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Quantitative evaluation of intracellular uptake of daunorubicin in acute myeloid leukemia: A method analysis*

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Summary. Critical technical parameters to establish a reliable method for quantifying the intracellular content of anthracyclines were evaluated in patients with acute myelocytic leukemia (AML); two methods were used for the isolation of leukocytes from the peripheral blood and two methods, for the extraction of daunorubicin (DNR), daunorubicinol (DOL), and doxorubicin (DOX) from these cells, followed by drug analysis using high-performance liquid chromatography (HPLC). At 0–4 °C the recovery of leukocytes after methylcellulose separation was low (64%). Cold hypotonic lysis gave better recovery (100%) when performed at the same temperature. After low-volume (2 ml extraction mixture) drug extraction from isolated leukocytes, the recoveries of DNR, DOL, and DOX from the cells were low, and they were inversely related to the cellularity of the sample, irrespective of the amount of drug in the cells. With high-volume extraction (5 ml extraction mixture) the recoveries were better (up to 95%), but they remained dependent on the cellularity. A correction factor accounting for these cellularity-related recoveries was applied to calculate the DNR and DOL contents of the leukocytes. Finally, using this information, plasma and cellular DNR and DOL levels were measured in seven patients with AML during their first course of remission induction therapy. The cellular DNR levels appeared to vary over a broad range and did not correlate with plasma pharmacokinetics.

Introduction

Daunorubicin (DNR) is a major drug in the treatment of acute myelocytic leukemia (AML). In current treatment protocols the drug is frequently administered during remission induction (RI), consolidation (C), and maintenance (M) therapy. The clinical response of patients with AML treated with anthracycline-containing chemotherapy varies considerably. Although the complete remission rate of AML is high (60%–70%) [8], a significant proportion of cases is primarily refractory. In the responding patients the relapse rate of AML is high, owing to incomplete eradication of leukemic cells. The duration of response may vary from 2 months to several years. The reasons for this considerable variation in therapeutic effect may relate to different biological properties of the leukemic cells per se and their susceptibility to the cytoreductive effect of the drug, or to interindividual differences in drug penetration into the leukemic cell. Monitoring of daunorubicin and doxorubicin (DOX) plasma levels in AML patients during RI has yielded little information to add to our understanding of the reasons for the interindividual variation in initial response and eventual relapse [13]. Moreover, the plasma concentrations of DNR and its major metabolite daunorubicinol (DOL) were not correlated with the concentrations of the drug in leukemic cells obtained from AML patients [6, 11]. Since DNR apparently exerts its major effect by intercalation into DNA [1, 7, 19], the nucleus of leukemic cells can be regarded as the major target of DNR therapy. Determinations of DNR and its metabolite in leukemic cells, rather than in plasma, are likely to provide more important parameters for estimation of the response to the drug. However, little is known about the methodological factors of the assay, and therefore the results of the studies may suffer from inadequate technical performance or reproducibility. We report an analysis of certain factors which directly affect the outcome of the intracellular drug measurements. Different methods of sampling AML cells from the peripheral blood, followed by high-performance liquid chromatography for evaluation of the DNR content, were examined. The quality of isolation and separation of the leukemic blasts from other cells and the extraction method were investigated for maximal recovery and for avoidance of artefacts. The data obtained were then applied to a clinical study in seven newly diagnosed AML patients treated with daunorubicin as part of the remission induction chemotherapy regimen.

Materials and methods

Drugs and chemicals

Daunorubicin, daunorubicinol and doxorubicin were a gift from Farmitalia Carlo Erba (Milan, Italy). The drugs were freshly dissolved in methanol and diluted in water to give a final concentration of 200 μg/ml, and stored at −20 °C in the dark. The HPLC solvents were HPLC grade

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reagents (J. T. Baker Chemicals B. V., Deventer, Holland). Dulbecco's phosphate-buffered saline (PBS) at pH 7.3 had the following composition in deionized water: NaCl 136.8 m*M*, KCl 2.7 m*M*, Na2HPO4 8.1 m*M*, and KH2PO4 1.5 m*M*. Lysis buffer (290 mosmol) had the following composition in deionized water: NH4Cl 155.1 m*M*, NaHCO3 11.9 m*M* and EDTA 0.1 m*M*. Methylcellulose (Dow Methocel, viscosity 4000 cps) was dissolved in deionized water and Dulbecco's medium to prepare a final 2% solution (w/v).

