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Pharmacokinetics of erythrocyte-bound daunorubicin in patients with acute leukemia

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Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Oleksii A. Skorokhod[ABCDEFG], Tat'yana Ts. Garmaeva[ABCDEFG], Victor M. Vitvitsky[ABCDEFG], Valentin G. Isaev[BC], Elena N. Parovichnikova[BC], Valerii G. Savchenko[BC], Fazoil I. Ataullakhanov[ABCDEFG]

National Research Center for Hematology, Russian Academy of Medical Sciences, Moscow, Russia

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Summary

Background: The objective of the present study was *in vitro* and *in vivo* investigation of erythrocytes as vehicles for anthracycline antibiotics.

Material/Methods: The kinetics of daunorubicin binding with erythrocytes was studied in blood and in washed erythrocyte suspensions from healthy donors and patients with acute leukemia. The effect of daunorubicin on erythrocyte deformability was studied using cell filtration through membranes with 3 μm-diameter cylindrical pores. Erythrocyte-bound daunorubicin (EBD), prepared by equilibrating anticoagulated autologous blood with the antibiotic, was administered (45 or 60 mg/m^2 body surface) to 14 leukemic patients as part of the 7+3 or RACOP courses. The pharmacokinetics of daunorubicin and its tolerability were studied.

Results: Human erythrocytes bound daunorubicin (rubomycin) in citrated whole blood or in washed saline suspension. The equilibrium erythrocyte/medium daunorubicin concentration ratios (attained in 30–60 min at 37°C) averaged 2.9±0.5 (n=13) in blood and 5.7±0.6 (n=8) in suspension ($p<0.001$), without any significant difference between the erythrocytes of donors and patients with acute drug-resistant leukemia or leukemic relapses. Incubation of patient blood with daunorubicin (0.5 mg/ml cells) did not affect erythrocyte deformability (filterability). After intravenous administration, the peak drug concentration and its elimination rate were lower for EBD than for free daunorubicin. The patients tolerated EBD better than its standard free form. In nine patients who received three EBD infusions, side effects were less frequent than in those treated with free daunorubicin.

Conclusions: Our results indicate that daunorubicin-loaded erythrocytes are promising for clinical application and deserve further clinical study.

key words: **daunorubicin • carrier erythrocytes • pharmacokinetics • acute leukemia**

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Author's address: Fazoil I. Ataullakhanov, National Research Center for Hematology, Novozykovsky proezd, 4a, Moscow, 125167, Russia, e-mail: fazli@bioscence.msk.su

BACKGROUND

Anthracycline antibiotics, including daunorubicin (rubomycin), are basic drugs in the therapy for acute leukemia [1,2]. However, their use is accompanied by significant cardiotoxicity [3–5]. The risk of anthracycline cardiomyopathy dramatically increases for patients receiving in total more daunorubicin than 55–650 mg/m^2 body surface. Therefore, this dose is considered to be the upper limit of the tolerable range. In addition, clinical use of anthracycline antibiotics is associated with severe side effects, such as nausea, vomiting, mucositis, alopecia, and myelosuppression [3,6]. Reducing the toxicity of antitumor drugs without decreasing their efficacy is a clinically and pharmacologically important goal. It can be approached either by prolonging the time the drug circulates in the blood so that its dosage can be reduced, or by routing the drug preferentially to the afflicted organs or cells. One way to decrease anthracycline antibiotic toxicity and to increase their specific activity is to use vehicles for antibiotic transport in organism. Different macromolecules [7–10], microparticles (liposomes) [6,11–14], as well as erythrocytes can be used as drug vehicles.

Erythrocytes are promising vehicles for drug delivery in view of their ideal biocompatibility and biodegradability. It was shown that blood erythrocytes themselves could play a significant role in the storage, transport, and metabolism of different anti-cancer drugs [15–17]. The techniques of erythrocyte loading with various drugs are relatively simple and quite efficient [18,19]; thus, erythrocytes readily bind anthracycline antibiotics in isotonic media [20,21]. The advantages of erythrocyte-bound anthracycline antibiotics over their free forms have been shown in animals and cultured cells [20,22–26]. Doxorubicin immobilized in erythrocytes by glutaraldehyde treatment has been applied in veterinary medicine to treat dogs with lymphosarcoma and in a human case of inoperable colorectal cancer with multiple hepatic metastases, refractory to previous chemotherapy [27,28]. In dogs, remission was achieved; no therapeutic effect was attained in the cancer patient, who only developed high fever. Furthermore, severe myelosuppression was observed in all cases after several infusions of doxorubicin-loaded erythrocytes. Conceivably, it was the glutaraldehyde treatment that was responsible for the toxic effects observed, as it might have substantially altered the properties of the drug, including its toxicity [29]. Loaded cells prepared by incubation with anthracycline antibiotics in an isotonic medium rather than treated with glutaraldehyde appear to be a more suitable therapeutic option. No side effects or complications were observed in three patients with lymphoproliferative diseases after infusion of such erythrocytes loaded with doxorubicin. The peak concentration of the latter was several times lower and its circulation half-life several times longer than those of the free form of the drug [30]. Therefore, the use of erythrocytes as vehicles may prolong the effect of these drugs while reducing their toxicity.

