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# Intracellular concentrations of anti cancer drugs in leukemic cells in vitro vs in vivo

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**Summary.** A comparison of intracellular concentrations of daunorubicin, doxorubicin and ara-C in myeloid blast cells was carried out in vivo and in vitro. In vivo, blood samples were obtained from 27 patients with acute non-lymphoblastic leukemia during and up to 4 days after drug infusion. Leukemic cells were isolated and drug concentrations were determined by HPLC. Before treatment, leukemic cells from 21 patients were isolated from blood and bone marrow, and in vitro incubations were done with anthracyclines for 1–3 h at concentrations of 0.1–1.0 μ*M* and with ara-C for 1 h to 5 days at concentrations of 0.5–5.0 μ*M*. The cells were cultured for 5 days, during which cell samples were taken for drug determination. The results showed that incubation with 0.2 μ*M* daunorubicin for 1 h and 0.2 μ*M* doxorubicin for 3 h and continuous exposure to 0.5 μ*M* ara-C gave intracellular concentration curves similar to those obtained in vivo. After 5 days' culture, the cytotoxic effect was determined by vital dye staining with fast green, the addition of an internal standard of fixed goose erythrocytes, cytospin centrifugation and counter-staining of living cells with haematoxylin/eosin (DiSC). Incubations at the above-mentioned concentrations exerted a cytotoxic effect of approximately 50%. We conclude that in mimicking the in vivo situation, it is important to consider differences in intracellular pharmacokinetics.

## Introduction

In cancer chemotherapy, tumors initially show variable sensitivity to cytostatic drugs and the development of drug resistance is common. The toxicity of these drugs and the poor prognosis for non-responding patients make it important to choose the optimal drug combination from the start. Consequently, many efforts have been made to develop in vitro methods for predictive drug testing, and these methods are also used for screening new anti-cancer drugs [7]. Although correlations to clinical response have been reported [16], thus far it has not been feasible to use these tests in clinical practice as a basis for selecting drugs for the individual patient. In predictive drug tests such as the human tumor stem cell assay [17] or the differential staining cytotoxicity (DiSC) assay [18–20], tumor cells are often incubated in a standardized way, e. g. for 1 h at 1/10 of the plasma peak concentration [18]. However, we have previously shown that there is no simple relationship between plasma and intracellular concentrations for many anti-cancer drugs [9, 13, 14]. Differences in intracellular drug retention and intracellular metabolism are important for the activity of these drugs, and in clinical practice various anti-cancer drugs are given on separate dose schedules.

Furthermore, the action of some drugs is specific to the cell cycle phase and other agents are prodrugs that form their active metabolites intracellularly. An example of the latter is ara-C, which undergoes phosphorylation to ara-CTP, which is considered to be its active metabolite [21]. To increase the accuracy of in vitro testing to predict drug response, it appears important that the incubation procedure be modified such that the intracellular concentration of the drug or its active metabolite in the malignant cell mimic in vivo concentrations obtained after administration of the drug to the patient. The aim of this study was therefore to compare the in vitro concentrations of anthracyclines and ara-C in leukemic blast cells with the intracellular drug concentrations obtained in vivo in patients with acute nonlymphoblastic leukemia.

## Patients and methods

*In vivo pharmacokinetics.* Studies of intracellular drug uptake in vivo were carried out in 27 patients (ages 18–80 years) with previously untreated acute nonlymphocytic leukemia (ANL). The patients had normal liver and renal function and had a peripheral white cell count in the range of $4.0–204 \times 10^9$ cells/l, with >80% blast cells.

Venous blood samples were drawn at intervals during and after the rapid infusion of 60 mg/m$^2$ doxorubicin or daunorubicin or the continuous infusion of 100 mg/m$^2$ ara-C over 24 h. The blood samples were collected in heparinized test tubes and immediately cooled on ice. The leukemic cells were separated on sodium metrizoate/ficoll (Lymphoprep; Nyegaard & Co, Oslo) [5] and washed twice in ice-cold phosphate-buffered saline (PBS, pH 7.4); thereafter, they were resuspended in 1 ml PBS and immediately stored at −20° C until the day of analysis [12]. In samples used for the determination of intracellular ara-CTP, acid-soluble nucleotides were extracted from the leukemic cells

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with 0.8 *M* PCA and, after centrifugation (5 min, 1000 *g* at 4° C), the supernatant was neutralized with 1.2/0.5 *M* $KOH/NH_4H_2PO_4$ before freezing [8].

