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Uptake and Metabolism of Daunorubicin by Human Leukemia Cells

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Summary. *Radiolabeled daunorubicin was used to study in vitro uptake of daunorubicin (DNR) by the human promyelocytic leukemia cell line HL-60 and by leukemic cells from five previously untreated patients with acute nonlymphocytic leukemia (ANLL). Uptake of the metabolite daunorubicinol (DOL) and the metabolism of DNR were examined using high-performance liquid chromatography (HPLC). Uptake of DNR and DOL by HL-60 and ANLL cells exhibited a similar kinetic pattern. The uptake of DOL was 35%–50% of the uptake of DNR at the same test concentration in both HL-60 and ANLL cells. Approximately 5%–10% of intracellular DNR was metabolized to DOL by HL-60 and ANLL cells after 24 h of drug exposure. Measurements of DNR or DOL derived from liquid scintillation spectrometry and HPLC permit a sensitive and accurate assessment of the pharmacokinetics of these drugs in human leukemia cells. In addition, the HL-60 cell line can be used as a model for studying in vitro pharmacokinetics of the anthracyclines.*

Introduction

The anthracycline antibiotic daunorubicin (DNR) is commonly used in the treatment of patients with acute leukemia [20, 21]. When patients with normal liver function are given an IV bolus dose of DNR, plasma DNR rapidly declines to either low or undetectable levels [1, 13, 17]. 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) [1, 13, 17]. In contrast to DNR levels in plasma, DNR levels in leukemic cells decline more slowly [1, 17], although some intracellular conversion of DNR to DOL does occur [10, 13]. These observations have led to the speculation that intracellular levels of DNR and its metabolites may indicate target tissue effect more reliably than plasma anthracycline levels [1, 2, 10, 11, 17] and thus have greater clinical relevance.

The availability of experimental cell lines has made it possible to study the cellular pharmacodynamics of the anthracycline drugs. The characteristics of DNR uptake, metabolism, and cytotoxicity in leukemic cells in vitro have been examined by several groups of investigators [1, 2, 4, 14–19, 22]. Koeffler et al. have recently reported that the human acute leukemic cell line, KG-1, could be used as an in vitro model for developing and testing chemotherapeutic agents [14]. They compared KG-1 cells with normal human myeloid colony-forming cells to test their proposed model. The human acute promyelocytic leukemia cell line HL-60 has been widely used for in vitro studies of granulocyte differentiation [8, 9]. This cell line is easily maintained in suspension culture and has been well characterized. To explore whether the HL-60 cell line can be used as a pharmacologic model, we examined the uptake kinetics of DNR and the uptake of its major metabolite DOL by these cells, as well as the intracellular metabolism of DNR. For comparison we studied the in vitro uptake and metabolism of DNR and the uptake of DOL by cells isolated from five untreated patients with acute nonlymphocytic leukemia (ANLL). Results of these studies show that HL-60 can serve as a model system for studying the in vitro pharmacokinetics of DNR in human leukemia.

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Methods

Drugs. Radiolabeled daunorubicin hydrochloride (^{14}C-DNR) (NSC 82151) produced by the Stanford Research Institute (SRI), Palo Alto, CA, 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-Pulec, Paris, France. Two other metabolites, 7-deoxydaunorubicinol and daunorubinone, were gifts from the SRI. The purity of these metabolites was confirmed by HPLC in our laboratory. Drugs were stored at −20° C in the dark. Test concentrations were prepared in normal saline on the day of the experiment.

HL-60 Cells. The human acute promyelocytic cell line HL-60 was the generous gift of Dr Steven Collins of the National Cancer Institute and was maintained in continuous suspension culture at 37° C in an atmosphere of 5% CO_2 in air, as previously described [8]. Cells were passaged weekly using RPMI 1640 medium supplemented with 15% fetal calf serum (Sterile Systems, Inc., Logan, UT), 2m*M* L-glutamine, and gentamicin 50 μg/ml (RPMI-FCS). Drug uptake studies were performed on cells in the logarithmic growth phase at a density of 0.5–1.0 × 10^6 cells/ml.

