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Uptake and metabolism of daunorubicin by human myelocytic cells

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Summary. Daunorubicin uptake and metabolism were studied in vitro with human myeloid leukemia cell lines (KG1, ML1); erythroleukemia cell line (K562); and myeloblasts from two untreated patients with acute myelogenous leukemia (AML). Uptake of daunorubicin by all the above was very similar, but metabolism of daunorubicin to daunorubicinol and the levels of reductase activity were extremely variable. We believe that this heterogeneity accurately reflects the in vivo situation in humans with acute leukemia. In vivo anthracyclines are subject to extensive metabolism, and the majority of patients do metabolize the drug to some extent; it is important, therefore, to use cell lines that reflect the in vivo metabolism. Conversely, rodent cell lines, which apparently lack one of the two major classes of daunorubicin reductase and do not appreciably metabolize daunorubicin, appear to be inadequate as models for studies designed to evaluate the enzymatic mechanisms of daunorubicin metabolism.

Introduction

Anthracycline antibiotics are selectively toxic against acute leukemia and certain solid tumors such as breast carcinomas and osteosarcomas [10]. In leukemic patients, daunorubicin is rapidly reduced to its metabolite daunorubicinol within 1 h of treatment [15]. This metabolic conversion is catalyzed by a class of enzymes referred to as aldoketoreductases [6–8], and we have shown in previous work that human and rabbit livers contain multiple daunorubicin reductases [1, 2]. In addition to the known pH 8.5 aldehyde reductase [13], we identified a new class of anthracycline antibiotic reductases not found in rat or mouse liver. This new reductase activity has an acidic optimum (approximately pH 6.0) and is clearly distinguishable from the pH 8.5 daunorubicin reductase by ion exchange, gel filtration chromatography, isoelectric focusing and use of inhibitors [1–3]. We have shown that the pH 8.5 daunorubicin reductase is inhibited by barbital but not pyrazole, clearly distinguishing it from pyrazole-sensitive, barbiturate-insensitive alcohol dehydrogenase and from the pH 6.0 daunorubicin reductase, which is insensitive to both barbital and pyrazole [3].

It is clear, therefore, that reduction of daunorubicin to daunorubicinol in human or rabbit liver is mediated by at least two classes of enzymes [1–3], the aldehyde (pH 8.5) daunorubicin reductase [13, 14, 27], and the ketone (pH 6.0) reductase [1–3]. This indicated to us a complexity that was not originally appreciated when all reductase activities were determined at pH 7.4, and this complexity in turn suggested that variations in either class of reductase could influence overall metabolism of daunorubicin and hence chemotherapeutic response. Since it is our desire to study anthracycline antibiotics to provide information that will be applicable to their use in the treatment of human leukemia, it became of utmost importance that we study parameters of drug action such as uptake, metabolism, retention, and efflux in an in vitro model that is as closely analogous as possible to the in vivo situation. In vivo for example, anthracyclines are subject to extensive metabolsim [5, 9, 25, 26, 28], whereas there is no significant metabolism in vitro using rodent cell lines such as Ehrlich ascites and P388 during short-term incubations [22, 24]. This probably reflects a lower degree of metabolism and the lack of pH 6.0 reductase in rodents and therefore makes rodent cell lines inadequate for the study of anthracycline metabolism. Yet, uptake and metabolism of anthracyclines have been studied extensively in rodent cell lines [11, 17, 18, 24], but few studies have used human cells or cell lines [12]. To explore whether the latter can be used as a pharmacologic model, we examined the daunorubicin uptake and reductase profiles in several human cell lines [16, 19, 20]. For comparison, we studied drug uptake and examined reductase profiles in myeloblasts isolated from two untreated patients with acute myelogenous leukemia (AML). It is our conclusion that some human cell lines can be used effectively to study the actions of anthracycline antibiotics in vitro.

Materials and methods

Chemicals. Daunorubicin was obtained from Ives Laboratories, Inc., New York, NY; NADPH was from Sigma Chemicals Co., St. Louis, Mo; [^{3}H]daunorubicin was purchased from New England Nuclear, Boston, Mass; and silica gel plates with fluorescent indicator were from Eastman Kodak Co., Rochester, NY.

