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Kinetics and sensitivity of daunorubicin in patients with acute leukemia

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Summary. *Leukemia cells isolated from eight patients with acute leukemia before treatment were examined for in vitro uptake of daunorubicin (DNR) and inhibition of DNA synthesis. In addition, plasma and cellular levels of DNR and daunorubicinol (DOL) were examined in six of the eight patients. Inhibition of DNA synthesis was determined with a ^{3}H-thymidine incorporation assay. In vitro cellular ^{14}C-DNR was quantified by means of liquid scintillation spectrometry, whereas in vivo DNR and DOL concentrations were determined by high-performance liquid chromatography. In vitro intracellular plateau concentrations of DNR were achieved within 1–2 h after continuous exposure to 0.01, 0.1, and 1.0 ug/ml in the majority of cases. Based on our in vitro studies, a dose–response curve was found between increasing intracellular DNR and incorporation of ^{3}H-thymidine. Peak intracellular levels of DNR after treatment occurred immediately after administration of the drug, whereas intracellular DOL levels accumulated over several hours. Plasma concentrations of DNR and DOL were not useful in estimating target tissue concentrations or inhibition of ^{3}H-thymidine incorporation. Extrapolation of in vivo cellular DNR concentrations to the in vitro dose–response curve allows an estimate of DNR sensitivity.*

Introduction

Standard induction chemotherapy for patients with acute nonlymphocytic leukemia (ANLL) usually includes both daunorubicin (DNR) and cytosine arabinoside (Ara-C) [22]. When patients with normal liver function are given an IV bolus dose of DNR, plasma concentrations rapidly decline to either low or undetectable levels [1, 4, 10]. This rapid decline can be attributed to cellular uptake of the parent drug by many tissues and is associated with the appearance in the plasma of the major metabolite, daunorubicinol (DOL). In contrast to plasma DNR levels, DNR concentrations in leukemic cells decline more slowly, and some intracellular conversion of DNR to DOL occurs [1, 10]. It is likely that intracellular levels of DNR and its metabolites correlate with target tissue effect more reliably than plasma anthracyline levels and thus have greater clinical relevance [2, 6, 7].

Park et al. [8] and others [9, 11] have attempted to use in vitro cytotoxicity models to predict response in patients with acute leukemia. There are many methodologic and practical problems, however, with the assay systems [12, 15, 20]. In the present study, we have used leukemic cells from patients with acute leukemia to examine both in vitro and in vivo kinetics of DNR and inhibition of DNA synthesis. The purpose of this preliminary study is to determine whether a correlation exists between in vitro and in vivo kinetics of DNR and drug sensitivity in leukemic cells.

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Materials and methods

Radiolabeled daunorubicin hydrochloride (^{14}C-DNR) (NSC 82151) produced by the Stanford Research Institute (SRI), Palo Alto, Calif, USA, was obtained from the National Cancer Institute. The purity of ^{14}C-DNR was documented by SRI using radioautography and confirmed by high-performance liquid chromatography (HPLC) in our laboratory. The major metabolite, DOL, was a gift from Rhone-Poulence, Paris, France. Two other metabolites, 7-deoxydaunorubicinol and daunorubicinone, were gifts from SRI. The purity of these metabolites was confirmed by HPLC in our laboratory. All drugs were stored at −20° C in the dark. Test concentrations for in vitro studies were prepared in normal saline on the day of the experiment.

Isolation of human leukemic cells. Leukemic cells were obtained from eight patients with acute leukemia. In vitro uptake and inhibition of ^{3}H-thymidine incorporation were measured in bone marrow cells of two patients and in cells from peripheral blood (> 90% blasts) of the remaining six patients. All samples were depleted of erythroid cells by cold hypotonic lysis [5].

In vivo kinetic and inhibition of ^{3}H-thymidine incorporation studies of DNR were performed in cells isolated from peripheral blood samples. To summarize, EDTA was used to anticoagulate blood samples (5ml) at several time points and these were rapidly cooled to 4° C. Each sample was centrifuged at approximately 600 rpm for 5–10 min, and platelet-rich plasma was centrifuged at 3,400 rpm for 10 min to remove the remaining leukocytes and platelets. Buffy coats were resuspended in 1.5 ml PBS, and the cell number was determined with a Coulter counter. Samples (1 ml) of plasma and cells were then processed for HPLC.

