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Vinorelbine high-affinity binding to human platelets and lymphocytes: distribution in human blood

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Abstract. Using [^{3}H]-vinorelbine, we demonstrated the presence of saturable and time-dependent high-affinity binding sites on human platelets and lymphocytes. The dissociation constant and binding-site values observed were 200 ± 38 nM, 20.0 ± 2.2 amol/platelet, and 155 ± 20 amol/lymphocyte, respectively. Among other blood components, saturable low-affinity binding of vinorelbine to alpha$_1$-acid glycoprotein, serum albumin, and lipoproteins was observed. The binding to erythocytes was nonsaturable. Given the relative concentrations of these carriers, vinorelbine mainly distributes in the platelet compartment in blood (>70%), and the amount of free vinorelbine in plasma relative to the total amount in blood is <2%. It is suggested that because of the preferential retention of vinorelbine by platelets, variations in the platelet count are very likely to produce changes in the free blood fraction of vinorelbine.

Introduction

Vinca alkaloids are cytotoxic drugs used extensively in the treatment of certain neoplastic diseases, particularly lymphomas. Vinorelbine (Navelbine) is a semisynthetic vinca alkaloid with a marked lipophilicity that produces characteristic pharmacokinetic features [5]. It has been established that vinblastine and vincristine bind reversibly to platelets [3] and to serum proteins [2, 7]. Moreover, it is generally accepted that the free drug concentration is more closely related to the pharmacologic or toxicologic response than to the total drug concentration, since drug delivery from the blood to cells and tissues occurs from the free pool of drug in the blood [8].

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The aim of the present study was to investigate the protein and blood-cell binding of vinorelbine, including serum binding in cancer disease and drug interactions at the protein-binding level.

Patients and methods

Chemicals. All chemicals were of analytical grade. Unlabeled navelbine ditartrate (batch RU 11) was supplied by P. F. Médicament. Tritium-labeled vinorelbine ditartrate reference TMM 1140, batch 190-1; 507.13 Ci/mol or 0.47 Ci/g) was supplied by C. E. A (Saclay, Gif-sur-Yvette, France). The drug was stored at –20° C until use.

Human proteins. Human serum albumin A-1887 (Sigma), human serum albumin A-8763 (Sigma; molar ratio NEFA/HSA = 4.5), alpha$_1$-acid glycoprotein (Behring, 99% pure), gamma globulins (Sigma Cohn's fraction II, 99% pure), and lipoproteins [very-low-density lipoprotein (VLDL) fraction, Sigma L-2264; low-density lipoprotein (LDL) fraction, Sigma L-2136; and high-density lipoprotein (HDL) fraction, Sigma L-2014] were dissolved in Sörensen's phosphate buffer (pH 7.4).

Serum collection from cancer patients. Sera were obtained from patients with clinical and histologically proven neoplasia. Blood samples were collected into vacuum glass tubes without the addition of anticoagulant, were allowed to clot for 1 h, and were then centrifuged to yield serum. The mean age of the patients was 65 ± 14 years (range, 39–88 years). The mean concentration of alpha$_1$-acid glycoprotein was 31.1 ± 13.3 μM (range, 13.7–60.5 μM) and that of albumin was 447 ± 69 μM (range, 343–597 μM).

Blood-product preparation. Blood was drawn from the antecubital vein of a healthy volunteer and collected in tubes containing ethylenediaminetetraacetic acid (EDTA) disodium salt (20 mg/10 ml blood). Plasma was separated from erythrocytes by centrifugation at 3,000 rpm for 3 min. Erythrocytes were washed twice with NaCl (9 g/l) before use. Plasma or erythrocytes were used on the day of collection or on the following day. Blood was centrifuged for 5 min at 130 *g* to yield platelet-rich plasma (supernatant), which was centrifuged for 20 min at 1,000 *g*. The pellet of platelets was then washed in PBS (phosphate buffer plus NaCl, 9 g/l) by recentrifugation (5 min, 50 *g*), and the supernatant containing platelets was centrifuged for 10 in at 1,000 *g*. The final pellet was dispersed in incubation buffer (PBS containing 0.5 g glucose/l). To obtain lymphocytes, blood was mixed with the same volume of Hanks' solution and the mixture was centrifuged on Ficoll/Paque density gradient (25 ml diluted blood and 10 ml Ficoll) at 400 *g* for 35 min using the

method of Böyum [1]. The lymphocyte ring was removed and washed twice in Hanks' solution. The lymphocytes were finally resuspended in the incubation buffer for binding experiments. Lymphocytes and platelets were counted using a Malassez cell with trypan blue.