*In vitro pharmacokinetics.* Studies of intracellular drug uptake in vitro were conducted on leukemic cells from 21 adult patients with previously untreated ANL. The peripheral white cell count ranged from 5.0 to $204 \times 10^9$ cells/l with >80% blast cells.

Leukemic cells from blood and bone marrow were isolated before the start of treatment, washed and resuspended in RPMI 1640 with 10% fetal calf serum and 1% L-glutamine. The cells were incubated in sealed polypropylene tubes in a shaking water bath at 37° C for 1 h to 5 days at a cell concentration of $5 \times 10^5$ cells/ml with either 0.2 and 0.5 μ*M* doxorubicin for 3 h or 0.2 and 1.0 μ*M* daunorubicin for 1 h or with continuous exposure to 0.5 μ*M* ara-C. The incubations were terminated by centrifugation (400 *g* for 10 min at 20° C). The cells were resuspended in fresh medium without washing and allowed to grow in suspension culture in a humidified $CO_2$ incubator (ASSAB) with 5% $CO_2$ at 37° C. Samples used for the determination of intracellular drug concentrations were taken from the incubated cells at the end of the incubation and at intervals during the culturing period. Then, 5 ml ice-cold PBS was added to each tube, which was immediately centrifuged (1000 *g* for 5 min at 4° C). The samples were washed twice and resuspended in 2 ml PBS, frozen and stored at −20° C until the day of analysis. In samples used for the determination of ara-CTP, acid-soluble nucleotides were extracted as described above.

*In vitro cytotoxicity.* After the complete culturing period, the chemosensitivity of the leukemic cells was determined using the differential staining cytotoxicity (DiSC) assay, which was carried out according to Weisenthal et al. [18–20], with slight modifications. After incubation and cultivation for 5 days, 0.2 ml from each sample was vital-stained with 0.2 ml 2% fast green together with an internal standard of fixed goose erythrocytes. After 10 min, a 0.2 ml sample of this suspension was cytospin centrifuged (1300 rpm, 7 min) and the living cells were counter-stained with haematoxylin-eosin. The cytotoxic effect of the tested drug was determined by the ratio of living cells to goose erythrocytes and expressed as the percentage of the same ratio in a drug-free control.

*Drug determination.* The intracellular and plasma concentrations of anthracyclines in vivo were determined by HPLC according to Baurain et al. [3, 4]. After thawing and sonication (75 W, 20 kHz, 30 s) a 0.2 ml aliquot of the cell sample was added to 0.2 ml 0.1 *M* borate buffer (pH 9.8) containing either 0.2 ml 1.0 μ*M* doxorubicin as the internal standard if the patient had received daunorubicin or the same amount of daunorubicin if the patient had received doxorubicin. Since no intracellular anthracycline metabolites are detected in vitro, these samples were analysed by a fluorometric method [11]. The cell samples were sonicated and the drugs were extracted with 27% trichloroacetic acid (TCA). The drugs were assayed by fluorometry using a model RF-510 Shimadzu spectrofluorometer (excitation and emission wavelengths, 485 and 560 nm, respectively). The drug concentrations in each sample were calculated by comparison with identically treated standard solutions.

The intracellular concentrations of ara-CTP were determined by HPLC; the chromatographic procedure was carried out as described elsewhere [8]. Aliquots (50–200 μl) of the nucleotide extracts were injected into a Hewlett-Packard 1084B liquid chromatograph equipped with a Partisil 10 SAX anion-exchange column (Whatman, 4.6 mm × 25 cm). Ara-CTP was quantitated by measuring its absorbance at 280 nm using a Beckman 160 UV detector. As the mobile phase, $NH_4H_2PO_4$ was used at two concentrations in a gradient system (0.005 *M*, pH 2.8; 0.75 *M*, pH 3.7). The concentrations of ara-CTP were determined by comparing the peak heights of the sample with those of standard mixtures. Protein concentration was determined according to Lowry et al. [10] using bovine serum albumin (BSA) as a standard.

