EPR), a cytostatic antibiotic, with plasma proteins (PP), namely α-HSA, γ-HSG, α+β-HSG and with isolated human red blood cells (RBCs) was investigated and further correlated with the *in vivo* pharmacokinetics and binding of EPR and two of its metabolites, 13-dihydroepirubicin and 7-deoxydoxorubicinone to RBCs. The *in vitro* encapsulation rate in isolated erythrocytes amounts to 52.9 ± 2.8% and remains constant within the range of studied concentrations (2.5–20 μg/ml). EPR was found to bind differently to the various PP *in vitro*. Binding to α-HSA amounted up to 51.0 ± 7.10%, to α+β-HSG 79.45 ± 2.7%, to γ-HSG 57.1 ± 2.8%. The *in vivo*-binding rate of EPR, dihydroepirubicin and deoxydoxorubicinone to RBCs after 5 min of injection was 32 ± 6.96%, 11.6 ± 3.1% and 10.05 ± 3.5% respectively, their availability in serum was 42.6 ± 11.8%, 2.4 ± 0.4% and 1.2 ± 0.67% respectively.

Introduction

Since it is known that plasma proteins can play an effective role as a subcompartment of the blood, it will be extremely useful to study the protein binding of drugs in order to obtain further information about its clinical pharmacokinetics and drug distribution (Colombo *et al.,* 1983). Blood components may also function as a storage vehicle for drugs, erythrocytes for example possess desirable properties of a drug carrier through which an intravasal depot may be achieved (Pitt *et al.,* 1983). Epirubicin, a cytostatic antibiotic from the anthracycline family represents the 4′-epimer of doxorubicin. The mechanism of its antitumor activity is similar to that of doxorubicin which intercalates into DNA and blocks therefore protein synthesis (Yoa-Pu Hu *et al.,* 1989) or inhibits the activity of the DNA topoisomerase II (Le Bot *et al.,* 1988).

EPR seems to have a better therapeutic index than doxorubicin, due to its lower myelosuppressive and lower cardiotoxic effects at equal doses (Weenen *et al.,* 1986). This can be explained by the difference in pharmacokinetics and metabolism revealing a lower half-life of elimination for EPR due to a unique glucuronidation pathway in human (Van der Vigh *et al.,* 1990).

Due to the fact that RBCs play an active role in drug distribution, metabolism and elimination it was necessary to carry out this study to investigate how far RBCs do interfere in the distribution and metabolism of EPR. The mechanism of interaction between EPR and RBCs might be an electrostatic one, similar to that of doxorubicin (Suillerot *et al.,* 1988) and/or interaction with the lipid components of the RBC membrane. The fact that metabolites were found in RBCs indicates penetration of EPR inside the RBCs and thus supposing the presence of lipid areas inside the blood cells.

Two metabolites were detected with the system we applied, 13-dihydroepirubicin and 7-deoxydoxorubicinone. 13-Dihydroepirubicin is formed through reduction of the C_{13} carbonyl function by an aldoketo reductase, 7-deoxy-doxorubicinone through reductive and hydrolytic cleavage of the amino sugar moiety to yield the anthraquinone aglycone (Robustelli, 1989) as shown in Fig. 1.

The 13-dihydro metabolites of the anthracyclines are generally considered as active as the parent compound in contrast to the inactive aglycones