The objective of the present study was to further assess erythrocytes as vehicles for anthracycline antibiotics. The kinetics of daunorubicin binding with erythrocytes was studied in blood and in washed erythrocyte suspensions from healthy donors and patients with acute leukemia resistant to chemotherapy or with leukemic relapses. Anthracycline antibiotics can decrease erythrocyte deformability [31]. Therefore erythrocyte-bound daunorubicin (EBD) can disturb circulation in small tissue capillaries. In this connection, we examined the possible effect of daunorubicin on erythrocyte deformability (filterability) under conditions simulating EBD preparation and administration. Finally, we studied the kinetics of daunorubicin in the blood and plasma of patients with acute leukemia after its administration as free or erythrocyte-bound form (EBD). We also evaluated tolerability of EBD.

MATERIAL AND METHODS

The pharmaceutical preparation of daunorubicin used in this study was rubomycin hydrochloride, a product of FAO Ferein (Moscow, Russia).

The kinetics of daunorubicin binding to erythrocytes was studied in either whole blood or a suspension of washed cells. Healthy donor or patient blood was collected in the glucose-cytrate preserving solution Glugicir at a blood/anticoagulant volume ratio of 4: 1. To wash, suspend, and incubate erythrocytes, we used isotonic phosphate-buffered saline (PBS, pH 7.4) of the following composition: 140 mM NaCl, 9 mM Na_2HPO_4, 1.3 mM NaH_2PO_4, and 5 mM glucose. Daunorubicin was dissolved in PBS and added to the blood or erythrocyte suspension to a final concentration of 0.15–0.65 mg/ml and the mixture was continuously agitated at 37°C. Samples were drawn at intervals to measure the total and extracellular daunorubicin concentrations. The intracellular daunorubicin concentration was calculated as:

$$Ci = (100Ct - Ce(100 - Ht))/Ht$$

where Ct, Ce, and Ci denote the total, extracellular, and intracellular drug concentrations, respectively, and Ht is the hematocrit (the percentage of the total blood or suspension volume occupied by erythrocytes).

To examine the effect of daunorubicin on erythrocyte deformability, patient Glugicir-preserved blood was continuously agitated in the presence of daunorubicin (0.25, 0.5, or 1.0 mg/ml cells) for 1 h at 37°C. Then the erythrocytes were washed three times with PBS. Prior to measurement, the erythrocytes were diluted in PBS to a hematocrit of 1%. Erythrocytes of the same patient isolated from blood not exposed to daunorubicin served as the control. The erythrocyte deformability was assessed at 21°C by the filtration technique using the equipment and methods described elsewhere [32]. Briefly, aliquots (250 μl) of PBS and the erythrocyte suspension were allowed to pass through a filter with cylindrical pores 3 μm in diameter, and their passage times (T_{PBS} and T_{susp}, respectively) were recorded. The T_{PBS}/T_{susp} ratio was used to characterize erythrocyte deformability.

A total of 26 patients with acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL), relapsed or

Table 1. Parameters of the patients involved in the study (range and median in parentheses). The total number of patients is 30 because four patients (numbers 8, 9 10, and 14) participated in two cycles of daunorubicin administration with different forms of daunorubicin.

Sequence of daunorubicin treatment	Free Free EBD	EBD EBD EBD	Free Free Free
Number of patients	N=5	N=10	N=15
Age (years)	15–48 (32)	15–60 (40)	15–60 (35)
Sex (M/F) – number	M-3, F-2	M-4, F-6	M-6, F-9
Hemoglobin (g/l)	77–134 (100)	48–112 (106)	76–129 (100)
WBC ($10^3/mm^3$)	2.5–16 (4.3)	2.4–108 (4)	2–120 (4)
Platelets ($10^3/mm^3$)	28–515 (186)	38–396 (200)	26–540 (140)
Bone marrow blast cells (%)	2.8–70 (3,6)	1–50 (2.4)	1.6–84 (4)
Creatinine (mmol/l)	0.07–0.08 (0.07)	0.06–0.13 (0.08)	0.06–0.1 (0.08)
Bilirubin (mmol/l)	5–13 (7)	3–20 (7)	3–18 (7)
Serum protein (g/l)	64–78 (73)	66–78 (72)	60–81 (71)
Infections (cases)	0	0	3
Organomegalia (cases)	1	4	4
Lymph nodes enlargement (cases)	0	0	1
Hemorrhage (cases)	0	0	1

resistant to chemotherapy, were included in the study of EBD pharmacokinetics and its tolerability. Fourteen patients were investigated in both the pharmacokinetic and the clinic study, the other 12 patients only in the clinic study. Eligibility requirements included a life expectancy of 4–6 weeks and recovery from toxic effects of previous chemotherapy. Patients were required to have adequate liver function, adequate renal function, and normal metabolic parameters. Patients should not have evidence of active infection at the time of protocol entry (excluding 3 patients in the group treated with free daunorubicin). A left ventricular ejection fraction measured by echocardiogram (LVEF) of >–60% was required. Patient parameters are listed in Table 1.

The experimental protocol was approved by the Scientific Council of the National Research Center for Hematology (Russian Academy of Medical Sciences), methods and risks were explained, and written consent forms were obtained from every patient prior to the beginning of the study. Daunorubicin was administered at a standard dose of 45 or 60 mg/m^2 as part of the conventional 7+3 protocol of chemotherapy for AML [1] and the RACOP (cytarabine, daunorubicin, cyclophosphamide, prednisone, and vincristine) protocol of chemotherapy for ALL [2]. According to these protocols, daunorubicin was administered once daily for the first three days of the cycle.