Collection and isolation of human peripheral blood leukocytes

Human leukocyte suspensions were prepared from buffy coats, which were obtained as a byproduct following single donor platelet pheresis (Aminco, Celtrifuge 1), with ACD-A as an anticoagulant. We wished to achieve maximal leukocyte recovery from the blood for measurement of drug uptake and compared two separation methods:

A. Sedimentation of the cells in methylcellulose. One part of the blood was mixed with one part of ice-cold PBS and methylcellulose 40:1 (v/v) in a siliconized tube and placed on ice. After 20 min the leukocytes were harvested. The same procedure was repeated for the residual cells of the pellet. Immediately after collection, the cells were placed on ice. Then, the cells were washed with PBS at 4 °C to remove the remaining methylcellulose (10 min, 450 *g*). Finally, the leukocytes were resuspended in PBS.

B. Separation by hypotonic lysis. After washing the cell suspension with PBS to remove the plasma, the cells were resuspended in a small volume of PBS. One part of the cell suspension was mixed with four parts of lysis buffer and placed on melting ice. After 10 min the suspension was centrifuged, the supernatant was removed, the leukocytes were washed with PBS to remove the remaining lysis buffer, and finally the leukocytes were resuspended in PBS.

Then the cell suspension was diluted with PBS to final concentrations of 1, 3, 10, and 30×10^9 cells/l. Total cell counts following separation were compared with the initial count of the blood sample before separation. DNR, DOL, and DOX (50 µl of each) were added to 2 ml of each leukocyte suspension to give final concentrations of 0.1, 0.5, and 2.0 µg DNR, DOL, and DOX per ml suspension. After 5 min the samples were stored at −20 °C until further processing.

Collection and isolation of whole blood, plasma and leukocytes from AML patients

The criteria of the French-American-British working party were used to diagnose AML [4]. The patients were treated according to the EORTC LAM-6 protocol. Remission induction chemotherapy consisted in DNR 45 mg/m^2 body surface on days 1, 2 and 3 by IV bolus, cytosine arabinoside (ara-C) 200 mg/m^2 IV on days 1–7 and vincristine 1 mg/m^2 IV on day 2.

Blood samples (10 ml) were drawn into tubes with EDTA as the anticoagulant, immediately before and at 5, 10, 20, 30 min and 1, 4, 8, and 24 h, after the first dose of DNR was administered during the first course of RI treatment. The samples were cooled to 4 °C, the cells were counted, and 2 ml whole blood was taken from each sample and frozen. Another part of each of these samples was centrifuged at 4 °C, after which the plasma was collected and frozen. The buffy coat was depleted of erythrocytes by cold hypotonic lysis. The remaining leukocytes, usually myeloblasts, as determined by microscopic examination of stained cells, were resuspended in PBS, counted, and also frozen.

Therapeutic end-points of remission induction regiments

The criteria for evaluating response were those established by the Cancer and Acute Leukemia Group B [14]. In brief, complete remission (CR) or partial remission (PR) is achieved when after one or two RI courses the proportion of leukemic cells has fallen to <5.0% (CR) or to 5.1%–25.0% (PR) while hematopoiesis and peripheral blood counts have returned to normal.

Failures of the induction treatment were characterized according to Preisler et al. [12]: Type 1: absolute drug resistance; type 2: relative drug resistance; type 3: regeneration failure; type 4: hypoplastic death; type 5: early death; type 6: extramedullary leukemic persistence.

Extraction

A. Low-volume extraction. Borate buffer (0.2 ml; 0.5 *M*, pH 9.8) and 2 ml extraction mixture (chloroform: methanol 4:1) were added to 2.0 ml cell suspension in a siliconized tube. The tubes were closed with silicone corks and vortexed for 2 min. After centrifugation at 1000 *g* for 10 min, 100 µl of the lower organic layer were injected into the column.