Isolation of Human Acute Leukemia Cells. Leukemic blast cells were obtained from five patients with ANLL. Bone marrow samples obtained from four patients were depleted of erythroid cells by cold hypotonic lysis [5]. A peripheral blood

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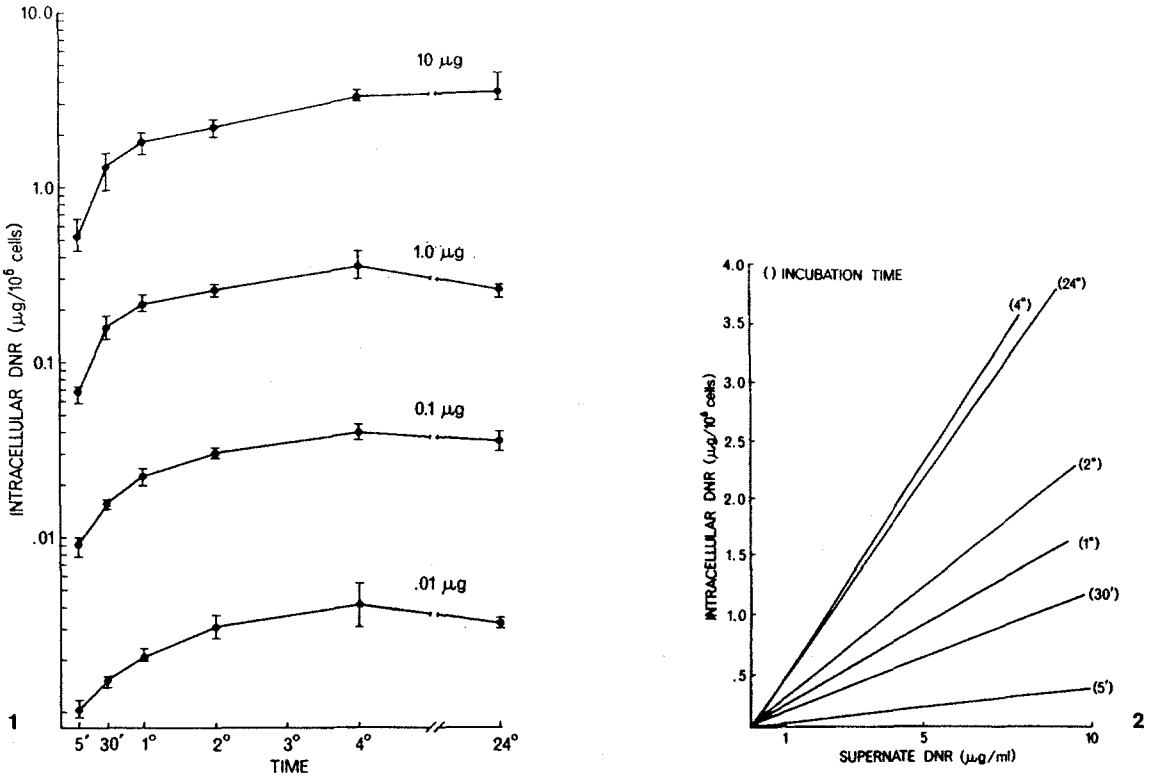

Fig. 1. Uptake of DNR by HL-60 at four test concentrations (0.01, 0.1, 1.0, 10.0 μg/ml). Each *point* represents the mean of triplicate experiments (range is indicated by *bars*)

Fig. 2. Relationship of intracellular versus supernate concentration for each time point. This relationship is derived by taking intracellular concentrations achieved for a given time of drug exposure and applying the linear regression equation. The correlation coefficient was > 0.986 for all lines