Free daunorubicin was administered intravenously in 50 ml of 0.9% NaCl over 5–10 min. To prepare EBD, 300–400 ml of patient blood was collected in a Hemacon PVC blood pack (Russia) containing 100 ml of Glugicir or a PVC blood pack (Baxter) containing 63 ml of CPDA-1. Daunorubicin solution (20–50 ml) was added to the blood pack to give a final dose of 45 or 60 mg/m^2 of patient body surface (0.3–0.6 mg/ml cells in the pack), and the mixture was incubated at 37°C with constant agitation for an hour, the time needed to attain equilibrium binding. The EBD was administered to patients through a central venous catheter for 1.0–1.5 h.

Response to the therapy was determined according to the following criteria: remission was defined as a reduction of bone marrow blast cells to less than 5%, blood granulocyte concentration not less than 2500/ mm^3, hemoglobin not less than 100 g/l. Otherwise the patient was considered as no responding to the therapy. Toxicity and side effects were classified according to NCI Common Toxicity Criteria (NCI CTC).

Blood samples for measuring the daunorubicin concentration were drawn from the central venous catheter. The blood-preserving solution Glugicir was used as an anticoagulant. Under free daunorubicin administration, the first sample was drawn immediately upon completing its infusion. The subsequent samples were drawn after 0.1, 0.25, 0.5, 1, 2, 6, 18, and 24 h. Under EBD administration, the first sample was drawn immediately upon completing its infusion, and the others after 0.5, 1, 2, 6, 18, and 24 h. In several cases a sample was drawn 30 min after the start of EBD infusion, before its completion. After the third (last) administration of daunorubicin, samples were also drawn daily for the subsequent five to seven days. By this time, the blood and plasma daunorubicin concentrations had dropped to its quantitation threshold (the background level of about 10 ng/ml).

Bone marrow and spinal fluid samples were taken from several patients after the third daunorubicin infusion. Bone marrow samples (1.5–2 ml) were taken by sternum puncture to sample tubes containing blood-preserving solution Glugicir. Spinal fluid samples were taken to dry sample tubes.

Daunorubicin was extracted from the samples using chloroform, and its concentration in extracts was measured by spectral methods based on the procedures described elsewhere [29,33–36]. During *in vitro* experiments aimed at evaluating the drug binding to erythrocytes, the daunorubicin concentration in chloroform extracts was high enough to be determined spectropho-

Table 2. Equilibrium ratios of erythrocyte daunorubicin concentration to medium daunorubicin concentration in blood and suspension of healthy donor and patient erythrocytes.

Donor/Patient	Blood	Washed erythrocytes in PBS	Hematocrit, (%)	Total daunorubicin concentration, (mg/ml)	Equilibrium erythrocyte/medium concentration ratio
Donor 1	+	–	35	0.65	7.4
Donor 2	+	–	30	0.25	3.5
Donor 3	+	–	35	0.20	4.3
Donor 4	+	–	37	0.33	2.8
	–	+	33	0.33	8.1
Donor 5	+	–	37	0.30	1.5
	–	+	37	0.30	4.0
Donor 6	+	–	43	0.30	2.0
	–	+	43	0.30	4.0
Donor 7	+	–	42	0.17	3.2
	–	+	42	0.17	5.5
Patient A	+	–	18	0.21	2.2
	–	+	23	0.20	5.0
Patient B	+	–	26	0.26	2.9
	--	+	35	0.22	7.4
Patient C	+	–	22	0.33	3.5
	–	+	22	0.33	6.9
Patient D	+	–	31	0.15	0.4
Patient E	+	–	35	0.20	1.3
Patient F	+	–	20	0.26	2.4
Patient G	–	+	28	0.30	4.8

tometrically at the absorption maximum of 486 nm. In pharmacokinetic samples, daunorubicin was assayed spectrofluorimetrically, using the excitation and emission wavelengths of 476 and 588 nm, respectively. The lower limit of quantitation for daunorubicin in pharmacokinetic samples was 10–20 ng/ml, depending on the background turbidity and fluorescence of blank plasma samples of individual patients.

The time dependencies of the blood and plasma doxorubicin concentrations obtained in the pharmacokinetic studies were approximated by a sum of two exponentials according to the following expression:

$$C=Cb + A1exp(-t/T1) + A2exp(-t/T2)$$

Here, C is blood or plasma daunorubicin concentration at time *t* after the end of infusion; Cb is the lower limit of daunorubicin quantitation in samples (background level); A1 and A2 are coefficients; and T1 and T2 are the time constants of the first and the second exponentials, respectively (specific times of daunorubicin disappearance). The values of T1 and T2 were determined using the Microcal Origin 3.5 software (Microcal Software, Inc.).

The time constants (T1 and T2) and the areas under the concentration curve for the first and the second antibiotic injections were determined using the experimental points between the first and the second injections and between the second and the third injections, respectively (within 24 hours). For the third injection

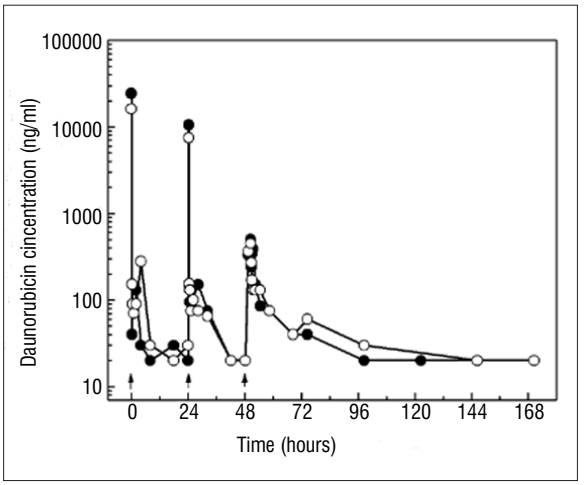

Figure 1. Kinetics of the blood (closed circles) and plasma (open circles) daunorubicin concentration in patient 1 (see Table 3), treated with standard free daunorubicin on days 1 and 2 and erythrocyte-bound daunorubicin on day 3 of the chemotherapy cycle (protocol 7+3) at a dose of 45 mg/m^2 per infusion. Arrows indicate the moments of the start of daunorubicin infusion.

these parameters were determined using all experimental points from the third injection up to the end of the observation.