B. High-volume extraction. Borate buffer (0.2 ml; 0.5 *M*, pH 9.8) and 5 ml extraction mixture were added to 2.0 ml leukocyte suspension in a 30-ml glass bottle and shaken for 20 min at 300 rpm on a Mini-shaker (MSR, Salm and Kipp, Breukelen, Holland) in the dark. After this, 1 ml of the organic phase was dried with a gentle stream of nitrogen. Dried samples were reconstituted with 300 µl chloroform: methanol (4:1) and 100-µl aliquots were then analyzed by HPLC.

The patient plasma samples were extracted using the low-volume extraction technique, and the whole blood and leukocyte samples were extracted using the high-volume extraction method, with DOX added as internal standard.

High-performance liquid chromatography (HPLC)

HPLC separation (adapted from Baurain et al. [2]) was accomplished using a Waters Associates M-510 pump and a Waters Associates automatic sample injector (Model 710B). The stationary phase consisted of 7-µm silica gel particles prepacked into a 250 × 4.6 mm stainless steel column (Lichrosorb Si-60-7, Chrompack). The mobile phase consisted of chloroform, methanol, acetic acid, water and 3 m*M* MgCl2 solution in water (720:210:40:24:6 by volume), filtered (Millipore FH 0.5 µm) and used at a flow rate of 0.8 ml/min. Fluorescent detection was accomplished using a Gilson Spectra-Glo fluorometer at 480 and 560 nm for the excitation and emission wavelengths, respectively, and equipped with a 45-µl flow cell. The integration of the peak areas was performed by a Shimadzu Model CR3A integrator (United Technologies Packard, Delft, Holland).

The linearity and sensitivity of the method were determined from serial concentrations of DNR, DOL, and

DOX in chloroform: methanol (4:1). Linear calibrations were obtained for all three compounds in the concentration range of 12.5–400 mg/ml (Fig. 1) (coefficient of variation 5%–7% at 50 mg/ml).

The lower detection limit of DNR in plasma was 5 ng per injection of 100 µl and after extraction from biological fluids, 25 ng/ml biological fluid or 5 µg/10^9 leukocytes.

Mathematical modeling

The data from the seven AML patients were analyzed by fitting a two-compartment model to the observed plasma and intracellular DNR concentrations [15]. The first (central) compartment represents the plasma distribution of DNR. The second (peripheral) compartment represents the concentration-time curves of DNR in tissues, including the cellular elements in blood. The plasma and intracellular concentration–time disappearance of the parent drug (DNR) are described by the following equations, respectively:

$$C(t) = A \cdot e^{-\alpha \cdot t} + B \cdot e^{-\beta \cdot t} \quad (1)$$

$$C(t) = A \cdot [e^{-\beta \cdot t} - e^{-\alpha \cdot t}] \quad (2)$$

where C(t) is the plasma (Eq. 1) or intracellular (Eq. 2) level of DNR at each time t after the injection, A and B are constants, and α and β are first-order elimination rate constants.

The DNR area under the concentration–time curves (AUC) in plasma and leukocytes, the volume of the central (plasma) compartment (V_1), the apparent distribution volume (Vd_{area}), and the total-body clearance (Cls) were calculated from this model.

Results

Separation of human leukocytes

For determination of DNR and DOL concentrations attained in vivo in leukemic cells of AML patients, it is necessary to use a separation method which provides pure leukocyte fractions without contamination of erythrocytes or selective loss of leukocytes. The separation was performed at 0–4 °C, to prevent metabolism of DNR [16]. Other possible degradation of DNR was avoided by working in reduced daylight and using siliconized tubes [17, 18]. We compared two procedures for cell separation. In the first place, methylcellulose was used for sedimentation of leukocytes. Secondly, using the lysis buffer, the leukocytes were isolated following hypotonic lysis of the erythrocytes. The recoveries of leukocytes and erythrocytes were expressed relative to the total number of leukocytes and erythrocytes before separation (set at 100%). The leukocyte purification factor designates the ratio of the percentage recovery of leukocytes in the leukocyte pellet and in the whole blood sample.