## Results

*Intracellular in vivo pharmacokinetics and in vitro concentrations of daunorubicin.*

Drug concentrations in the leukemic cells after a 10-min infusion of 60 mg/m$^2$ daunorubicin in seven patients with ANL are shown in Fig. 1. There was a rapid intracellular accumulation and a slower elimination. The peak concentration was around 0.5 nmol/mg cell protein, and 50 h later there were still detectable intracellular concentrations. The main metabolite, daunorubicinol, was present at low concentrations. Figure 2 shows intracellular in vitro concentrations after a 1 h incubation of leukemic cells from five patients with 0.2 and 1.0 μ*M* daunorubicin. The higher incubation concentration gave a 10-fold higher peak concentration and about a 25-fold higher AUC compared with the in vivo results. A concentration curve that more closely resembled the in vivo situation was obtained after a 1-h incubation with 0.2 μ*M* daunorubicin. The peak levels in vitro ranged from 0.3 to 1.3 nmol/mg protein, with a mean value of 0.9 nmol/mg protein.

*Intracellular in vivo pharmacokinetics and in vitro concentration of doxorubicin*

Figure 3 shows the intracellular drug accumulation in vivo in five patients with acute leukemia who received a rapid infusion of 60 mg/m$^2$ doxorubicin. The mean peak concentration for doxorubicin (0.13 nmol/mg cell protein) was lower than that for daunorubicin. However, doxorubicin was retained longer: after 50 h, the intracellular drug level was still 0.1 nmol/mg protein. No metabolites were detected intracellularly. The intracellular uptake of doxorubicin in vitro after a 3-h incubation of leukemic cells from nine patients with 0.2 and 0.5 μ*M* doxorubicin is presented in Fig. 4. The in vitro peak levels obtained with 0.2 μ*M* varied from 0.10 to 0.35 nmol/mg protein, which was similar to that obtained in vivo.

*Intracellular in vivo pharmacokinetics and in vitro concentrations of ara-CTP.*

Figure 5 shows the concentrations of ara-CTP in leukemic cells from 17 patients with acute leukemia during the continuous infusion of 100 mg/m$^2$ ara-C. The concentrations were around 100 pmol/mg cell protein. To obtain a similar intracellular concentration of ara-CTP in vitro, a continuous incubation of cells was carried out at a concentration of 0.5 μ*M* ara-C (Fig. 6). After a 3-h incubation with

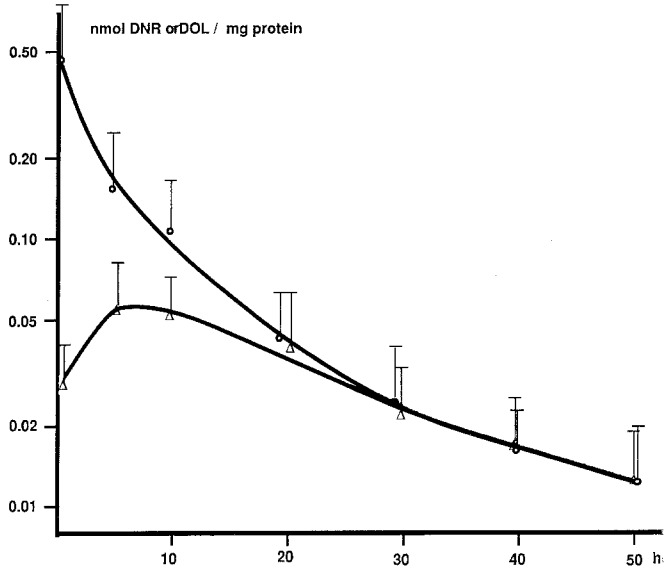


**Fig. 1.** Intracellular concentrations (± 1SD) of daunorubicin (○) and daunorubicinol (△) in leukemic cells from 7 patients with acute leukemia after the rapid infusion of 60 mg/m$^2$ daunorubicin