In vitro uptake of DNR. Leukemic cells isolated from patients before treatment were exposed to DNR in suspension culture to determine the cellular uptake of DNR as previously

reported [5]. ^{14}C-DNR was used as a tracer to measure total intracellular DNR. The uptake of DNR by leukemia cells was determined at three drug concentrations, 0.01, 0.1, and 1.0 μg/ml. At the beginning of each experiment, the appropriate amount of drug was added to 5 ml cell suspensions, which were incubated at 37° C in 5% CO_2 in air. At several time intervals (5, 15, 30, 60, 120, or 240 min), cultures were rapidly cooled to 4° C and a measurement of the total drug associated with the cells was made. ^{14}C-DNR radioactivity was quantified in each sample by liquid scintillation spectrometry.

In vivo quantification of DNR and DOL. Quantification of DNR in leukemic cells and plasma samples from patients following treatment was performed by an HPLC method previously reported [5]. The HPLC system consisted of a DuPont model 850 HPLC equipped with a Lichrosorb RP-18 column (5 u*M*, 250 mm × 4.6 mm), a Gilson-GLO fluorometer, and a Spectra-Physics 4100 integrator. An isocratic system with a mobile phase of 50% acetonitrile, 35% water, and 15% 0.1 *M* phosphoric acid was used at a flow of 1.2 ml/min at room temperature. An internal standard (IS), adriamycin, was added to plasma and cell samples, which were extracted twice with a chloroform/methanol (4:1) mixture. The extraction yielded approximately 70% drug recovery for cells and 80% for plasma. The lower phase from each sample was dried and stored at −20° C in the dark until the time of analysis (usually 12−14 h). Samples were reconstituted with 100 μl chloroform, and aliquots (50−75 μl) were injected for analysis. Concentrations of DNR and DOL were quantitated with a standard curve of DNR/IS peak height ratios determined on the day of analysis for known DNR concentrations. Cellular and plasma concentrations of DNR (1−10 μg vs 0.020−2μg) were significantly different and required that individual standard curves be developed for cell and plasma samples.

Inhibition of DNA synthesis. Inhibition of DNA synthesis by DNR was examined with a modified ^{3}H-thymidine method previously reported [17]. To summarize, triplicate samples (0.5 to 1 × 10^6 cells/culture flask) were exposed to 0.01, 0.1, and 1.0 μg/ml, DNR for 1 h at 37° C in 5% CO_2 in air. At the end of the 1 h incubation period the cells were centrifuged and washed twice with PBS. Cells were resuspended in RPMI culture medium with 10% fetal calf serum and then incubated in 35 mm tissue culture dishes for 48 h. The cells were incubated for an additional 24 h with 5 μCi ^{3}H-thymidine (New England Nuclear, specific activity 2 mCi/mmol) and then cooled to 4° C for 10 min to prevent further incorporation. Cell samples were centrifuged and washed twice with cold PBS. The resulting cell pellets were completely dissolved in 1 ml 1*N* NaOH for 20 min at 37° C, and 1 ml 1*N* HCl and 2 ml cold 25% trichloroacetic acid were added to each tube. The samples were shaken vigorously for 1 min, allowed to stand at 4° C for 15 min and collected on filters. The filters were transferrerd to scintillation vials containing 10 ml Liquaflor and radioactivity determined with a Beckman LS−1215 scintillation counter. The percentage of incorporation of ^{3}H-thymidine was calculated by dividing the cpm in the drug-exposed cells by cpm in control cells and multiplying by 100. Incorporation of ^{3}H-thymidine was assessed on day 3 both for in vitro exposure to DNR and for samples obtained from patients following treatment.