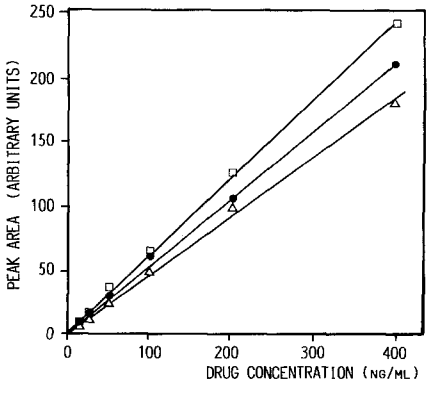

Fig. 1. Calibration of peak height versus concentration of DNR (●) DOL (□), and DOX (△) in chloroform: methanol (4:1); sample analyzed in duplicate

Table 1. Recoveries of leukocytes and erythrocytes in human leukocyte pellets after separation[a]

Separation method	N[b]	Recovery of leukocytes (%) (mean ± SD)	Recovery of erythrocytes (%) (mean ± SD)	Leukocyte purification factor (mean ± SE)[c]
Methyl-cellulose	86	65 ± 24	3 ± 3	66 ± 15
Lysis buffer	67	106 ± 16	2 ± 3	490 ± 102

[a] Before separation the leukocyte concentration of the blood samples varied from 0.25 to 170 × 10^9 cells/l (set at 100%)

[b] Number of experiments

[c] The leukocyte purification factor designates the ratio of the percentage recovery of leukocytes in the leukocyte pellet and in the whole blood sample

As shown in Table 1, the recovery of leukocytes after hypotonic lysis was complete (106%) and superior to that after methylcellulose separation (65%). The recovery of erythrocytes was equivalent with both methods (2%–3%), which indicates almost complete removal of erythrocytes from the leukocyte pellets after separation with either method. In further experiments we used hypotonic lysis for cell separation.

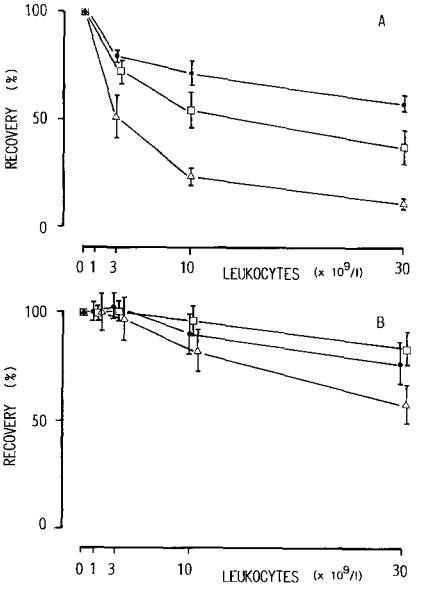

Fig. 2 A, B. Recovery of DNR (●), DOL (□), and DOX (△) after extraction from different concentrations of leukocytes, using low-volume extraction **(A)** and high-volume extraction **(B)** methods. Means ± SD of **(A)** and 12 **(B)** experiments

Table 2. Patient characteristics

Patient	Sex	Age	FAB classification	WBC ($\times 10^9$/l) at initial diagnosis	% Blasts in peripheral blood at initial diagnosis	Dose of DNR (mg/m^2)	Dose of DNR (mg)	Number of induction courses	Outcome of remission induction therapy
1	M	42	M1	1.44	7	45	75	2	CR
2	M	73	M1	13.0	75	45	80	1	CR
3	F	65	M1	1.406	0	45	70	2	CR
4	M	45	M4	4.1	84	45	90	2	PR
5	M	37	M4	107.3	76	45	90	2	PR
6	M	26	M4	170.0	99	45	80	2	PR
7	M	17	M2	6.5	79	45	80	2	Type 1 failure