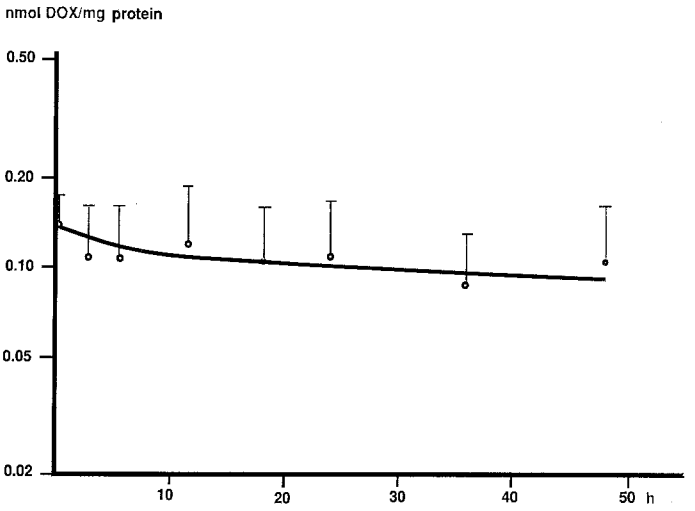


**Fig. 3.** Intracellular concentrations (± 1SD) of doxorubicin in leukemic cells from 5 patients with acute leukemia after the rapid infusion of 60 mg/m$^2$ doxorubicin

0.5 μ*M* ara-C and a subsequent change to fresh medium, there were no detectable ara-CTP concentrations after 22 h. Incubation with 5 μ*M* ara-C for 3 h resulted in a 10-fold higher peak concentration after 3 h than did the continuous infusion, but with rapidly decreasing ara-CTP concentration.

*Intracellular concentrations vs plasma concentrations in vivo.*

No significant correlation was found between the plasma pharmacokinetics and intracellular pharmacokinetics of the drugs. The correlation coefficient for plasma peak vs intracellular peak levels was 0.12 for daunorubicin and 0.55 for doxorubicin. The drugs were markedly concentrated intracellularly. Assuming that 1 mg cell protein corresponds to a volume of 5 μl, the intracellular peak concentration of daunorubicin vs plasma was around 250 times higher. After 10 h the mean intracellular concentration was 1,000 times higher, and after 20 h, 2,700 times higher than that of plasma. For doxorubicin, the corresponding increase was initially 20 times, which increased to 550 and 500 times after 10 and 20 h respectively.

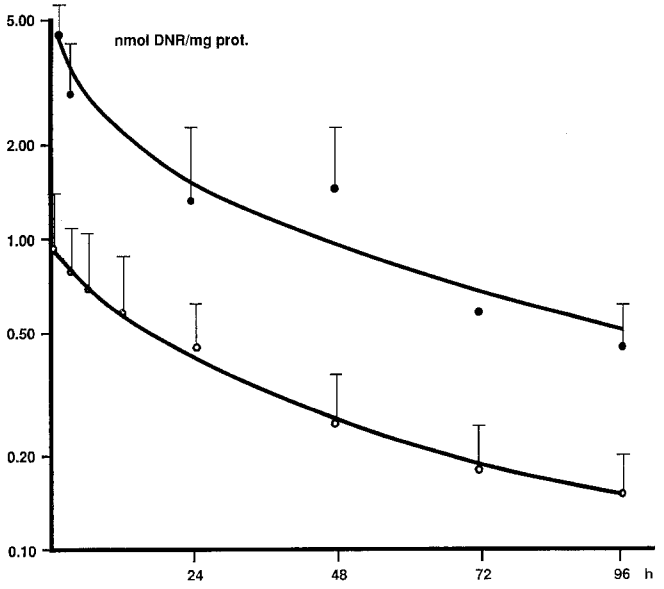


**Fig. 2.** Intracellular concentrations (± 1SD) in vitro in leukemic cells incubated with 0.2 (○) and 1.0 μM (●) daunorubicin for 1 h

*In vitro cytotoxicity.*

Cells from different patients responded very individually to the cytotoxic effect exerted by the drugs. Incubation with 0.2 μ*M* daunorubicin for 1 h gave a cytotoxic effect of 50.8% (± 19.8%) living cells after 4 days' cultivation. At the higher concentration of 1.0 μ*M* for 1 h, the cytotoxicity increased to 18.8% (± 12.1%) living cells. After incubation with doxorubicin, the cytotoxicity showed similar values: 0.2 μ*M* doxorubicin for 3 h gave 59.7% (± 25.3%) and 0.5 μ*M* for 3 h, 39.6% (± 24.3%) living cells. The mean cytotoxic effect after continuous incubation with ara-C was 45.7% (± 29.4%) living cells.

