Alternatively, it is known that there are at least two cytochrome P450 enzymes responsible for the conversion of encainide to ODE in human liver microsomes [12]. Should similar enzymes be present in the rat, it is possible that one of them may have been selective for the metabolism of (−)-encainide. Studies are now in progress to distinguish these possibilities.

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Binding of retelliptine, a new antitumoral agent, to serum proteins and erythrocytes

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Retelliptine hydrochloride (Fig. 1) is a new antitumoral derivative in the ellipticine series exhibiting high cytotoxicity against several experimental tumor models in mouse and rat without serious side effects [1]. Its antitumor efficiency may be due to intercalation into DNA which could alter secondly topoisomerase II activity and involve single and/or double strand breaks [2, 3].

At physiological serum pH, this drug is a lipophilic, weak basic molecule ($pK'_a = 7.84$) and it is predominantly in an unionized form.

No previous assays about serum protein binding of this intercalant class has been published. So, before beginning phase I trial and in order to better understand their binding and blood distribution, we have investigated the binding of retilliptine to blood components.

Materials and Methods

Chemicals. Radiolabelled retelliptine hydrochloride ([^{14}C]3′propyl; 11.34 mCi/mmol) was gently provided by SANOFI Recherche (Montpellier, France). Purity checked by mass spectrometry was above 98%. Stock solution was prepared in distilled water (0.5 mM). Stability in water has been established previously. At pH < 8, aqueous solubility was excellent up to 4 mM.

Blood fractions. All the proteins used were from human origin. They included human serum albumin (HSA, Sigma 1887), α_1-acid glycoprotein (AAG, Behring), polyvalent γ-globulins (Sigma, G 2388) and lipoproteins (HDL, LDL, VLDL: Sigma, L 2014, L 2139, L 2264). Erythrocytes were prepared from fresh human blood samples. Heparin was added to blood prior to centrifugation. Red cells were washed out twice by large volumes of isotonic saline solution to eliminate residual plasma.

Binding assay. Methods usually used for measuring protein binding as equilibrium dialysis or ultrafiltration were not appropriate for retilliptine because of very important non-specific adsorption on cellulose dialysis membranes. So, the binding of retilliptine to proteins and erythrocytes

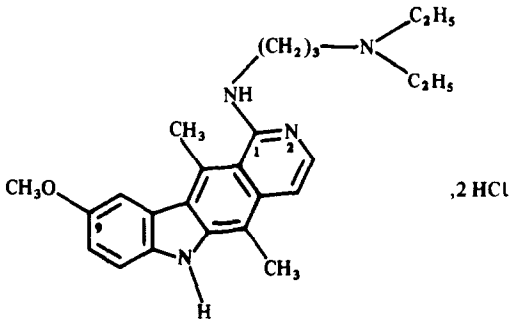

Fig. 1. Chemical structure of retelliptine.

was studied by an erythrocyte partitioning method. This technique assumes that free concentration of drug (C_f) in plasma is in equilibrium with that in erythrocytes.

Erythrocyte suspension was prepared in diluted serum (10–90% of serum by increments of 10%) or in protein solutions containing various amounts of HSA, AAG, γ-globulins or lipoproteins. All dilutions were achieved by isotonic phosphate saline buffer (0.067 M Sorensen's buffer plus 0.15 M NaCl; pH 7.4). Serum pH was readjusted to 7.4 before using by lactic acid 1 N (10 μL for 1 mL). Fifty μL of stock solution of [^{14}C]retelliptine was added to 2.25 mL of erythrocyte suspension.

For the erythrocyte binding study, erythrocytes were suspended in phosphate buffer saline containing increasing concentrations of radiolabeled retelliptine. Then, triplicate samples were incubated at 37° for 30 min with gentle orbital shaking in a Brunswick water bath. Preliminary distribution studies showed that drug equilibrium between erythrocytes and protein medium was achieved within 20 min and was constant for at least 1 hr. The drug did not bind to the flasks used for all experiments (polypropylene flasks, Packard Ref. 6000178).

At the end of incubation, hematocrit (H) was measured and triplicate 25-μL aliquots taken for counting whole suspension (WS). It was around 20% for all experiments except for total serum protein binding study in which it had a physiologic value of 40%.