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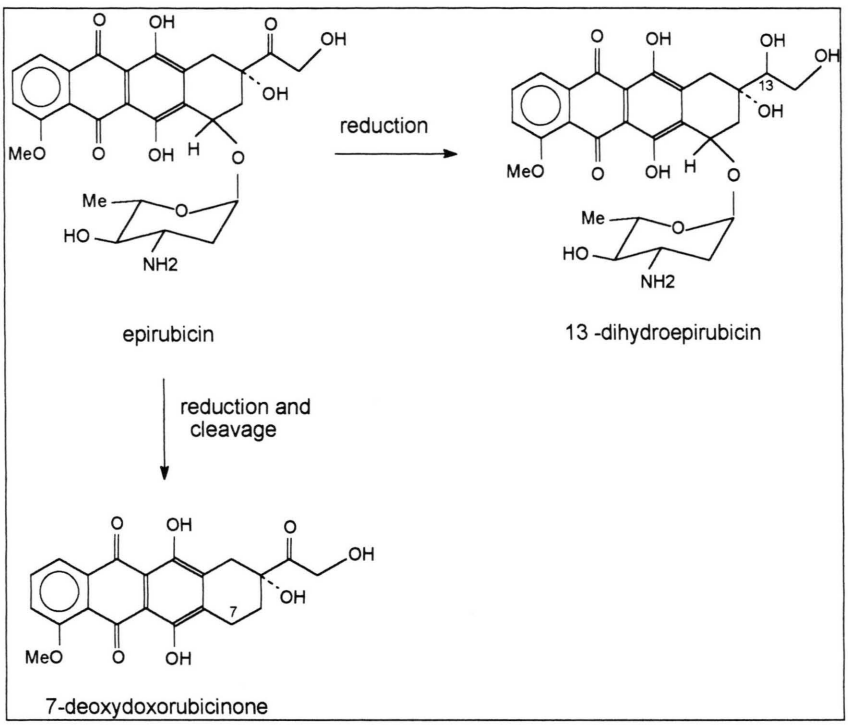

Fig. 1. Metabolization pathway of epirubicin.

or glucoronides (Schott and Robert, 1989). However, very few data are available on their activity.

Materials and Methods

Chemicals

Epirubicin hydrochloride (Farmorubicin 2 mg/ml) was supplied as a lyophilized powder for injection, by Farmitalia Carlo Erba AG (Vienna, Austria), 13-dihydroepirubicin and 7-deoxydoxorubicinone were supplied by Farmitalia Carlo Erba AG (Milano, Italy). Isoton II was obtained from Hellige (Germany). Erythrocyte concentrate from human volunteers was kindly supplied by the surgery department of the Hospital Rudolfstiftung (Vienna).

All reagents were of HPLC grade: methanol LiChrosolv, Merck (Darmstadt, Germany), bidistilled water, crist. potassium dihydrogen phosphate, Riedel-de Haën AG (Hannover, Germany), disodium hydrogen phosphate, Merck (Darmstadt, Germany).

The PP, α-HSA (96–99% albumin), γ-HSG (99%) and α+β-HSG were obtained from Sigma Chemical Co. (St. Louis, U.S.A.).

For the separation of the proteins Centrisarts ultrafiltration tubes with a MWCO of 10,000 from Sartorius AG (Göttingen, Germany) were used.

Chromatography

The amount of EPR was determined using a high performance liquid chromatographic method as described (Czejka and Georgopoulos, 1988) and which we further modified. The system used consisted of solvent delivery system Model 420 Kontron (Austria), an automatic autosampler injector Model 460 Kontron (Austria), Shimadzu RF-551 fluorimetric detector Shimadzu (Japan), a Nucleosil C18 column (5 μm), length 12.5·0.5 cm and a W&W recorder model 312. The fluorimetric detection followed at an excitation wavelength of 480 nm and an emission of 560 nm, with a range of ×4 and gain ×4. The system was operated at a flow rate of 1 ml/min, 110 bar pressure and 22 °C adjusted by a column oven 830 Kontron (Austria).

Sample clean up

Bond Elut C 18 cartridges (packing volume 100 mg, 30 μm mean particle diameter) Varian

Analytichem (Vienna) were used for solid phase extraction. The extraction procedure was carried out by use of a vacuum extraction unit from Vac Elut Analytichem Int. (U.S.A.).

Ultrafiltration

Preparation of erythrocytes: Heparinized fresh human blood was centrifuged at 1500×*g* for 20 min at 4 °C and the plasma was separated (Zocchi *et al.*, 1989). The erythrocytes were washed three times with an Isoton II solution, pH 7.4 and resuspended in Isoton II in the ratio 1:2.5 to yield a haematocrit of 0.4.