Statistical significance was analyzed using two-sample t-test.

RESULTS

Daunorubicin added to the blood or washed erythrocyte suspension progressively disappeared from the extracellular medium, but its total concentration remained unchanged, suggesting that the drug bound to erythrocytes. The equilibrium of daunorubicin between cells and medium was achieved in 30–60 min and demonstrated daunorubicin accumulation in erythrocytes (Table 2). Thereupon, the equilibrium erythrocyte/plasma concentration ratio was, on average, 3.5±0.7 (n=7) for donor blood and 2.1±0.5 (n=6) for patient blood; note that in one case this ratio was less than unity (Table 2). This difference was not significant ($p>0.1$). In the suspensions of washed erythrocytes, the mean ratio was 5.4±1.0 (n=4) for donor erythrocytes and 6.0±0.7 (n=4) for patient erythrocytes. This difference was also not significant ($p>0.5$). In each particular case, however, erythrocytes bound more antibiotic in saline suspension compared with the whole blood. The average erythrocyte/plasma concentration ratio in blood of all donors and patients was 2.9±0.5 (n=13). In the suspensions of washed erythrocytes the mean erythrocyte/medium ratio calculated for all donors and patients was significantly ($p<0.001$) increased to the value of 5.7±0.6 (n=8).

The results of filtration experiments are in good agreement with the data reported earlier for donor erythrocytes [31]. The T_{PBS}/T_{susp} ratio was 0.91±0.01 (n=3) at 0.25 mg/ml cells daunorubicin, 0.92±0.02 (n=8) at 0.5

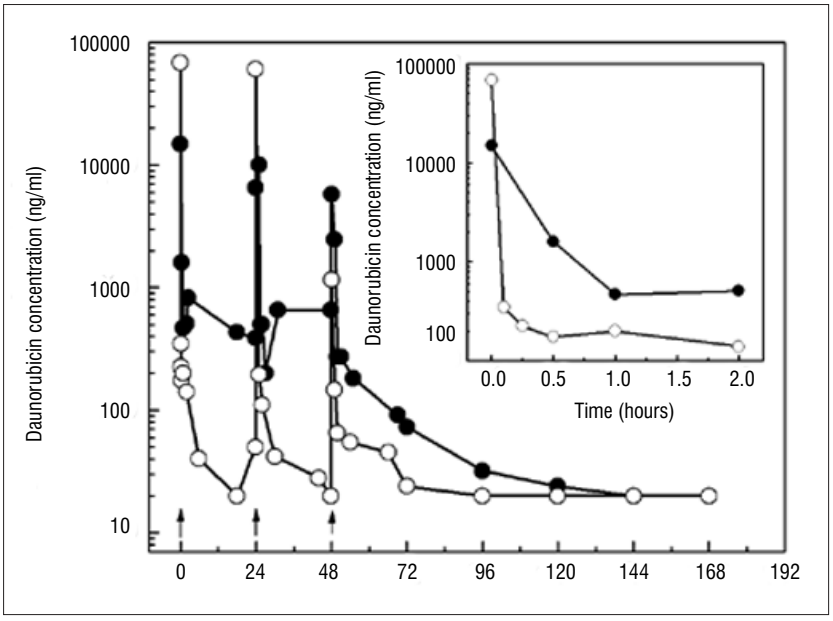

Figure 2. Kinetics of the plasma daunorubicin concentration in patient 10 (see Table 3) upon three consecutive infusions (protocol 7+3) of standard free daunorubicin (open circles) or erythrocyte-bound daunorubicin (closed circles). The cycle with erythrocyte-bound daunorubicin infusions was conducted 21 days after the standard cycle with the use of free daunorubicin. Daunorubicin dose was 60 mg/m^2 per infusion. Arrows indicate the moments of the start of daunorubicin infusion. Inset: the first two hours after free and erythrocyte-bound daunorubicin infusions.

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mg/ml cells daunorubicin, and 0.57±0.08 (n=5) at 1.0 mg/ml cells daunorubicin for daunorubicin-treated patient erythrocytes; and 0.84±0.05 (n=10) for the control erythrocytes of the same patients. The filterability of erythrocytes incubated at daunorubicin concentrations of 0.25 and 0.5 mg/ml cells was not significantly different from the control erythrocytes ($p>0.45$ and $p>0.18$, respectively). After incubation at a daunorubicin concentration of 1.0 mg/ml cells, erythrocyte filterability was significantly decreased ($p<0.01$). Such a relatively high daunorubicin concentration, however, was not used for EBD preparation. In this way our filtration experiments demonstrate that the EBD preparation does not cause a significant irreversible decrease in erythrocyte deformability.