## Discussion

The development of reliable in vitro tests is important for screening new anticancer drugs and for optimizing the treatment of the individual patient. There are several reasons for the difficulties in correlating in vitro results to the clinical outcome of the treatment. Such factors include the clinical status of the patient, systemic drug metabolism and the use of multidrug treatment.

Another factor that may be important is the intracellular pharmacokinetics. The results of the in vivo studies showed substantial differences in the intracellular pharmacokinetics of the various drugs. The anthracyclines, daunorubicin and doxorubicin, were retained in the tumor cells much longer than was ara-CTP. In vitro, the active in-

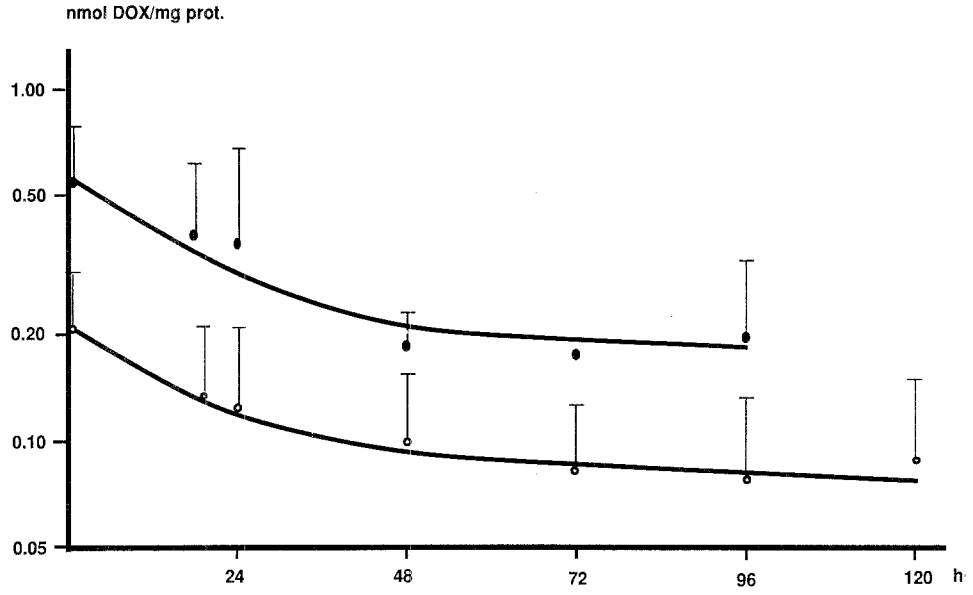


**Fig. 4.** Intracellular concentrations (± 1SD) in vitro in leukemic cells incubated with 0.2 (○) and 0.5 μM (●) doxorubicin for 3 h

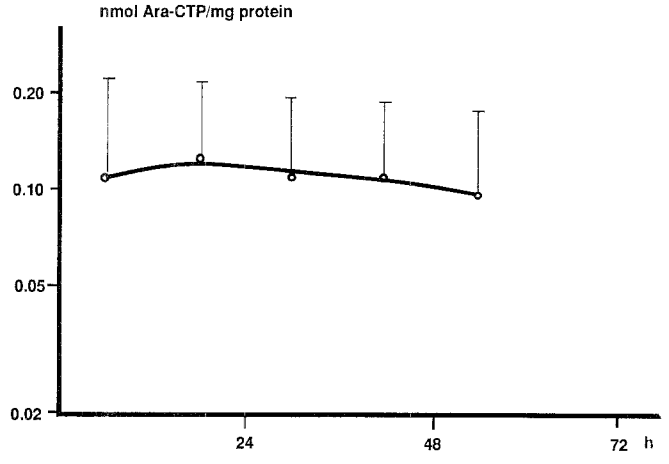


**Fig. 5.** Intracellular concentrations (±1 SD) of ara-CTP in leukemic cells from 17 patients with acute leukemia after the continuous infusion of 100 mg/m$^2$ ara-C