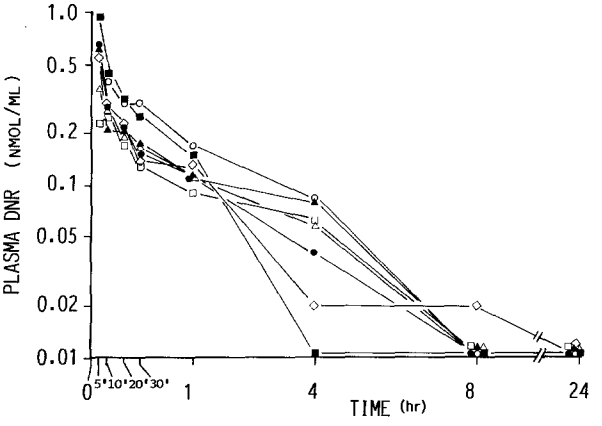

Fig. 3. Plasma concentration-time curves of DNR after first DNR IV bolus injection of DNR in seven AML patients during first RI treatment. Patients (see Table 2) are indicated by the following symbols: 1, ○–○; 2, ●–●; 3, ◇–◇; 4, □–□; 5, ■–■; 6, △–△; 7, ▲–▲

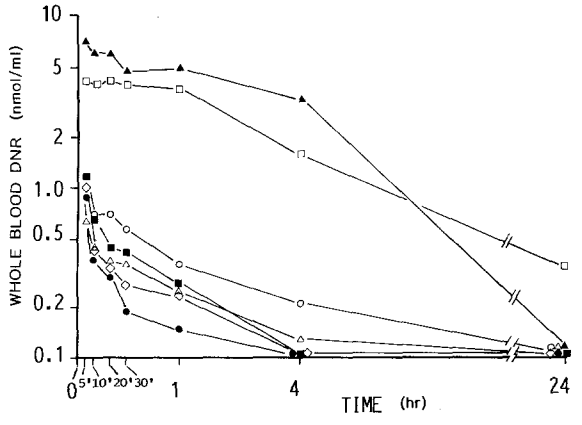

Fig. 4. Whole blood concentration-time curves of DNR after first IV bolus injection of DNR in seven AML patients during first RI treatment. Patients (see Table 2) are indicated by the following symbols: 1, ○–○; 2, ●–●; 3, ◇–◇; 4, □–□; 5, ■–■; 6, △–△; 7, ▲–▲

Recovery of DNR, DOL, and DOX after extraction from leukocytes

Low-volume extraction of DNR, DOL and DOX from biological samples, such as plasma and urine, yields recoveries of approximately 100% [2, 3]. When this extraction method is applied to biological samples containing variable concentrations of cells, it appears that the recoveries of DNR, DOL, and DOX depend on the cellularity of the extracted sample, regardless of the amount of drug added to the cells. As shown in Fig. 2A, the recovery of DOX is very low compared with that of DNR and DOL, especially in samples with large amounts of cells. Since DOX has been added as an internal standard for the quantification of DNR and DOL, better recovery is necessary.

Large-volume extraction with a long period of extraction gives better recoveries of DNR, DOL, and DOX (Fig. 2B). Nevertheless, again the recovery of DOX is lower than that of DNR and DOL. The recovery of all drugs depends on the cellularity of the sample. These results indicate that determination of the DNR content of human leukocytes may be subject to variation, depending on the method of isolation, the volume and duration of extraction, and the cell concentration of the suspension.

Pharmacokinetics of DNR in AML patients

Table 2 shows certain characteristics of seven previously untreated patients with AML. At diagnosis a large variation in the number of peripheral white blood cells (WBC) was apparent (range $1.4-170 \times 10^9$ cells/l). For calculation of DNR in whole blood and in leukocytes, correction factors were used to account for the cellularity of the samples. As shown in Fig. 3, the plasma concentrations of DNR rapidly declined to minimal or undetectable levels within 4–8 h after administration. This rapid decline was associated with the appearance of the major metabolite DOL in the plasma. Generally, DOL plasma concentrations exceeded DNR levels within 1 h after administration, and at 4 h after administration the DOL concentration was 80% or more of the total amount of detectable enthracycline (data not shown). With our method of drug determination no metabolites other than DOL were found.