After infusion of daunorubicin to patients, the drug partitioned almost equally between erythrocytes and plasma, whether infused free or bound to erythrocytes. Figure 1 shows representative curves for daunorubicin concentration versus time in blood and plasma of a patient who received two consecutive infusions of free daunorubicin followed by one EBD infusion. The pharmacokinetic data obtained in this study are summarized in Table 3. Our data on the pharmacokinetics of free daunorubicin were consistent with the literature [37–40]. Immediately after daunorubicin infusion, a wide scatter in its blood and plasma concentrations (peak concentrations) was observed (Table 3). The drug concentrations dropped rapidly during the first 10 to 30 min by one or two orders of magnitude. Thereupon, its concentration declined slowly (usually over several hours) to the lower limit of its quantitation. When EBD was administered to a patient, the drug reached relatively high blood and plasma concentrations before the end of infusion, namely, the daunorubicin concentration in the sample drawn 30 min after the start of infusion was close to the peak concentration observed immediately upon completion of the infusion. Note that the peak daunorubicin concentrations were, on average, significantly lower after EBD than after free daunorubicin infused in equivalent doses (Table 4), presumably because EBD infusions were of longer duration than those of free daunorubicin. In any case, the administration of EBD achieves a decrease in maximum daunorubicin concentration in patient blood and plasma.

The kinetics of daunorubicin clearance from the blood and plasma were similar. Two phases, fast and slow, could be distinguished in daunorubicin clearance whether the antibiotic was infused in the free or the EBD form. The characteristic times of these phases (T1 and T2) were on average longer after EBD administration (Table 4). In other words, daunorubicin remained longer in the circulation if it was administered in the erythrocyte-bound form. Figure 2 shows the plasma daunorubicin concentration as a function of time after three consecutive daunorubicin infusions given in two cycles of chemotherapy to the same patient. Free daunorubicin was infused in the first cycle and EBD in the second cycle. As one can see, the pharmacokinetics of the drug in the course of three EBD infusions followed the pattern observed after a single EBD infusion. Note that no evidence of drug accumulation was seen in the patient's blood or plasma following repeated EBD infusions. Although the difference between the mean characteristic times of daunorubicin clearance obtained with the free antibiotic and with EBD was great, its statistical significance was low in a number of cases (Table 4). The reason is that these parameters varied several fold from patient to patient. Despite great individual variations in daunorubicin binding to erythrocytes and in its pharmacokinetics, the changes in the pharmacokinetics observed in the case of EBD were common for all patients. The prolonged presence of daunorubicin in the circulation and the reduction in its peak concentration observed after EBD infusion produce offsetting effects on the mean area under the concentration curve.

Table 3. Pharmacokinetic parameters for free and erythrocyte-bound (EBD) forms of daunorubicin in blood and plasma (parenthesized) of the patients.