Equilibrium dialysis. Aliquots of protein solutions were added to Teflon cells (0.25 ml/side) and dialysis was performed against Sörensen's buffer (67 m*M*, pH 7.4) containing various concentrations of vinorelbine (mixture of radiolabeled and unlabeled drug at a constant ratio). The dialysis was performed at 37° C for 3 h under constant stirring (20 rpm), with no apparent accumulation of fluid on the protein or serum side of the dialysis chamber. The two chambers were separated by a semipermeable membrane (Visking; pore diameter, 15–20 Å; cutoff, 12,000 Da). Previous studies had shown that equilibrium with respect to the free drug fraction was achieved within 2 h. When serum was used, the dialysis was performed against Sörensen's buffer (67 m*M*, pH 7.1) to restore physiologic pH in the system [10] (the pH of frozen serum is approx. 8) and the concentration of vinorelbine was 1,000 n*M*.

Binding to erythrocytes. Washed erythrocytes were resuspended (hematocrit, approx. 0.5) in PBS. Variable amounts of vinorelbine were added to glass flasks containing 1 ml of erythrocyte suspension to produce concentrations of 0.4–19 μ*M*. The samples were incubated for 30 min under gentle orbitary shaking at 37° C in a Brunswick water bath. Preliminary distribution studies showed that equilibrium between the erythrocytes and the medium was achieved within 10 min and remained constant for at least 1 h. At equilibrium, aliquots of whole suspension and supernatant were removed, and the vinorelbine concentration in erythrocytes was obtained from the following formula:

$$E = \frac{WS - P \cdot (1 - H)}{H}, \quad (1)$$

where *E* represents the erythrocyte drug concentration, *WS* represents the whole suspension, *P* indicates the supernatant (after centrifugation), and *H* represents the hematocrit.

Binding to platelets and lymphocytes. Binding experiments were carried out in glass tubes in a total volume of 1 ml containing various concentrations of labeled vinorelbine and platelet or lymphocyte suspension in PBS plus 0.5 g glucose/l. After incubation, cold (4° C) TRIS buffer (5 m*M*) was rapidly added to stop the reaction. Bound and free ligands were separated by rapid filtration through Whatman GF/B glass-fiber filters. Each filter was washed with an additional volume of 2 × 5 ml ice-cold 5 m*M* TRIS buffer (pH 7.4) at 4° C and the radioactivity was counted in a liquid scintillation counter. Specific binding was defined as the difference between the binding observed in the presence versus the absence of 100 μ*M* unlabeled vinorelbine. First, the time to reach the binding equilibrium was determined in glass flasks containing 10 ml suspension, and the linearity of binding was then determined using various concentrations of cells or platelets. These experiments were done using approximately 25–45 n*M* vinorelbine.

Determination of labeled drug concentration. Concentrations of [^{3}H]-vinorelbine in buffer, protein solutions, cell suspensions, and filters were determined in a Packard liquid scintillation counter (Tri-Carb 460 CD). For these determinations, erythrocyte suspensions were bleached, whereby 25 μl erythrocyte suspension was mixed with 200 μl sodium hypochlorite by a 10-s vortex agitation; after 10 min, 3 ml liquid scintillation solution was added to the resulting sample for counting.

Calculations. According to mass-balance considerations and using our estimated binding parameter, we made some theoretical calculations to simulate the fractional amount of vinorelbine bound to each blood fraction (f_{bi}) as well as the fractional amount of free drug (f_u) [9].

Analysis of data. The binding data were analyzed according to previously described models [8] by an iterative nonlinear regression program using the least-squares criterion (MicroPharm, INSERM 1990). All values are presented in the form of a mean or estimate, standard deviation (SD), and sample number (*n*).