1) Binding to erythrocytes: 900 µl of the washed erythrocytes were spiked with 100 µl EPR in Isoton II (pH 4). The tubes were kept under protection from light for 0, 15, 30, 60 and 120 min in a Techne Dri-Block DB-1 at 37 °C in order to determine the time dependency of the encapsulation. Following concentrations of EPR were measured: 2.5, 5.0, 10 and 20 µg/ml. Three experiments were carried out with each concentration. Solutions of EPR in Isoton II were handled under identical conditions for control. After incubation the samples were centrifuged for 5 min at 4000×*g* using a Labofuge I Heraues, Christ (Austria). The supernatant was then removed and chromatographed as described previously.

2) Binding to plasma proteins: Physiological concentrations of the PP in buffer solution (19.7 ml 0.9% KH_2PO_4 and 80.3 ml 1.2% Na_2HPO_4, pH 7.4) α-HSA, α+β-HSG: 4 g/100 ml, γ-HSG 1 g/100 ml were freshly prepared as given in the literature (Ciba Geigy Tables, 1979).

The ultrafiltration membrane of the tubes was purified with distilled water for 20 min from adherent contaminants. The membrane was then preconditioned for 5 min (until saturation) with the solution which had to be ultrafiltered. In accordance to the RBCs the following concentrations of EPR solution were measured: 2.5, 5.0, 10 and 20 µg/ml. 900 µl protein solution was spiked with 100 µl EPR solution and kept at room temperature for 30 min. Three experiments were carried out with each concentration. After various incubation times 15, 30, 60 and 120 min samples were centrifuged for 5 min at 4000×*g* and the supernatant was analyzed as described.

3) In vivo binding to RBCS: EPR was injected as an i.v. bolus (60 mg/m^2) over 2 min in four patients suffering from colorectal cancer. Mean dose EPR injected was 110 ± 5.80 mg. Blood samples were drawn from patients after 5 min of injection, when α-phase of EPR distribution from blood and tissues into blood subcompartments has been terminated. Samples were centrifuged at 2500×*g* for 5 min. EPR concentration was determined in serum and in RBCs.

Solid phase extraction

The RP-18 extraction cartridges were preconditioned thrice with 1 ml of methanol and conditioned with 2 ml phosphate buffer (pH 4.0). 1 ml erythrocyte suspension was lysed with 1 ml of distilled water and centrifuged at 4000 r.p.m. The supernatant was then washed with methanol/buffer. EPR was extracted from the supernatant and serum with 1 ml methanol/phosphate buffer and analyzed.

Biometric calculations

As known, there exists a balance between free, RBC-bound and PP-bound drug concentration in the blood (Derendorf and Garett, 1987). Therefore the given coefficient of RBC partitioning defines the ratio between bound and free plasma drug concentration as in Eqn. (1):

$$K_{RBC} = \frac{C_{RBC}}{C_{Serum}}. \tag{1}$$

The whole amount of EPR in serum was determined according to Eqn. (2):

$$100\%\ EPR_{ser} = \%\ EPR_{free} + \%\ EPR_{RBC} + \%\ M1 + \%\ M2 + \%\ M1_{RBC} + \%\ M2_{RBC} + EPR_{PP}\,. \tag{2}$$

Besides, the distribution of the metabolites in RBCs formed in the liver should be considered.

Results and Discussion

Protein and RBC binding of a drug represents an important factor of its serum pharmacokinetics due to distribution into subcompartments of the blood. Anyhow through binding to RBCs and/or PP a retarded circulation of the drug results, which is undesirable with anthracyclines due to the permanent cardiotoxicity of the circulating drug. In the present study we wanted to elucidate the extent of EPR and its metabolites binding to RBCs

and PP, which is still up till now not investigated. Differences in binding to PP are due to i) a difference in the concentration of binding protein, ii) a difference in the characteristics of the binding protein itself (binding site, binding affinity) (Taeyuki *et al.*, 1991), iii) the chemical structure of the drug.