Results

Table 1 shows the characteristics of eight patients with acute leukemia. Cells from eight patients were studied for in vitro uptake of DNR, seven (patients 1, 2, 3, 4, 6, 7, and 8) were examined for in vitro incorporation of ^{3}H-thymidine, four (patients 1, 2, 3, and 5) were studied for in vivo plasma kinetics, and six (patients 1, 2, 3, 5, 7, and 8) for cellular kinetics. Four patients (patients 1, 2, 7, and 8) were studied for in vivo incorporation of ^{3}H-thymidine. Graphic data for patient 8 are not included in Figs. 1−4 but the observed and predicted inhibition of ^{3}H-thymidine incorporation is shown in Table 2.

Figure 1 shows the in vitro uptake pattern in cells from seven patients. As previously reported [7, 14, 16−19] greater than 90% of intracellular DNR is reached within 2 h in the majority of cases. The pattern of drug uptake suggests that DNR is taken up by passive diffusion. The cellular concentration of DNR for the seven patients is quite variable even though the pattern of uptake is similar in all cases.

Figure 2 shows the relationship between intracellular concentration of DNR and incorporation of ^{3}H-thymidine. The slopes of the curves show that a wide range of variability exists between cells in terms of the effective cellular concentration of DNR needed to inhibit incorporation of ^{3}H-thymidine. The cells from the five newly diagnosed patients had a similar degree of inhibition at the highest exposure concentration and

Table 1. Patient characterstics

Patient	Sex/age	Type of leukemia[b]	WBC (× 10^3/μl)	% Blast	% Neutrophils	Stage of disease[c]	Previous DNR (mg/m^2)	Dose of DNR (mg/m^2)
1	F/29	AMMoL (M4)	50.0	62	18	N/D	0	50
2	F/67	AML (M1)	88.0	91	6	N/D	0	30
3	F/76	AMMoL (M4)	70.0	90	6	1st relapse	200	50
4	M/14	ALL (L1)	114.0	97	0	N/D	0	50
5	F/37	AML (M1)	20.9	86	0	N/D	0	50
6	F/55	AML (M2)	214.0	99	0	N/D	0	30
7	F/65	AML (M1)	140.0	95	0	N/D	0	30
8[a]	F/14	CML	108.0	48	32	Blast crisis	0	45

[a] Blood samples were processed with Ficoll Hypaque density centrifugation. Final percentages of blasts were greater than 75%
[b] Classification by the French-American-British (FAB) scheme
[c] N/D, newly diagnosed

were different from cells tested in the patient who was previously treated (patient 3).

Figure 3A shows the in vivo cellular kinetics of DNR and DOL in the patients following treatment. Similar to in vitro kinetics, the in vivo intracellular levels of DNR were quite variable among patient samples. The peak cellular DNR concentrations occurred immediately after the administration of drug in all patients. Intracellular DOL was also detected but was lower than the DNR concentration during the study period.

Figure 3B shows that the plasma concentrations of DNR rapidly declined to either very low or undetectable levels within 1 h of administration. Generally, DOL plasma concentrations exceeded DNR levels within 30 min of administration. Plasma concentrations of DNR and DOL could not be used to predict cellular levels of DNR or DOL.

By determining in vivo cellular DNR concentrations, we compared the in vivo kinetic studies with the in vitro uptake and inhibition of ^{3}H-thymidine incorporation studies. When in

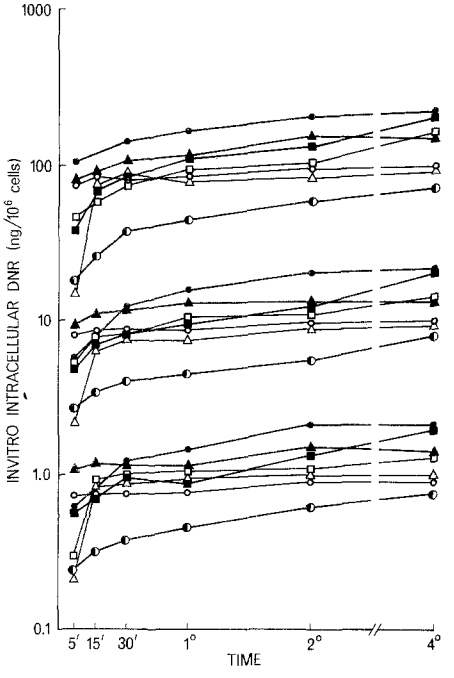

Fig. 1. Uptake of DNR by leukemic cells at three test concentrations, 0.01, 0.1 and 1.0 μg/ml. Patients 1–7 are represented by symbols ●——●, ○——○, ▲——▲, △——△, ■——■, □——□, and ◐——◐, respectively. Each *point* represents the mean of triplicate experiments