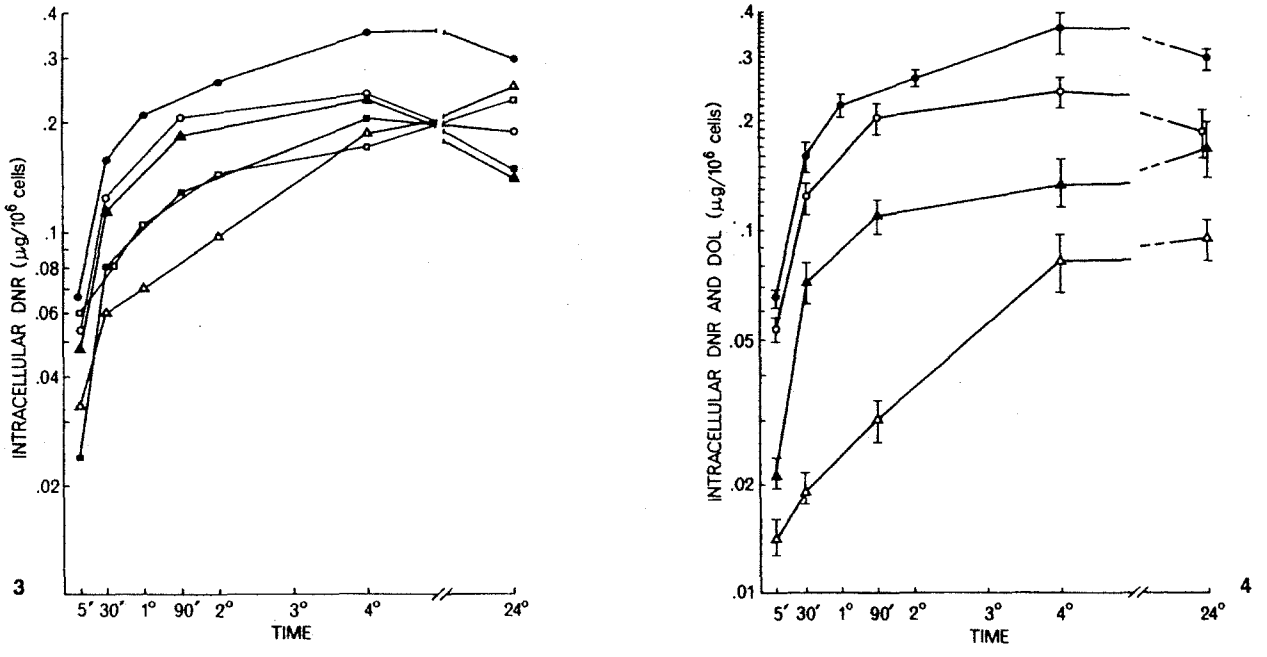

Fig. 3. Uptake of DNR by ANLL (○, ▲, △, ■, □) and HL-60 (●——●) cells. Each *points* represents the mean of triplicate or duplicate experiments

Fig. 4. Uptake of DNR and DOL by HL-60 and by ANLL cells of one patient at a test concentration of 1 μg/ml. DNR in HL-60 (●——●), DNR in ANLL cells (○——○), DOL in HL-60 (▲——▲), and DOL in ANLL cells (△——△). Each *point* represents the mean of triplicate experiments (range is indicated by *bars*)

sample anticoagulated with EDTA was obtained from one patient with leukocytosis of 40,000 cells/μl (> 95% blast cells). This peripheral blood sample was first centrifuged to remove platelet-rich plasma and the mononuclear leukocytes were then isolated by Ficoll-Hypaque density gradient centrifugation [7]. Leukocytes isolated from the marrow and peripheral blood samples were washed with phosphate-buffered saline and suspended in RPMI-FCS at $0.5-1.0 \times 10^6$ cells/ml for the in vitro studies. Viability of the isolated cells was ≥ 90% by erythrosin B dye exclusion.

Uptake of DNR and DOL by Leukemic Cells. HL-60 cells and blast cells from patients with ANLL were exposed to DNR or DOL during suspension culture to determine the cellular uptake of these compounds in vitro. ^{14}C-daunorubicin was used as a tracer to measure intracellular DNR. The uptake of DNR by HL-60 cells was determined at four drug exposure concentrations, 0.01, 0.1, 1.0, and 10 μg/ml. Daunorubicin uptake by ANLL cells and DOL uptake by both HL-60 and ANLL cells were examined at an exposure concentration of 1.0 μg/ml. At the start of each experiment the appropriate amount of drug was added to replicate 5-ml cell suspensions, which were then incubated at 37° C in 5% CO_2 in air. At several time points triplicate cultures were rapidly cooled to 4° C and measurements of the amount of drug in the supernate and in the washed cell pellets were made. ^{14}C-Daunorubicin radioactivity in each fraction was quantitated by liquid scintillation spectrometry. Daunorubicinol uptake was quantitated by HPLC. The appearance of DOL in cells exposed to DNR was also determined by HPLC analysis.

High-Performance Liquid Chromatography. Quantitation of DOL and identification of DNR metabolites were accomplished using a modification of previously reported techniques [3, 6, 12]. To summarize: A DuPont model 850 gradient pump equipped with a Lichrosorb RP-8, 5 μ*M*, 250 mm × 4.6 mm column, a Gilson-GLO fluorometer, and a Spectra-Physics 4100 integrator were used in the detection of the fluorescent compounds. An isocratic system with a mobile phase of 50% acetonitrile, 40% water, and 10% 0.1 *M* phosphoric acid was used at a flow of 1.5 ml/min.

To quantitate intracellular DOL, the washed cell pellets were resuspended in 1 ml RPMI-FCS and a known amount of DNR was added as an internal standard (IS). The suspension was then extracted twice with 4 ml chloroform methanol (4 : 1) and the combined organic phases were dried with a stream of nitrogen. The extraction efficiency was approximately 90% using this method. The samples were then stored at −20° C in the dark until the day of analysis. Dried samples were reconstituted with 100 μl chloroform, and 25-μl aliquots were then analyzed by HPLC. Concentrations of DOL were quantitated using a standard curve of DOL/IS area under the curve ratios previously determined for known DOL concentrations.