In Fig. 4 the whole blood concentration-time curves for DNR are shown. When they are compared with those for plasma, it is obvious that a more variable decline can be observed. At 4 h after administration of the drug two patients had high DNR (60%) and DOL (40%) blood levels, while the other patients had low or undetectable levels.

DNR accumulated extensively in the leukocytes, at concentrations which were even more variable than those in plasma and blood (Fig. 5). Maximum cellular DNR concentrations were reached almost immediately after administration of the drug in all patients. Intracellular DOL was present at much lower concentrations than in plasma (<20%).

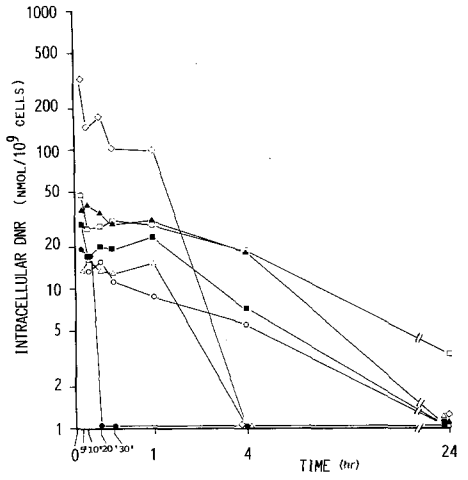

Fig. 5. Leukocytes concentration-time curves of DNR after first IV bolus injection of DNR in seven AML patients during first RI treatment. Patients (see Table 2) are indicated by the following symbols: 1, ○–○; 2, ●–●; 3, ◇–◇; 4, □–□; 5, ■–■; 6, △–△; 7, ▲–▲

Table 3. Plasma pharmacokinetic parameters of daunorubicin in AML patients after IV bolus injection at t = 0

Patient	$t_{1/2\alpha}$ (h)	$t_{1/2\beta}$ (h)	V_1[a] (l)	Vd_{area}[b] (l)	Cls[c] (l/h)
1	0.07	1.04	0.12	0.57	0.38
2	0.07	0.77	0.23	0.34	0.31
3	0.07	2.17	0.13	0.64	0.20
4	0.10	0.48	0.12	0.32	0.47
5	0.18	0.81	0.60	0.74	0.46
6	0.02	0.61	0.01	0.25	0.28
7	0.01	0.76	0.01	0.33	0.30

[a] Volume of the central (plasma) compartment
[b] Apparent distribution volume
[c] Total-body clearance

Table 4. Area under the plasma and leukocyte concentration – time curves (AUC) for daunorubicin in AML patients after IV bolus injection at t = 0

Plasma AUC (nmol × h/ml)				
Patient	0–4 h	0–8 h	0–24 h	0–∞ h
1	0.35	0.37	0.37	0.37
2	0.47	0.49	0.49	0.49
3	0.49	0.61	0.65	0.65
4	0.36	0.36	0.36	0.36
5	0.27	0.27	0.27	0.27
6	0.54	0.54	0.54	0.54
7	0.50	0.51	0.51	0.51
Leukocyte AUC (nmol × h/10^9 cells)				
1	4.61	4.61	4.61	4.61
2	33.61	44.72	50.02	50.07
3	175.20	175.43	175.43	175.43
4	60.83	82.92	95.28	95.48
5	104.04	158.67	214.41	218.95
6	106.88	157.34	199.94	202.10
7	24.81	26.14	26.21	26.21

Tables 3 and 4 show the calculated plasma and cellular pharmacokinetic parameters of DNR in these seven AML patients. Clearly, the wide interindividual variation in the AUCs for DNR in leukocytes is not reflected by the AUCs in plasma of the same patients. Thus, the data suggest that the leukocytes should be regarded as a separate compartment.