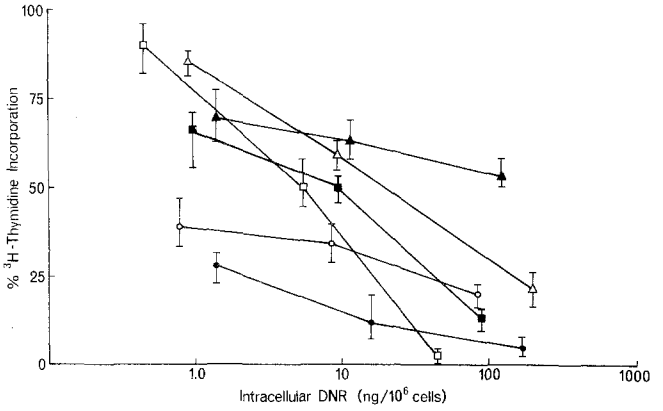

Fig. 2. In vitro incorporation of ^{3}H-thymidine after cells were exposed to DNR in patients 1, 2, 3, 4, 6, and 7 ●——●, ○——○, ▲——▲, △——△, ■——■, and □——□, respectively. Intracellular concentrations were based on the 1-h time point seen for uptake studies (Fig. 1). Each *point* represents the mean of triplicate experiments evaluated on day 3 after exposure to drug. Leukemic cells from patient 5 failed to survive for the 3-day period and were eliminated from this study