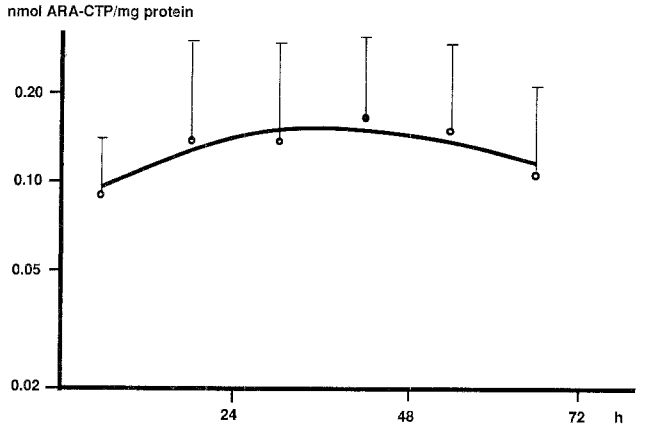


**Fig. 6.** Intracellular concentrations (±1 SD) in vitro in leukemic cells continuously incubated with 0.5 μ*M*

tracellular metabolite of ara-C was not detectable 24 h after the incubation had been terminated. This drug is S-phase-specific and, with a short incubation time, cells in other cell-cycle phases might not be affected [21]. To obtain a significant cytotoxic effect of ara-C in vitro, it thus appears important to extend the incubation, which is in accordance with clinical practice in which ara-C is given as continuous or repeated infusions.

There were also differences in the intracellular pharmacokinetics of daunorubicin and doxorubicin in vivo as well as in vitro. The intracellular steady-state level of doxorubicin was reached at a slower rate but was retained longer than that of daunorubicin. These results are in accordance with our previous findings [15]. The incubation times of 1 h for daunorubicin and 3 h for doxorubicin were chosen since they gave intracellular steady-state concentrations for the respective drugs [12]. The results showed that for doxorubicin, incubation with 0.2 μ*M* for 3 h closely mimicked the in vivo situation with regard to the intracellular drug concentration. For daunorubicin, even 0.2 μ*M* resulted in somewhat higher intracellular concentrations than were obtained in vivo. To a small extent this difference was compensated by the intracellular levels of daunorubicinol that were found in vivo, since the intracellular cytotoxicity of this metabolite is similar to that of the parent drug [1]. Furthermore, in some treatment schedules such as the TAD regimen [6], daunorubicin is given as repeated infusions, which may justify the somewhat higher in vitro concentrations.

The DiSC assay was carried out after all incubations to determine whether the concentrations found gave a plausible cytotoxic effect. The mean cytotoxicity was around 50%, which should enable the discrimination between sensitive and resistant cells in vitro as well as enabling the investigation of combinations of anticancer drugs; theoretically, the latter is the most realistic in vitro test for patients treated with a multidrug regimen.

Compared with what has often been used, our concentrations were lower and, for some drugs (doxorubicin and ara-C), the incubations were longer. This is partly in ac-

cordance with previous findings that longer incubation periods at lower drug concentrations are more cytotoxic to $CFU_{GM}$ than are brief incubations at high concentrations [2].

The purpose of this study was to expose malignant cells to intracellular drug concentrations similar to those used in the clinical situation, thus minimizing one factor that may influence the predictive value of in vitro tests. We found that there were differences between various anticancer drugs according to their cellular pharmacokinetics, which must be considered in predictive drug testing if we are to mimic in vivo conditions. The present study does not answer the question as to whether this adaption of the intracellular concentrations improves the accuracy of in vitro tests in predicting treatment results. However, a study is under way in which we are comparing the results of in vitro tests with the clinical outcome for patients with acute nonlymphocytic leukemia. Preliminary results indicate a good correlation.

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