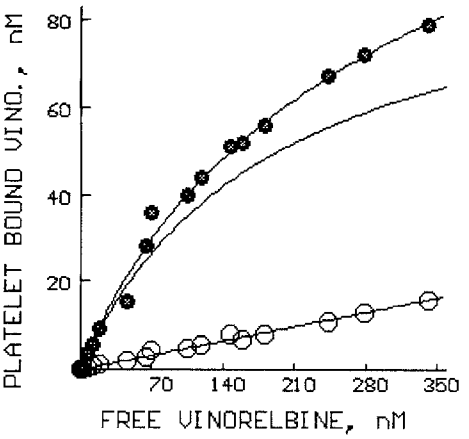

Fig. 1. Binding of vinorelbine to platelets at 37° C. Platelets (5 · 10^6/ml) were incubated for 1 h in the incubation buffer. *Lower straight line*, nonspecific binding; *upper curve*, total binding; *curve in between*, specific binding

Table 1. Parameters of vinorelbine binding to platelets and lymphocytes

	B_{max} (n*M*)	K_D (n*M*)	Nonspecific binding (slope of linear binding)
Platelets (5 · 10^6/ml)	100 ± 11	200 ± 38	0.048 ± 0.007
Lymphocytes (8.5 · 10^5/ml)	132 ± 17	235 ± 50	0.019 ± 0.008

B_{max}, Concentration of sites; K_D, dissociation constant.
Note that at the physiologic concentration of platelets (4 · 10^5/mm^3), B_{max} = 8,000 n*M* and the slope of nonspecific binding = 3.84, and at the physiologic concentration of lymphocytes (4,000/mm^3), B_{max} = 621 n*M* and the slope of nonspecific binding = 0.089

Results

Determination of equilibrium time for cell binding

The vinorelbine concentration in lymphocytes or platelets was measured as a function of time and association rate constants (k_{+1}) were determined, being 1.0 ± 0.1 μ*M*$^{-1}$ · min^{-1} for platelets and 0.54 ± 0.11 μ*M*$^{-1}$ · min^{-1} for lymphocytes. The binding equilibrium was reached at 20 min for 5 · 10^6 platelets/ml and for 833,000 lymphocytes/ml. The binding of vinorelbine to platelets or lymphocytes was linear in the range of 2–20 · 10^6 platelets/ml or 0.1–1 · 10^6 lymphocytes/ml.

Vinorelbine binding to platelets and lymphocytes

Vinorelbine bound to platelets (Fig. 1) and lymphocytes according to a saturable process with a high affinity and a high binding-site concentration [K_D, 200 ± 38 n*M* and 235 ± 50 n*M*, respectively; B_{max}, 100 ± 11 n*M* (corresponding to 5 · 10^6 platelets/ml) and 132 ± 17 n*M* (corresponding to 850,000 lymphocytes/ml), respectively], showing a high level of specific binding at the K_D value:

Table 2. Parameters of vinorelbine binding to isolated plasma proteins

Protein (mM^{-1})	n	K_A (mM^{-1})	$n \cdot K_A$
Albumin[a]	1.09 ± 0.16	1.31 ± 0.25	
Albumin[b]	1.02 ± 0.22	1.18 ± 0.33	
Alpha$_1$-acid glycoprotein	0.12 ± 0.03	616 ± 309	16 ± 2
Gamma globulins	1.87 ± 0.32	2.69 ± 0.83	
HDL	45.2 ± 32.1	0.98 ± 0.88	
LDL	759 ± 156	2.46 ± 0.79	
VLDL	1,690 ± 110	9.03 ± 1.43	

[a] Pure defatted human serum albumin; nonesterified fatty acids/albumin = 0.04 (mol : mol)

[b] Human serum albumin; nonesterified fatty acids/albumin = 0.48 (mol : mol)

n, Number of sites; K_A, association constant; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein

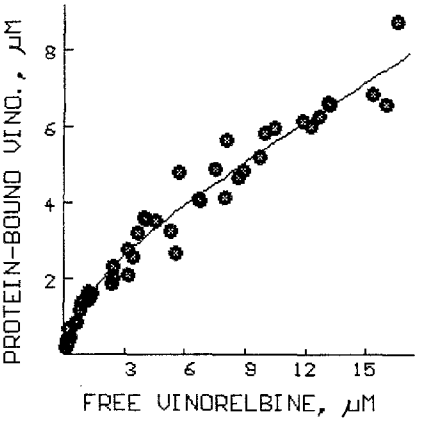

Fig. 2. Binding of vinorelbine to alpha$_1$-acid glycoprotein at 37° C. The alpha$_1$-acid glycoprotein concentration in solution in phosphate buffer at pH 7.4 was 20 μM. The data plotted were derived from equilibrium-dialysis experiments

>80% for platelets and >90% for lymphocytes. The results are summarized in Table 1.