Discussion

For better understanding of the wide variation in individual response to DNR in AML, individual drug monitoring in the leukemic cells is of interest. Therefore, we decided to develop an efficient and reliable method that would be useful for measuring concentrations of DNR and DOL in the cells of AML patients. Methylcellulose or Ficoll-Isopaque are frequently used for separation of leukocytes. We found that the recovery of leukocytes after methylcellulose separation when applied at 0–4 °C is low (64%), which is most likely due to the relatively low temperature used for the procedure. At 0–4 °C methylcellulose is a gel with increased viscosity, which may interfere with proper separation of blood cells [6]. At 20 °C methylcellulose forms a colloid solution, and at this temperature the recovery of leukocytes is better (95%). Obviously, this temperature, although permitting satisfactory cell recoveries, has the disadvantage of being associated with the metabolic degradation of DNR to DOL by aldoketoreductase, an enzyme which is present in leukocytes and in myeloblasts [9, 10]. Cold hypotonic lysis gave clearly superior results. It could be performed at 0–4 °C, with excellent separation of white and red blood cells and 100% recovery of all nucleated cells.

The extraction method is shown also to be critical. The recovery of DNR depends on the volume of extraction and the duration of extraction (Fig. 2A, B). Owing to the use of large volumes in bottles, the surface exposures of the two liquid phases are increased and a better transfer of the drugs into the organic phase is obtained. Extraction times up to 20 min improved drug recoveries, but extractions beyond 20 min did not contribute further.

Even under conditions of optimal cell isolation and drug extraction the recoveries of DNR and DOX remain dependent on the cellularity of the sample. This is the case irrespective of the amount of drug in the cells. Thus, the number of cells submitted to extraction is inversely related to the fraction of intracellular DNR and added DOX to enter the organic phase. In other words, the number of leukocytes correlates negatively with the partition coefficients of DNR and DOX. It remains to be determined whether this is caused by fractional binding of the drugs to cell fragments, or whether the cell fragments mechanically prevent a proper exchange of the drug molecules between the two liquid phases. In calculations of the DNR content of leukocytes, it is therefore still necessary to correct for cellularity of the sample. This correction factor is variable and is derived from the concentration of cells in the sample. It might be that excessive denaturation of DNA, for example by pretreatment with silver nitrate, improves the extraction ratio of DNR from DNA binding sites. To avoid heterogeneous recoveries from different samples, we standardized the patient blood samples and leukocytes with PBS at final concentrations between 1 and 10×10^9 cells/l before starting on the extraction procedure.

With the above-defined criteria it is possible to monitor the in vivo plasma and cellular pharmacokinetics of DNR and DOL in patients in a reproducible way during treatment. In the small series of patients with AML treated with DNR during remission induction and with variable numbers of circulating leukemic cells, it was found that: (1) plasma levels of DNR and DOL do not correlate with blood and leukocyte concentrations of DNR and DOL; (2) plasma pharmacokinetic parameters do not correlate with cellular pharmacokinetic parameters; (3) in vivo cellular levels of DNR are considerably more variable than in vivo DNR levels in plasma or whole blood; (4) the pattern of DNR metabolism is different in the leukocytes and the plasma.

Although these conclusions are based on a small number of patients, they demonstrate that the plasma concentration of DNR and DOL provides little information on the drug concentrations attained in the leukocytes. These findings are in agreement with the results of DeGregorio et al. [6], who indicated that plasma concentrations of DNR and DOL were not useful for estimation of the in vitro inhibition of DNA synthesis. Our findings are also consistent with the conclusion of Preisler et al. [13], who showed that there was no significant correlation between plasma levels of anthracyclines and clinical response to therapy in patients with AML. Therefore, to analyze the clinical effectiveness of DNR it may be useful to pursue the pharmacokinetics of DNR and metabolites not only in plasma but particularly in leukocytes in blood and bone marrow.

It remains to be investigated whether the determinations of drug concentrations in the leukocytes will indeed provide prognostic indicators of the variable outcome of remission induction therapy and the duration of complete remission in acute leukemia patients. As yet, our studies described here may be of use to set a methodological baseline for these approaches.

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