Cell lines. The cell lines used were ML1, which is a myelocytic cell line; KG1, composed predominantly of myeloblasts and promyelocytes [21]; and the erythroleukemia K562 cell line, which is composed of undifferentiated blast

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cells [4]. They were maintained in continuous suspension culture in different media: ML1 in RPMI 1640; KG1 in the alpha modification of Eagle's medium (αMEM) containing 50 μg/ml garamycin; and K562 in Dulbecco's modification of Eagles medium (DMEM), each of which was supplemented with 10% fetal bovine serum. Cultures were maintained at 37°C in an atmosphere of 5% CO_2 in air, and cells were passaged twice weekly. Cells were used for studies while they were in the logarithmic growth phase.

Isolation of myeloblast. Myeloblasts were separated from bone marrow aspirate by diluting 1 vol bone marrow sample with 3 vol Hank's balanced salt solution (HBSS). Aliquots of 10 ml were each added to 5 ml sodium metrizoate, and components were separated by centrifugation at 1200 rpm for 20 min [23]. The monolayer was removed, washed three times with HBSS, and scored. More than 90% of the cells were blasts.

Enzyme preparation. All solutions for enzyme preparations were maintained at 0–4°C. Cells were sonicated four times (15 s each) in 0.002 *M* Tris-HCL, pH 7.4, using a Branson sonicator at a setting of 5 and a direct current of 4 amp. This type of homogenate served as the source of enzyme activity for all experiments described in these studies. Protein determinations were performed by the Bio-Rad assay, using bovine serum albumin as the reference protein (Bulletin no. 1051 from Bio-Rad Laboratories, Richmond, Calif).

Daunorubicin reductase activity. The reaction mixture, in a final volume of 0.5 ml, contained 0.5 m*M* NADPH, 0.65 m*M* daunorubicin, and buffers as indicated. The reductases were studied under optimal conditions and with linear kinetics. The reaction was initiated by addition of the enzyme and run at 37°C; background rates were determined in the absence of NADPH and subtracted from those in the presence of NADPH. Daunorubicin and daunorubicinol were separated with $CHCl_3$:CH_3OH:H_2O (80:20:3; vol/vol/vol) on silica gel plates (250 μm with fluorescent indicator) that had been activated at 120°C for 30 min before application of the samples [6, 8]. After chromatography, the fluorescent areas were detected with ultraviolet lights and scraped from the plates into test tubes, after which the fluorescent materials was eluted from the silica gel with 2 ml 6.54 N H_2SO_4 in 95% ethanol. The concentrations of daunorubicin and its metabolite were determained with a Perkin-Elmer luminescence spectrometer (excitation wave length of 470 nm and emission spectrum at 585 nm) and a daunorubicin standard curve.

Determination of pH profile. The pH profile of daunorubicin reductase was determined in different buffers at concentrations giving conductivity readings of 8500–9500 mΩ. Buffers used were citrate phosphate (pH 4.5–6.0), potassium phosphate (pH 6.0–7.5), Tris-HCl (pH 7.5–8.8). Otherwise, assay conditions were identical to those described above under daunorubicin reductase activity.

Isoelectric focusing. Isoelectric focusing was performed as previously described [1, 2] using an LKB 8101 electrofocusing column (100 ml capacity with 1% carrier ampholyte (pH range, 3.5–10) and a stabilizing sucrose gradient containing 0.5 m*M* dithiothreitol. Samples were introduced into the dense solution of the sucrose gradient of the column, and electrofocusing lasted about 24 h at 4°C with an intial power of 5.0 W. After electrofocusing was completed, the contents of the column were collected in 2-ml fractions at a flow rate of 60 ml/h; pH measurements and reductase assays were performed immediately.