Patient number, sex/age, diagnosis	Sequence of daunorubicin treatment	Dose per infusion, (mg/m^2)	Peak concentration, (μg/ml)		T1, (h)		T2, (h)		Area under concentration-time curve, (μgxh/ml)	
1	Free	45	24.4	(16.1)	0.1	(0.1)	8.2	(14.7)	3.9	(3.4)
M/31	Free		10.6	(7.5)	0.1	(0.1)	3.5	(6.6)	2.8	(2.1)
AML (M2)	EBD		0.5	(0.5)	1.3	(2.1)	55.5	(–)	5.9	(9.5)
2	Free	45	2.7	(3.2)	0.1	(0.1)	5.8	(12.0)	1.3	(2.2)
M/16	Free		2.4	(4.9)	0.2	(0.6)	4.2	(2.7)	1.9	(3.2)
ALL	EBD		0.6	(0.4)	3.1	(0.6)	18.6	(17.8)	6.0	(5.7)
2*	Free	45	13.0	(16.3)	0.2	(0.2)	2.4	(2.8)	9.2	(9.6)
M/16	Free		33.9	(31.7)	0.3	(0.2)	9.8	(17.4)	20.0	(19.5)
ALL	EBD		2.1	(3.6)	0.8	(1.7)	17.0	(16.9)	13.5	(9.9)
3	Free	45	17.5	(20.0)	0.1	(0.1)	2.2	(1.8)	8.4	(9.7)
F/48	Free		21.6	(21.1)	0.1	(0.1)	4.4	(4.5)	13.7	(15.3)
AML (M2)	EBD		6.2	(7.0)	0.2	(3.7)	2.9	(5.7)	14.6	(18.8)
4	Free	45	17.0	(15.0)	0.1	(0.1)	1.5	(6.5)	2.5	(3.2)
M/15	Free		3.8	(3.0)	0.1	(0.1)	13.3	(6.2)	2.8	(1.7)
ALL (T-type)	EBD		0.6	(0.7)	6.2	(3.8)	23.8	(13.2)	8.7	(7.7)
5	Free	60	7.9	(7.2)	0.3	(0.3)	3.0	(1.7)	4.8	(7.0)
F/50	Free		1.2	(1.6)	0.3	(0.2)	11.9	(5.8)	2.5	(4.0)
ALL (pre-B)	EBD		6.8	(7.4)	0.3	(0.3)	8.5	(21.4)	11.2	(11.9)
6	EBD	45	10.2	(12.4)	0.1	(0.1)	2.2	(3.1)	5.8	(7.0)
M/60	EBD		11.1	(11.7)	0.2	(0.2)	8.6	(3.3)	5.8	(5.9)
AML (M2)	EBD		12.3	(–)	0.2	(0.1)	18.4	(9.5)	13.3	(18.5)
7	EBD	45	0.5	(0.6)	1.1	(1.3)	5.1	(4.3)	4.5	(4.3)
F/42	EBD		1.4	(1.2)	0.6	(0.6)	14.4	(–)	2.5	(1.8)
ALL (pre-B)	EBD		0.9	(1.0)	0.3	(0.2)	13.3	(3.2)	2.9	(3.3)
8	EBD	45	1.6	(1.4)	0.5	(0.1)	3.0	(3.2)	2.8	(2.2)
F/31	EBD		1.5	(1.9)	0.3	(0.1)	10.1	(2.2)	3.0	(3.3)
ALL	EBD		0.6	(1.9)	0.9	(0.3)	13.3	(13.5)	3.3	(4.0)
9	EBD	45	5.0	(5.5)	0.1	(0.1)	2.6	(2.7)	6.0	(5.5)
F/48	EBD		5.0	(4.0)	0.1	(0.1)	2.5	(4.5)	10.0	(8.0)
ALL (pre-B)	EBD		2.1	(3.1)	0.4	(0.7)	6.6	(11.8)	10.0	(9.2)
10	EBD	60	–	(14.8)	0.1	(0.2)	13.2	(20.9)	22.9	(14.3)
F/42	EBD		6.9	(10.0)	0.2	(0.2)	9.3	(13.0)	14.3	(18.3)
AML (M6)	EBD		6.0	(5.8)	1.0	(1.1)	13.1	(18.4)	11.0	(10.6)
11	EBD	60	3.7	(5.0)	0.9	(0.4)	–	(10.7)	5.7	(5.7)
M/48	EBD		3.6	(4.1)	0.1	(–)	4.2	(2.6)	4.9	(5.0)
AML (M2)	EBD		8.8	(8.8)	0.1	(0.1)	10.0	(4.3)	12.5	(13.5)
12	EBD	60	0.7	(0.6)	0.2	(0.1)	3.0	(2.8)	4.0	(1.8)
M/31	EBD		5.9	(8.5)	0.1	(0.1)	5.8	(2.6)	6.6	(6.9)
AML (M4)	EBD		4.2	(6.6)	0.1	(0.1)	13.0	(4.3)	4.6	(7.4)
13	EBD	60	3.4	(1.5)	0.1	(0.2)	12.0	(12.0)	4.4	(2.4)
F/15	EBD		0.2	(0.2)	0.5	(0.7)	8.4	(36.0)	1.7	(1.6)
AML (M5)	EBD		3.3	(2.4)	0.5	(0.5)	20.4	(16.5)	5.2	(4.1)
14	EBD	60	3.0	(3.1)	0.1	(0.1)	1.0	(1.0)	3.9	(3.5)
M/31	EBD		7.5	(4.0)	0.1	(0.1)	1.0	(1.0)	3.3	(2.9)
AML (M4)	EBD		2.7	(1.6)	0.2	(0.2)	58.0	(9.7)	12.1	(7.9)
8*	Free	45	34.0	(20.0)	0.1	(0.1)	0.8	(0.5)	1.8	(1.1)
F/31	Free		7.0	(5.5)	0.1	(0.1)	10.0	(10.0)	0.5	(0.8)
ALL	Free		61.8	(26.1)	0.1	(0.1)	1.0	(1.1)	4.4	(3.9)
9*	Free	45	1.3	(0.9)	0.1	(0.1)	4.8	(4.0)	2.4	(3.3)
F/48	Free		1.1	(2.1)	0.1	(0.1)	18.0	(10.2)	4.8	(5.4)
ALL (pre-B)	Free		1.7	(2.7)	0.1	(0.1)	6.9	(4.3)	1.7	(1.8)
10*	Free	60	56.8	(68.2)	0.1	(0.1)	1.0	(1.6)	4.8	(4.6)
F/42	Free		49.2	(60.5)	0.1	(0.1)	12.0	(1.3)	26.1	(8.2)
AML (M6)	Free		–	(1.2)	0.1	(0.1)	11.0	(9.2)	3.9	(2.8)
14*	Free	60	16.5	(13.0)	0.1	(0.1)	0.2	(0.2)	1.4	(1.4)
M/31	Free		8.9	(10.0)	0.1	(0.1)	0.4	(0.4)	0.8	(0.9)
AML (M4)	Free		8.4	(8.0)	0.1	(0.1)	0.2	(0.2)	1.0	(1.2)

* patients 2, 8, 9, 10, and 14 were studied during two cycles of daunorubicin administration. In all these cases except patient 2, in the first cycle daunorubicin was administered in standard (free) form. The second course was administered 21 days after the start of the first one

Table 4. Average pharmacokinetic parameters for the free and erythrocyte-bound (EBD) forms of daunorubicin in blood and plasma of the patients. Means ±SE are shown, with the number of measurements indicated in parentheses.

Dose per infusion (mg/m^2)	45				60			
Compartment	Blood		Plasma		Blood		Plasma	
Daunorubicin form	Free	EBD	Free	EBD	Free	EBD	Free	EBD
Number of patients	6	8	6	8	3	6	3	6
Number of infusions	16	17	16	17	8	16	8	16
Peak concentration (μg/ml)	16±4 (16)	3.7±0.9 (17)	12±2 (16)	3.6±0.9 (16)	21±8 (7)	4.4±0.6 (15)	21±9 (8)	5.0±1.0 (16)
Significance of the difference	0.006		0.003		0.008		0.03	
T1 (h)	0.13±0.02 2 (16)	1.0±0.4 (17)	0.14±0.03 (16)	1.0±0.3 (17)	0.15±0.03 (8)	0.29±0.07 (16)	0.14±0.02 (8)	0.29±0.07 (15)
Significance of the difference	0.04		0.02		0.21		0.15	
T2(h)	6±1 (16)	13±3 (17)	7±1 (16)	8±1 (15)	5±2 (8)	12±3 (15)	3±1 (8)	11±2 (16)
Significance of the difference	0.06		0.58		0.18		0.02	
Area under curve(μgxh/ml)	5±1 (16)	7±0.9 (17)	5±1 (16)	7±1 (17)	6±3 (8)	8±1 (16)	3.8±0.9 (8)	7±1 (16)
Significance of the difference	0.27		0.29		0.42		0.08	

Table 5. Daunorubicin accumulation in bone marrow of the patients receiving the standard (free) and EBD forms of daunorubicin. Patient numbers correspond to the numbers in Table 3.