Vinorelbine binding to isolated proteins

The binding of vinorelbine to each isolated protein was saturable according to a one-component class of sites. In the case of alpha$_1$-acid glycoprotein, a supplementary nonsaturable binding component was observed. The binding parameters obtained are presented in Table 2 and a representative experiment is shown in Fig. 2. No significant effect of nonesterified fatty acids on albumin binding was observed.

Vinorelbine binding to erythrocytes

Binding to erythrocytes was nonsaturable, and a binding constant (NK_E, ratio of the vinorelbine erythrocyte-associated concentration to the concentration in buffer) was obtained from partitioning of vinorelbine between erythrocytes and buffer. The NK_E value obtained was 1.118 ± 0.022.

Vinorelbine binding to sera from patients with cancer

Serum binding was studied in 24 cancer patients at the expected maximal therapeutic vinorelbine concentration (1 μM). The serum f_u value for vinorelbine ranged between 8.8% and 20.4% (binding, 91.2%–79.6%) and averaged 13.5% ± 3.6%.

Simulation of vinorelbine distribution in blood

Using the binding parameters of vinorelbine and given the platelet and lymphocyte counts and the typical concentrations of plasma proteins in cancer (biologic syndrome of inflammation, see Table 3), we calculated that platelets accounted for 78% of blood-bound vinorelbine and lymphocytes, for 4.8%, with the remainder being bound to plasma proteins and the blood f_u value being 1.78%. The assumption of platelet counts that were 50% higher (600,000/mm^3) or 50% lower (200,000/mm^3) than the physiologic values provided f_u values of 0.74% and 2.20%, respectively. Relative to total plasma vinorelbine, the plasma f_u value was 11.5%, and alpha$_1$-acid glycoprotein and the low-density fraction of lipoproteins (LDL) were the major carriers, accounting for 31.9% and 21.4% of plasma-bound vinorelbine, respectively.

Table 3. Blood distribution of vinorelbine expressed as a percentage of the total amount

Component	Concentration/count	% Associated vinorelbine
Human serum albumin	447 μM	1.0
Alpha$_1$-acid glycoprotein	31.1 μM	4.9
Gamma globulins	77 μM	0.7
HDL	13 μM	1.0
LDL	1 μM	3.3
VLDL	0.1 μM	2.7
Platelets	400,000/mm^3	78.0
Lymphocytes	4,000/mm^3	4.8
Erythrocytes	40%	1.3
None (f_u)		1.7

Discussion

The in vitro data obtained in the binding experiments were satisfactorily fitted to the models used and indicated that platelets were the main carrier in blood for vinorelbine. This finding is similar to previous observations of vinblastine and vincristine blood distribution [4].

Vinorelbine was rapidly taken up by either platelets or lymphocytes in less than 30 min, which is comparable with the platelet uptake rate previously found for vinblastine

(1 h) and faster than that reported for vincristine (8 h) [4]. Analogous to vinblastine, high concentrations of vinorelbine should be observed in the platelet and white blood cell compartments, whereas plasma concentrations should be rather low. It is likely that the fast distribution phase observed in the plasma pharmacokinetics of vinorelbine [5] could be related to this rapid uptake in platelets and white blood cells.

Our simulation on the blood distribution of vinorelbine showed that this drug was 78% platelet-bound and 14% serum-protein-bound, with 6% being bound to lymphocytes and a small part being bound to erythrocytes; the protein concentrations expected in cancer disease were used. The main binding proteins in serum were alpha$_1$-acid glycoprotein followed by low-density lipoproteins (LDL). This result is similar to that obtained by Steele et al. [7], who found that the alpha-globulin serum fraction and, particularly, alpha$_1$-acid glycoprotein were the most important binders of vinblastine. Moreover, a recent study [3] has shown that alpha$_1$-acid glycoprotein is a more specific carrier for vinca alcaloids than is albumin. These studies, however, did not assess the role of lipoproteins in the binding, and our observation that a nonnegligible fraction of plasma vinorelbine is LDL-bound indicates that the drug can be internalized into cells via the LDL receptor pathway [6, 11]. The serum f_u of vinorelbine (11.5%) that we simulated on the basis of our in vitro n and K_A estimates is in agreement with the mean value found in our collection of cancer sera (13.5% $\pm$ 3.6%). The free drug fraction in blood (the free amount in plasma divided by the total amount in blood) was lower (1.8%), mainly due to extensive binding of vinorelbine to platelets. Our simulations also show that changes in the platelet count are likely to produce strong variations in the blood f_u of vinorelbine to which tissue cells are exposed and are thus likely to modify the delivery of vinorelbine to tissues.

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