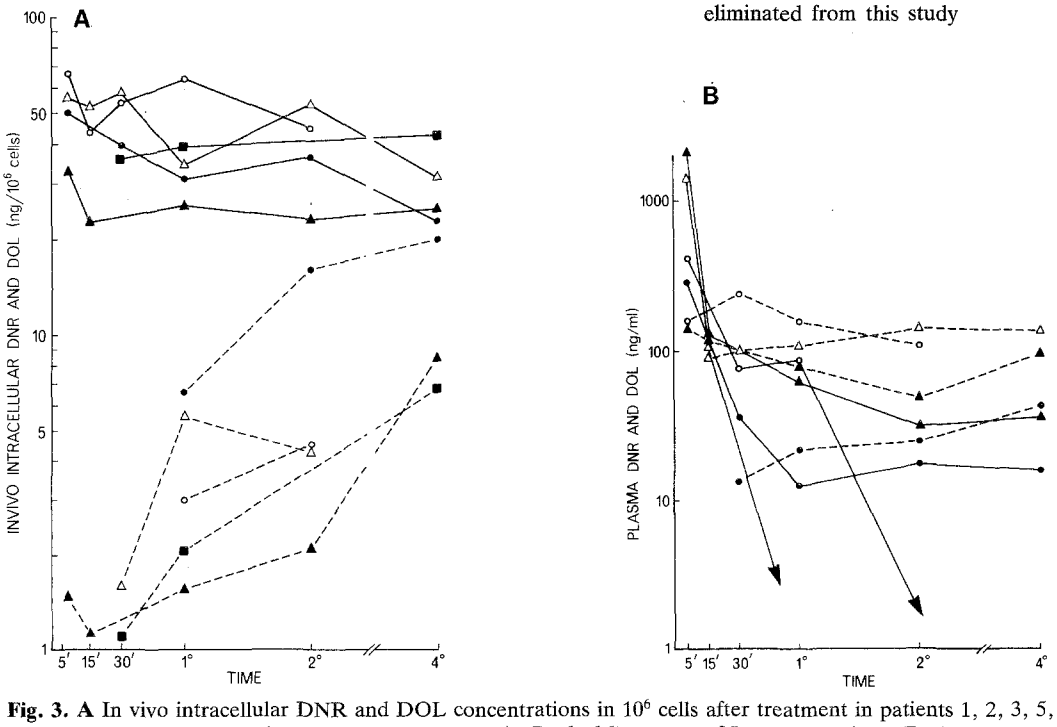

Fig. 3. **A** In vivo intracellular DNR and DOL concentrations in 10^6 cells after treatment in patients 1, 2, 3, 5, and 7 (●——●, ○——○, ▲——▲, △——△, and ■——■, respectively). *Dashed lines* are DOL concentrations; **B** plasma concentrations of DNR and DOL in patients 1, 2, 3, and 5 (●——●, ○——○, ▲——▲, and △——△, respectively). *Dashed lines* are DOL concentrations

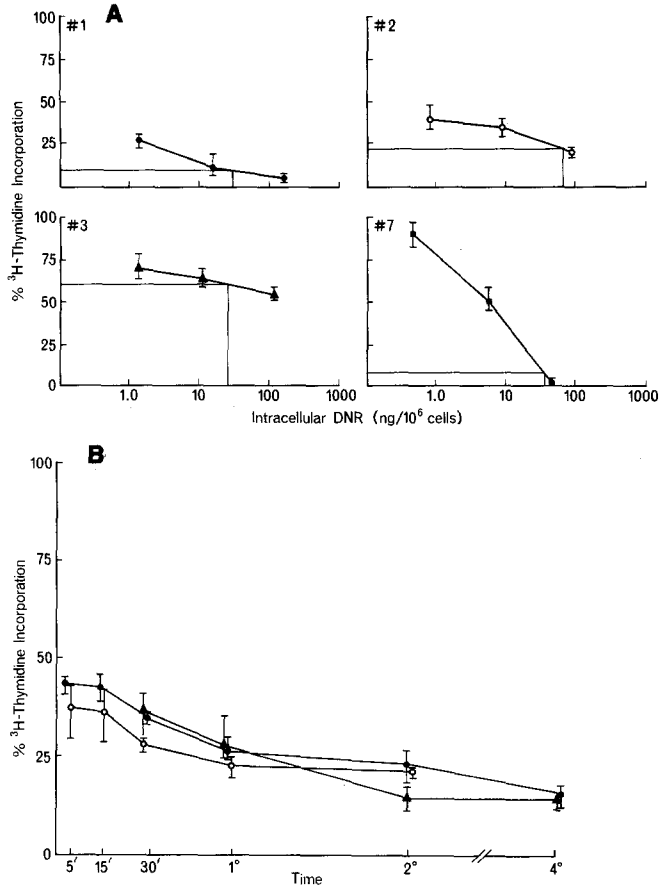

Fig. 4. A In vitro incorporation of ^{3}H-thymidine (Fig. 2), and predicted incorporation of ^{3}H-thymidine based on in vivo intracellular DNR concentrations in patients 1, 2, 3, and 7. Range is indicated by *bars:* **B** in vitro incorporation of ^{3}H-thymidine versus time after treatment in patients 1, 2, and 7 ●——●, ○——○, and ▲——▲, respectively. Each *point* represents the average of triplicate experiments (range indicated by *bars*)

Table 2. Predicted and observed response to daunorubicin

Patient	Predicted inhibition of ^{3}H-thymidine	Observed inhibition of ^{3}H-thymidine
1	10.0	22 (21–24)
2	22.0	20 (18–26)
7	7.5	15 (13–19)
8	13.0	24 (19–28)

vitro inhibition of ^{3}H-thymidine is plotted against in vitro cellular concentrations of DNR, a dose-response curve is obtained (Fig. 2). Extraplation of the in vivo cellular concentrations to the in vitro dose-response curve allows an estimate of ^{3}H-thymidine incorporation (Fig. 4A). The values for predicted inhibition of ^{3}H-thymidine for patients 1, 2, 3, and 7 were 10%, 22%, 60%, and 7.5%, respectively.