The ultrafiltration method results lead to the conclusion that EPR binds definitively and rapidly to PPs and human RBCs *in vitro* as well as *in vivo*. Sample clean up recovery of EPR was 97%, of 13-dihydroepirubicin 94%, of 7-deoxydoxorubicinone 88% and the absolute sensitivity 1 ng/ml. Among the studied protein fractions, α+β-HSG showed an unexpectedly high binding of 79.45 ± 2.7% to EPR, although this fraction mainly binds basic drugs. This is explicable by the fact that EPR may exist in an ionized form in blood having a pK_a value of 8.2 for the hydrochloride (Vigevani and Williamson, 1980). Binding to α-HSA amounted up to 51.0 ± 7.10%, and to γ-HSG 57.1 ± 2.8% as shown in Fig. 2. The *in vitro*-binding rate to RBCs seems to vary within a constant range of 52.9 ± 2.8% with a hematocrit of 0.4.

Table I shows the coefficients of partition for PP and RBCs *in vitro*. The *in vitro* interaction of EPR with PP and RBCs was independent either of PP, RBCs or EPR concentration within the studied range of concentrations (2.5–20 μg/ml).

The binding to RBCs and PP *in vitro* was independent of the temperature. Three experiments were carried out at 25 °C, with each concentration. Results did not show any significant difference to those experiments carried out at 37 °C. Binding to α-HSA was found to be 52.4 ± 5.5%, to α+β-HSG 76.1 ± 4.2%, to γ-HSG 59.5 ± 3.8% and to RBCs 54.6 ± 5.0%.

EPR shows a rapid distribution phase (α-phase) within the first 20 min, with half-life of 3 min (Eksborg, 1990), followed by an intermediate re-equilibration phase (β-phase) and a third phase of terminal elimination. Blood samples were drawn after 5 min of injection, because at this time point the distribution phase α is terminated and EPR can be found in serum and RBCs at high concentrations, before elimination and actual biotransformation take place. 13-Dihydroepirubicin (M1) and 7-deoxydoxorubicinone (M2) were detectable in serum and RBCs. The metabolites appear already within 5 min after injection, both in serum and RBCs, indicating thus a vivid biotransformation process for EPR. 42.6 ± 11.8% of EPR was found unbound in serum, 32.0 ± 6.9% was bound to RBCs and the rest was either bound to plasma proteins or metabolized.

Table II giving in the total 100% EPR as shown in Eqn. (2).

The concentration of the metabolites in RBCs is higher than in serum. The K_{RBC} for M1 after 5 min of injection was 4.83, for M2 8.0, indicating thus a very high affinity of the metabolites in serum to RBCs or a metabolizing process inside the erythrocytes. The very high K_{RBC} for M2 is due to its higher lipophilicity through its loss of the amino sugar group, and therefore more intensive interactions between it and the lipophilic RBC

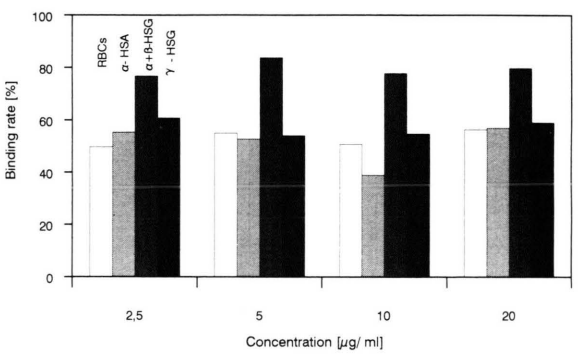

Fig. 2. *In vitro* binding of epirubicin to plasma proteins and red blood cells.

Table I. Coefficients of partition for epirubicin to red blood cells and plasma proteins *in vitro* with different epirubicin concentrations in buffer [μg/ml].

Conc. [μg/ml]	RBCs (M ± SD)	α-HSA	α+β-HSG	γ-HSG
2.5	0.96 ± 0.16	1.24 ± 0.5	3.29 ± 0.16	1.55 ± 0.17
5	1.22 ± 0.26	0.64 ± 0.04	3.33 ± 0.59	1.21 ± 0.22
10	1.06 ± 0.37	1.11 ± 0.09	5.15 ± 0.51	1.17 ± 0.28
20	1.29 ± 0.34	1.33 ± 0.49	3.93 ± 0.77	1.44 ± 0.21

Table II. Comparison of the percent concentration of epirubicin, dihydroepirubicin and deoxydoxorubicinone in serum and red blood cells *in vivo* (blood samples from patients) and *in vitro* (samples from test tube).