Intracellular DNR metabolites were identified by comparing their retention times with those of known metabolites. The percent metabolism was determined by dividing the area produced by the metabolite by the total area of parent plus metabolite.

Results

The in vitro uptake of DNR by HL-60 cells exposed to four different drug concentrations is shown in Fig. 1. At each concentration there was a rapid influx of drug during the first 1−2 h and a slower accumulation of drug thereafter. Based on a packed cell volume of 1 ml for 10^9 cells, the intracellular levels of DNR exceeded the supernate concentration by 450−550 times after 4 h of drug exposure. The levels achieved within the cells appeared to be dependent on the supernate concentration and time of exposure, as seen in Fig. 2.

Figure 3 shows the uptake of DNR by the cells isolated from patients with ANLL. Similarly to HL-60 cells, these leukemic blasts exhibited rapid uptake of drug within the first 1−2 h followed by a slower accumulation. A lower total intracellular concentration was seen in the ANLL cells than in HL-60 cells at the same DNR exposure dose.

The uptake of DOL at 1 μg/ml by HL-60 cells and by cells from three patients studied showed a similar kinetic pattern. Both HL-60 and ANLL cells exhibited an initial rapid uptake without any significant increase in concentration after 4 h of incubation with drug. An example of the uptake of DNR and DOL by HL-60 and by ANLL cells from one patient is shown in Fig. 4. As in the DNR uptake studies, HL-60 cells accumulated more drug than did the ANLL cells. Intracellular DOL levels achieved in both HL-60 and ANLL cells were 35%−50% of the intracellular DNR levels achieved at the same test concentration.

The intracellular metabolite DOL accounted for approximately 3%−5% and 5%−10% of intracellular anthracycline after 4 and 24 h of incubation in both HL-60 cells and ANLL blast cells.

Discussion

In the present study we examined the human promyelocytic cell line HL-60 and freshly isolated human leukemic cells to determine whether HL-60 cells could be used as a pharmacologic model. The kinetic patterns exhibited by HL-60 and by ANLL cells when exposed to DNR or DOL were similar, although HL-60 accumulated more drug. The pattern of intracellular accumulation of DNR by HL-60 cells is similar to that seen in other cells [2, 4, 14−16, 18, 19, 22]. This pattern is characterized by a rapid uptake within the first 1−2 h, followed by a slower accumulation thereafter. The intracellular level of DNR achieved by the HL-60 cells appears to be dependent on the supernate concentration and time of exposure (C × T). With C × T, targeted intracellular DNR levels can be achieved, and the relationship between intracellular drug and cytotoxicity [2] can then be accurately assessed.

An interesting observation is that in vitro intracellular levels of DNR exceeded the supernate concentrations by 450−550 times. A similar relationship between plasma and leukemic cell concentration exists in vivo [1, 13, 17]. Paul et al. reported that at the end of infusion, intracellular DNR levels exceeded plasma concentrations by a factor of 400 in two patients with acute leukemia [17]. Clearly, human leukemia cells exhibit a high affinity for DNR in both in vitro and in vivo environments. Whether in vitro ratios between leukemic cell and supernate concentrations can predict in vivo events remains to be determined.

In our studies, the uptake of DOL was between 35% and 50% of the uptake of DNR at the same test concentration, confirming the results of previous studies examining the cellular uptake of these two drugs [4, 22]. Bachur et al. have shown that more polar anthracyclines enter cells less readily [4] and propose that this may explain the differences in cellular uptake between DNR and DOL.

The intracellular metabolism of DNR to DOL we observed has been shown to occur in many tissues [10, 11, 15,

22]. In agreement with other investigators [2, 15], we found that less than 10% of intracellular DNR was converted to DOL. In a similar study, Yesair et al. incubated L-1210 leukemic cells with a lower test concentration than we used and found that approximately 14%–30% of DNR was metabolized to DOL after 2–4 h of incubation [22]. The difference between our results and theirs may be accounted for by the use of different cell lines and incubation concentrations, either of which may influence apparent reductase activity.

If intracellular levels of DNR and its metabolites are important clinically then it will be valuable to further explore the pharmacodynamics of anthracyclines. The use of our model and the methods described may enable us to determine concentrations of anthracyclines that, while cytotoxic to leukemic cells, will not produce unacceptable toxicities to other tissues.

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