In samples from four patients (1, 2, 7, and 8) who did not receive concurrent steroids, Ara-C, or prior hydroxyurea, we had an opportunity to compare these estimates with actual values for incorporation of ^{3}H-thymidine studies after treatment with DNR (Fig. 4B). By taking the predicted percent ^{3}H-thymidine incorporation for these four patients, a comparison of the percent ^{3}H-thymidine incorporation after treatment to predicted values can be made. Table 2 shows predicted and observed inhibition of ^{3}H-thymidine for patients 1, 2, 7, and 8. According to these data, a close correlation exists between in vivo and predicted drug sensitivity.

Discussion

In the present study, we examined the in vitro kinetics of DNR and its effect on ^{3}H-thymidine incorporation in leukemic cells isolated from patients with acute leukemia. In addition, in vivo plasma and cellular kinetics and ^{3}H-thymidine incorporation were measured during treatment in several patients. Our results show that (1) variability exists in uptake of DNR even though these cells exhibit a similar kinetic pattern; (2) an in vitro dose-response curve was obtained for inhibition of ^{3}H-thymidine incorporation versus intracellular concentrations of DNR; (3) in vivo cellular levels of DNR and DOL after treatment are quite variable; (4) plasma levels of DNR and DOL do not predict intracellular concentrations of DNR or degree of DNA synthesis; and (5) in vitro and in vivo cellular levels of DNR may be useful in predicting drug sensitivity.

Many in vitro models used for assessing the cytotoxic effects of anticancer agents have been reported [8, 9, 11, 13, 20]. The validity of using in vitro cytotoxicity assays in patients with acute leukemia still remains to be determined [15]. One of the major drawbacks on in vitro cytotoxicity assays is the difference between in vitro and in vivo pharmacokinetic events for the drugs tested. The selection of an in vitro concentration of drug to be used in cytotoxicity assays has been largely based on achievable peak plasma levels [15], which in the case of DNR may be of little value. For example, following IV administration of DNR, there is a rapid fall in plasma levels of the parent drug, associated with substantial intracellular accumulation in most tissues including leukemic cells. The kinetic pattern is further complicated by the fact that the major metabolite, DOL, which is produced by the reduction of the 13-carbonyl to 13-carbinol, retains much of the antitumor activity of the parent drug [3]. Furthermore, intracellular levels of DOL are initially low compared with DNR, but accumulate and can exceed intracellular DNR levels. Leukemia cells and other tissues may acquire DOL by cellular uptake as well as by intracellular metabolism [3, 5, 14, 21]. Interestingly, several investigators have associated a more favorable clinical response to higher DNR reductase activity (which is responsible for intracellular metabolism) in the leukemic cells [6, 7]. The association between intracellular levels of DOL and therapeutic response needs to be examined more thoroughly.

In addition to this complicated pharmacokinetic picture, the mechanism of action of the anthracycline antibiotics is unknown [18, 19]. Classically, the cytotoxic effects of DNR have been thought to act by way of high-affinity DNA binding, with intercalation of the aglycone moiety between adjacent base pairs and noncovalent interaction of the amino sugar with the phosphate backbone of DNA. Recent studies examining support-bound adriamycin (ADR) complexes have shown that ADR can be actively cytotoxic to cells without crossing the plasma membrane, thus suggesting the membrane as a target for ADR cytotoxicity [18, 19]. Thus, the anthracycline antibiotics may have two primary sites of action, DNA and the plasma membrane. Since both sites of action may produce cell death, we elected to examine total cellular DNR for our studies.

Taking the complicated in vivo kinetic picture into account, and the fact that DNA is probably not the sole site of action, how does one select and in vitro exposure concentration or exposure time of DNR? In an attempt to reflect in vivo conditions, we examined the relationship between in vitro and in vivo pharmacokinetics and sensitivity of DNR.

Based on in vitro uptake studies of DNR, we can examine drug sensitivity caused by an intracellular DNR concentration. We found a concentration-dependent inhibition of DNA synthesis with intracellular DNR. Since our studies go beyond examining in vitro kinetics and sensitivity to include in vivo cellular kinetics, we can estimate drug efficacy using our model by extrapolating in vitro studies to in vivo studies. Therefore, in vivo cellular drug levels can be measured and related to in vitro kinetic and sensitivity studies of DNR.

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