After centrifugation (3000 g, 3 min, in a Z 2320 K centrifuge, BHG, Gosheim, F.R.G.) at 37°, 25-μL aliquots of supernatant were counted (P). The ratio of drug concentrations in erythrocytes and protein solution (E/P) was calculated from the following equation:

$$E/P = [(WS/P) - (1 - H)]/H. \quad (1)$$

As we used only low concentrations of retelliptine, we may assume that the total and free concentrations of retelliptine in either protein solution or in erythrocytes are linearly related as follows:

$$C_p = (1 + nK_a \cdot P_t) \cdot C_f \quad (2)$$

$$C_e = (1 + NK_e) \cdot C_f \quad (3)$$

where C_p and C_e are the total concentrations of retelliptine in the protein solution and erythrocytes respectively, P_t is the total concentration of protein, C_f is the concentration of free retelliptine, nK_a is the total binding constant for retelliptine interaction with the protein (product of n, number of binding sites, by K_a corresponding association constant), and NK_e is the binding coefficient for retelliptine interaction with erythrocytes (dimensionless, product of N, concentration of erythrocyte binding sites, by K_e, association constant).

Dividing Eqn 2 by Eqn 3 gives:

$$C_p/C_e = (1 + nK_a \cdot P_t)/(1 + NK_e). \quad (4)$$

Accordingly, the C_p/C_e ratios were measured at a series of different protein concentrations (P_t).

Finally we may write:

$$C_b/C_p = (nK_a \cdot P_t)/[(nK_a \cdot P_t) + 1] \quad (5)$$

where C_b is the concentration of bound retelliptine to plasma protein.

Simulations of blood distribution. Some theoretical calculations were made to stimulate the fractional amount of retelliptine bound to each plasma and to erythrocytes in blood, as well as the amount of free drug (*fu*) as previously described [4].

Results

Binding to serum proteins. The NK value for retelliptine hydrochloride binding to normal serum is 21.88 ± 1.04. So, according to Eqn 5, the serum binding percentage is 95.6 ± 4.4% (bound concentration in serum relative to total concentration in serum).

Table 1 presents estimated nK values and corresponding binding percentages of this drug to plasma proteins. Figure 2 shows representative experimental data for retelliptine binding to HSA.

Binding to erythrocytes. Retelliptine hydrochloride is extensively bound to erythrocytes (96.8 ± 3.1%). The NK_e value is 30.84 × 0.55, 1.5-fold the serum NK value observed.

Simulations of blood distribution. Using the estimated binding parameters of retelliptine to the different blood fractions, we simulated its blood distribution at expected therapeutic levels (0.2–10 μM) in healthy condition or in cancer state. In the former, we chose physiologic hematocrit around 40% and physiologic protein concentrations (HSA = 600 μM; AAG = 20 μM, polyvalent γ-globulins 83.3 μM, HDL 4 g/L, LDL 3.5 g/L, VLDL 1 g/L). Inflammatory process and malnutrition are particularly frequent syndromes in cancer disease. So, we considered a higher α_1-acid glycoprotein concentration (40 μM), a lower hematocrit (35%) and a lower albumin concentration (400 μM). All results are reported in Table 2. In both cases, the free fraction is very low (2%) and the drug is extensively bound to erythrocytes (approx. 50%).

Discussion

The main feature of retelliptine hydrochloride binding is its high affinity for red cells and to a lesser extent for AAG and HSA. These results can explain the low free fraction of retelliptine in blood. Important binding to erythrocytes has been already investigated with other ellipticine derivatives such as elliptinium or 9-OH ellipticine. In previous *in*

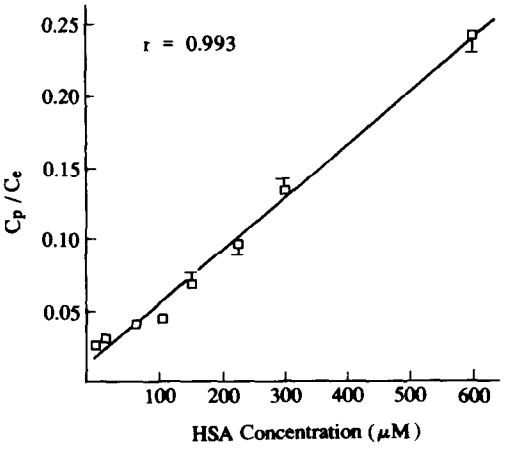

Fig. 2. Binding of retelliptine on HSA.

Table 1. nK_a values and corresponding binding percentage of retelliptine on plasma proteins

	HSA*	AAG*	γ-GLOB*	HDL†	LDL†	VLDL†
nK_a	$2.1 \times 10^4 \pm 3 \times 10^3$	$1.35 \times 10^5 \pm 4 \times 10^4$	$1.4 \times 10^4 \pm 4 \times 10^3$	0.85 ± 0.12	1.31 ± 0.04	0.71 ± 0.09
Binding %	92.6 ± 4.4	72.9 ± 5.4	53.8 ± 3.9	77.4 ± 10.9	58.1 ± 6.7	41.6 ± 5.3

* M^{-1}; † L/g.
Binding percentages are calculated at physiologic concentrations of proteins: HSA, 600 μM; AAG, 20 μM; γ-GLOB, 15 g/L; HDL, 4 g/L; LDL, 3.5 g/L; VLDL, 1 g/L.