Patient	Sequence of daunorubicin treatment	Time elapsed after daunorubicin infusion (min)	Bone marrow daunorubicin (ug/ml)	Blood daunorubicin (μg/ml)	Ratio of bone marrow to blood daunorubicin
7	EBD	60	0.68	0.18	3.8
	EBD				
	EBD				
8	EBD	60	0.34	0.21	1.6
	EBD				
	EBD				
11	EBD	60	0.32	0.22	1.5
	EBD				
	EBD				
12	EBD	60	0.24	0.17	1.4
	EBD				
	EBD				
12	Free	60	0.90	0.53	1.7
	Free				
	Free				
14	Free	5	0.29	0.73	0.4
	Free				
	Free				

Table 6. Number of non-hematological adverse effects (according to Common Toxicity Criteria (NCI CTC)) observed during treatment of the patients with the standard (free) and EBD forms of daunorubicin. The total number of patients is 30 because four patients (numbers 8, 9 10, and 14) participated in two cycles of daunorubicin administration with different forms of daunorubicin.

Symptom	CTC grade	Sequence of daunorubicin treatment		
		Free Free EBD	EBD EBD EBD	Free Free Free
		Number of patients		
		N=5	N=10	N=15
Nausea	Gastrointestinal symptom grade 2, 3	2	3	13
Vomiting	Gastrointestinal symptom grade 1,2, 3	1	0	8
Stomatitis	Gastrointestinal symptom grade 1,2, 3	0	2	6
Diarrhea	Gastrointestinal symptom diarrhea grade 2,3	0	2	5
Fatigue	Constitutional symptom grade 2, 3, 4	5	8	15
Headache	Pain symptom grade 2, 3, 4	4	8	15
Alopecia	Dermatology/skin syndrome grade 1, 2	2	3	8

On average, it turned out somewhat larger for EBD than for free daunorubicin (Table 4).

Daunorubicin accumulation in bone marrow was the same under administration of free daunorubicin and the EBD form (Table 5). In spinal liquid, daunorubicin was neither detected after free daunorubicin administration nor after EBD administration.

EBD was well tolerated in all cases. No immediate reactions of the patients to EBD administration were

Table 7. Number of hematological adverse effects (according to Common Toxicity Criteria (NCI CTC)), observed during treatment of the patients with the standard (free) and EBD forms of daunorubicin. The total number of patients is 30 because four patients (numbers 8, 9 10, and 14) participated in two cycles of daunorubicin administration with different forms of daunorubicin.

Symptom	CTC grade	Sequence of daunorubicin treatment		
		Free Free EBD	EBD EBD Free	Free Free Free
		Number of patients		
		N=5	N=10	N=15
Leukocytes <1000/mm^3	Blood symptom grade 4	4	9	15
Platelets <25000/mm^3	Blood symptom grade 3	5	7	15
Hemoglobin <65g/l	Blood symptom grade 4	0	5	9

Table 9. Infections observed during treatment of the patients with the standard (free) and EBD forms of daunorubicin. The total number of patients is 30 because four patients (numbers 8, 9 10, and 14) participated in two cycles of daunorubicin administration with different forms of daunorubicin.

Infection	Sequence of daunorubicin treatment		
	Free Free EBD	EBD EBD Free	Free Free Free
	Number of patients		
	N=5	N=10	N=15
Sepsis	0	2	3
Pneumonia	0	3	3
Necrotic enterocolitis	0	2	4
Herpes infection	0	2	0
Tonsillitis	1	0	0
Fever	2	3	4
Esophagitis	0	0	3
Local infections	1	1	1

observed. There was also no pronounced nausea, vomiting, severe stomatitis, or prolonged myelosuppression. Daunorubicin-related toxic effects (particularly nonspecific, non-hematological effects) were less pronounced in patients receiving EBD (Table 6). The two daunorubicin forms were similar with respect to the hematological toxicity (bone marrow suppression) they produced, judging by the time of onset, severity, and duration of cytopenia in the treated patients (Table 7,8). The intensity of replacement therapy (the amount of transfused platelets and erythrocytes) was similar for the patients treated with standard (free) and EBD forms of daunorubicin. None of the patients developed clinical or echocardiographic signs of direct cardiotoxicity. Five patients treated with EBD experienced infectious complications (one case of sepsis of mixed etiology, two cases of pneumonia, and two cases of abdominal syndrome with diarrhea).

Table 8. Time interval between the end of daunorubicin administration and myelotoxic agranulocytosis registration and the duration of neutropenia during treatment of the patients with the standard (free) and EBD forms of daunorubicin (range and median in parentheses). The following criteria were used for myelotoxic agranulocytosis registration: decrease in leukocyte number below 1000/mm^3, platelet number below 25000/mm^3, and hemoglobin below 65 g/l. The total number of patients is 30 because four patients (numbers 8, 9 10, and 14) participated in two cycles of daunorubicin administration with different forms of daunorubicin.