$n = 4$	% EPR	% M1	% M2
Serum	42.6 ± 11.8	2.4 ± 0.4	1.2 ± 0.67
RBC *in vivo*	32.0 ± 6.96	11.6 ± 3.1	10.5 ± 3.5
RBC *in vitro*	52.9 ± 2.8	–	–

membrane are facilitated. However, Suillerot *et al.* has proven that the prime interaction is that between doxorubicin and the negatively charged phosphate groups of the zeta potential, the fact which contradicted by the results we concluded showing that the more lipophilic metabolite deoxydoxorubicinone has a higher affinity to RBCs.

These results comply with the up-to-date scientific stand, supporting the fact that EPR and metabolites have a high affinity to RBCs (Czejka *et al.,* 1992). Czejka *et al.* found a K_{RBC} for EPR of 1.47 and for M2 3.27 after 5 min after an i.v. bolus of a mean dose of 101.67 ± 6.87 mg EPR.

Correlating *in vitro* with the *in vivo* binding of EPR to RBCs, the results show an *in vivo* K_{RBC} of 0.75, on the other hand an *in vitro* K_{RBC} of 1.12. That the *in vitro* EPR binding to RBC about 1.6-fold higher than *in vivo* is due to the fact that metabolizing enzymes, proteins and hepatic excretion are absent *in vitro.* The encapsulation or binding of EPR was completely fulfilled within 5 min, both *in vivo* and *in vitro* experiments. No metabolites were detected under *in vitro* conditions. De Loach *et al.* proved that the concentrations of doxorubicin higher than 3 mg/ml has a great influence on the amount of drug that can be encapsulated in RBCs (Magnani and Loach, 1992) the fact that we did not further investigate.

Extent of in vivo PP binding

It has been shown for some drugs that the extent of PP binding can be estimated using K_{RBC}, although this method is only applicable at high non-therapeutical concentrations (Marroum and Curry, 1993). The *in vivo* binding to PP can be calculated as follows:

$$f = 1 - \frac{K_{RBC\,vivo}}{K_{RBC\,vitro}}$$

where f = fraction of free drug.

The *in vivo* extent of protein binding was determined at a concentration of 1188.5 ± 702.26 ng/ml EPR in serum, resulting in a binding rate of 33.6%. Anyhow, although this method may not be applicable for anthracyclines, the result obtained can be in all cases reasonable as 42.6% of EPR was found unbound in serum and 32.0% bound to RBCs. Mean value of the *in vitro* binding of EPR to all PP studied fractions summed up to 62.5 ± 12.22%. As already mentioned, this difference in binding is caused by metabolizing processes, binding to other fractions (RBCs) and an assumed inactivation of EPR at blood pH 7.4.

Conclusions

The *in vivo* and *in vitro* determination of EPR in subcompartmental structures of blood allows to obtain further detailed information about the amount of EPR bound to tissues and the amount-free circulating in blood. Binding of EPR to PP and to RBCs is therapeutically not desirable, due to the resulting intravasal depot and the retarded circulation in organism. EPR undergoes a partial metabolic activation in blood through the formation of the dihydrometabolite. The use of RBCs in drug targeting as De Loach propagated for doxorubicin, may be useful just in the case the loaded RBCs can be transferred to the "site of action". However, this transfer action cannot be predicted, because it depends on the kind, stage and metastasization of the tumor. Encapsulation methods in RBCs in order to attain a better drug targeting were successfully achieved with antibiotics, such as gentamycin and anticoagulants, due to the enhanced RBCs concentration in the tumor area. It is still unforeseeable whether RBCs will prevail as drug carriers. Anyhow, RBCs should not be affected or damaged by the drug.

On the other hand, the present results show that PP and RBCs certainly act as a transport vehicle within the blood. Besides, the metabolization of EPR in RBCs should not be underestimated, as long as some of the formed metabolites, 7-deoxydoxorubicinone and glucuronides, are therapeutically insignificant and therefore contribute to the inactivation of EPR. These results are similar to those which we already observed with doxorubicin. In further experiments, the combination of EPR with biomodulating agents, for example interferon α will be examined relating to differences in metabolism, serum pharmacokinetics and distribution in subcompartmental structures.

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