Table 2. Comparison of computed distribution of retelliptine in blood of healthy individuals and cancer patients

Computed % bound to	Normal	Cancer
Plasma	48.7	52.2
Red cells	48.8	45
HSA	30.7	23.4
AAG	6.6	15
γ-GLOB	2.9	3.9
HDL	4.2	4.8
LDL	2.7	3.1
VLDL	1.7	2
Free fraction	2.5	2.8

See Results for hematocrit values and protein concentrations used in the calculations.

vitro studies on liposomes, cellular membranes and red cells, lipophilicity and high affinity for membrane phospholipids appeared as the most striking chemical property of these drugs [5–8]. Compared to elliptinium, another ellipticine derivative, 2-(diethyl amino 2-ethyl)9-OH ellipticinium chloride, presents a stronger antitumor efficiency on several experimental murine tumors. This greater cytotoxicity may be due to the presence of a side chain on the aromatic ellipticine ring which increases its lipophilicity and membrane interactions [9, 10]. In the same way, retelliptine has the same diethyl amino ethyl side group on the C1 position. So, we can expect, perhaps, a good diffusion across cytoplasmic membranes with an important intracellular uptake and especially, many membrane alterations increasing its antitumor effect [9, 10]. This very strong affinity for red cells also confirms preliminary pharmacokinetic data obtained in rat. Less than 5 min after short intravenous administration of the drug to CD (SD) BR rat (5 mg/kg), retelliptine was undetected in plasma but concentrations between 6 and 12 μM were measured in erythrocyte samples (unpublished data). Then 1 hr later, a slow release seems to occur in plasma, rapidly hidden by tissue distribution. Regarding its lipophilicity, confirmed by the high nK_a values obtained for all lipoproteins studied, this tissue distribution should be very large. The binding to erythrocytes is a non-saturable process for all concentrations tested (up to 200 μM) i.e. for all blood concentrations expected after therapeutic administration. As hematocrit values were low (20%) in the experiments, we can assume there will be no significant increase of free fraction in anemic cancer patient.

The serum binding profile of retelliptine looks like basic drugs, particularly because of high affinity binding to AAG. However, in plasma retelliptine is mainly bound to HSA because of the high concentration of this protein relative to AAG. Retelliptine presents a rigid structure including four aromatic cycles and possibly binds to AAG by hydrophobic interactions [11–14]. This binding could be a saturable process on one single site for concentrations above 20 μM. This saturation should not be observed at therapeutic concentrations.

We have compared blood distribution of retelliptine in healthy and cancer patients. In advanced disease, inflammatory process and malnutrition seem to be the most important factors modifying plasma protein concentrations and hematocrit value. Inflammatory anemia weakly increases distribution of the drug in plasma in which the major fraction of bound retelliptine is then associated to AAG. Nevertheless, the free blood fraction remains the same, this modification in blood distribution should not alter the pharmacokinetics of retelliptine.

Finally, the use of the erythrocyte partitioning method provides an interesting alternative to the classical equilibrium dialysis or to ultrafiltration techniques. It is particularly useful for protein binding studies of drugs such as retelliptine, exhibiting non-specific adsorption on cellulosic membranes.

In summary. The binding of retelliptine hydrochloride to isolated serum proteins and erythrocytes was studied in order to predict free plasma concentration and to understand its blood distribution. Because of very important non-specific adsorption on cellulose, retelliptine binding was studied by using an erythrocyte partitioning technique. In serum, retelliptine was mainly bound to albumin and α_1-acid glycoprotein. In blood, it was bound to erythrocytes via a non-saturable process up to 200 μM and 45–50% of the drug was associated to the red cells. This last result could be explained by a high affinity for phospholipid layers.

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Lack of effect of heparin on TXA2 binding to platelets

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Heparin was reported to potentiate platelet aggregation [1] and to interfere with the antiaggregating effects of PGI_2, PGD_2 [2] and of other prostanoid (PGE_1) and non-prostanoid compounds [3]. Heparin has been demonstrated to bind to specific receptors on the surface of intact human platelets [4] and to interact with other platelet receptors, such as alpha adrenoceptors so reducing their binding affinity [5]. However, no inhibitory effect of heparin on the PGI_2 and PGD_2 binding to platelets was demonstrated [2], so that the mechanism of the proaggregatory effect has not yet been clarified. Lewy *et al.* [6] reported an increase in TxB_2 plasma levels after heparin intravenous administration in humans so suggesting that heparin might accelerate TxA_2 mediated proaggregating effects or coronary vasocontriction.

The aim of the present study was to establish if the proaggregating activity of heparin is related to change in the binding characteristics of TxA_2 released during platelet