Parameter	Sequence of daunorubicin treatment		
	Free Free EBD	EBD EBD Free	Free Free Free
	Number of patients		
	N=5	N=10	N=15
Time interval between the end of daunorubicin administration and myelotoxic agranulocytosis registration, (days)	(4)	(4)	(3)
Duration of neutropenia, (days)	0–14 (12)	3–33 (9)	5–19 (13)

This did not exceed the incidence of such complications in patients on standard chemotherapy (Table 9).

It was not the goal of this study to evaluate the antileukemic efficacy of EBD. The group of patients was too small to correctly consider this problem. It is noteworthy, however, that four (one with leukemic relapse and three in remission) out of the five patients who received two infusions of free daunorubicin followed by a third infusion of EBD remained in remission. One patient with primarily resistant AML did not respond to chemotherapy. In the group of ten patients who had received three EBD infusions, eight patients achieved remission, and two patients did not respond to chemotherapy.

Discussion

Our data demonstrate that daunorubicin-loaded erythrocytes can be prepared using whole blood or washed erythrocytes obtained from healthy donors as well as from patients with acute leukemia. Daunorubicin-loaded erythrocytes can be prepared by incubating blood or washed erythrocytes with the antibiotic at 37° for an hour. Daunorubicin was shown to bind much better to washed erythrocytes than to erythrocytes in the blood. Its lower equilibrium concentration ratios observed in the blood are likely to result from the binding of a considerable fraction of daunorubicin with plasma proteins and lipoprotein complexes. Most of the daunorubicin, however, usually binds to erythrocytes. Therefore, we find it admissible to use patient whole blood for preparing EBD.

It was shown that preparation of EBD does not influence erythrocyte deformability (filterability). In fact, in our filtration experiments we simulated the procedure of EBD

preparation and the subsequent washing off of daunorubicin from the erythrocytes and its dilution after EBD infusion to the patient. Daunorubicin binding by erythrocytes is reversible [26,29]. In this way, during the preparation of erythrocytes for the filtration experiments (cell washing and dilution), cell daunorubicin concentration can be significantly decreased. In our preliminary experiments we found that 50–70% of initially bound daunorubicin remains in erythrocytes after the washing procedure. Cell daunorubicin concentration should drop significantly after dilution of erythrocytes to a 1% suspension prior to filterability measurements. Taking into account the erythrocyte/medium concentration ratios determined here for patient erythrocytes, we could evaluate the equilibrium cell daunorubicin concentration in the 1% suspension. In the cells preincubated at daunorubicin concentrations of 0.25–1.0 mg/ml cells, it should be close to the peak daunorubicin concentrations observed in blood and plasma after infusion of EBD or free daunorubicin to patients (Table 3). Erythrocytes incubated at the daunorubicin concentrations used in the EBD preparation procedure (0.25 and 0.5 mg/ml cells) possessed the same filterability as control cells. Erythrocyte filterability significantly decreased after incubation at the daunorubicin concentration of 1.0 mg/ml cells. Such a relatively high daunorubicin concentration, however, was not used for EBD preparation. In this way our filtration experiments demonstrate that the EBD preparation does not cause a significant irreversible decrease in erythrocyte deformability. Therefore, the erythrocytes infused to patients as EBD should not cause any disturbance of circulation.

When EBD was administered to patients, the antibiotic peak concentration was lower and it circulated longer compared with standard daunorubicin administration. The rate of doxorubicin clearance from the bloodstream is known to be negatively correlated with its therapeutic efficacy [41,42]. We can expect by analogy that the change in the pharmacokinetics of daunorubicin observed in its erythrocyte-bound form would enhance its therapeutic effect.

All pharmacokinetic parameters measured in this study demonstrate very high inter-patient variations. Actually, a very high inter-patient variation in pharmacokinetics is typical for anthracycline antibiotics, and the reason of this variability is largely unknown [40,43]. The fast phase of decrease in blood daunorubicin level is connected with drug distribution in tissues [40,44]. Thus, pharmacokinetic variability at the fast phase can be explained by inter-patient differences in body composition and in the permeability of inter-organ barriers to the antibiotic. The slow phase of blood daunorubicin pharmacokinetics is determined mainly by excretion of the antibiotic from the body with the bile acids [40,44]. In this way, pharmacokinetic variability at the slow phase can be explained by inter-patient differences in liver function. It should also be noted that anthracyclines are generally used in combination therapy, together with many other toxic drugs that can act as additional factors of pharmacokinetic variability.

Treatment of patients with EBD reveals its antileukemic activity. Moreover, compared with the standard daunorubicin form, EBD is better tolerated by patients. Daunorubicin-related toxic effects (particularly nonspecific effects) were less pronounced in patients receiving EBD. In this way, our results indicate that daunorubicin-loaded erythrocytes are promising for clinical application and deserve further clinical study.

CONCLUSIONS

1. Daunorubicin-loaded erythrocytes can be prepared by incubating blood or washed erythrocytes with the antibiotic at 37° for an hour. The mean equilibrium erythrocyte/medium concentration ratios are 2.8 in blood and 5.7 in suspension. Therefore, it is more expedient to load washed erythrocytes with daunorubicin than to use patient blood for preparing EBD.

2. Erythrocyte deformability is not affected by daunorubicin binding during the preparation of EBD.

3. The peak daunorubicin concentration and the rate of its clearance from the circulation are lower after EBD than after free daunorubicin infusions. As a result, the average plasma and blood daunorubicin concentrations over the course of chemotherapy prove to be considerably higher for the EBD than for its standard free form.

4. Compared with its standard free form, EBD is better tolerated by patients.

5. These results indicate that anthracycline antibiotic-loaded erythrocytes are promising for clinical application and deserve further clinical study.

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