Cellular uptake of [^{3}H]daunorubicin. Cells were washed three times with cold phosphate-buffered saline and resuspended in their respective media at a concentrations of 2×10^7 cells/ml. Cells (3×10^6) in 150 μl medium without serum were preincubated for 5 min at 37°C in a shaking water bath. [^{3}H]Daunorubicin (20μl) was added at two concentrations, 11.15 μ*M* and 111.5 μ*M* (0.3 μCi). At appropriate time points, duplicate aliquote (0.1 ml) were removed, transferred to ice-cold tubes containing 300 μl silicone oil + 300 μl phosphate-buffered saline as the top layer, and centrifuged immediately for 30 s in an Eppendorf model 3100 centrifuge. The upper layer was removed and rinsed three times with water, and the oil was carefully aspirated. The cell pellet was dissolved in 100 μl tissue solubilizer and neutralized with acetic acid, and the radioactivity was then determined. For determination of 'zero time' values, drug and cells were added simultaneously and processed as above. Drug uptake studies were performed on cells in the logarithmic growth phase at a density of 6×10^5 cells/ml.

Subcellular distribution of [^{3}H]daunorubicin. Cells were suspended in the appropriate medium and preincubated for 10 min at 37°C in a shaking water bath. The cells were then incubated in the presence of [^{3}H]daunorubicin, 0.1 μg/ml, for 1 h. After incubation, 2.0×10^7 cells were removed, washed three times with 0.9% NaCl, and suspended in 7 ml 2 m*M* $CaCl_2$ for 5 min at 37°C. The swollen cells were homogenized for 5 min, and then immediately the osmolarity was adjusted to normal by the addition of 1 ml 2 *M* sucrose. The nuclei were collected by centrifugation (600 *g* for 15 min) and washed twice with 8 ml 0.25 *M* sucrose – 2 m*M* $CaCl_2$. The supernatant and washings were combined and centrifuged at 2000 *g* for 10 min to obtain the mitochondrial fraction and then at 150000 *g* for 60 min to obtain the microsomes and plasma membranes. Radioactivity associated with these fractions and the cytoplasmic supernatant was determined relative to the radioactivity present in whole cells.

Results

Enzyme preparations from cells of the three cell lines and from myeloblasts of two AML patients were used to examine the pH activity profile of daunorubicin reductase. The KG1 cell line showed mainly the aldehyde (pH 8.5) reductase activity, and K562 showed a very low level of metabolism, suggesting low reductase activity (Fig. 1). The same profiles were obtained when the three cell lines were grown in the same medium, suggesting that these variations are not due to different culture media. The myeloblasts isolated from one patient (MNM) had substantial activity at pH 8.5 and essentially no activity at pH 6.0, while those from the other patient (KLM) had activities near, but shifted slightly away from, both these pH optima

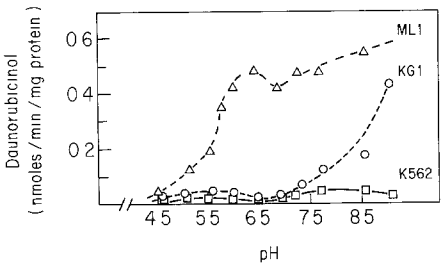

Fig. 1. Activity pH profile of daunorubicin reductase from different myelocytic cell lines: MLl (Δ – Δ), KGl (O – O), und K562 (□ – □). Buffers used were citrate phosphate (pH 4.5, 5.0, 5.5, and 6.0), potassium phosphate (pH 6.0, 6.6, 7.0, and 7.5), and Tris-HCl (pH 7.5, 8.0, 8.5, and 8.8). Assays were run immediately after enzyme preparation, as described in *Materials and methods*

(Fig. 2). Myeloblasts from three other patients were examined and the pH profiles were different from one another, suggesting heterogeneity in the reductase profiles of patient leukemic cells (data not shown).

The above reductase activity profiles were confirmed by isoelectric focusing (IEF). As shown in Fig. 3, only one peak of pH 8.5 reductase activity was detected in KGl cells by IEF; no pH 6.0 reductase activity was observed. IEF of the MLl cell homogenate, as expected, showed both pH 6.0 and 8.5 activities, and additional minor peaks of activity were also observed, suggesting that the reductases in leukemic cells exist in multiple forms (Fig. 3B). Similarly, IEF of the myeloblast homogenate of patient MNM showed only one peak of reductase activity at pH 8.5, while that of patient KLM showed peaks of activity at both pH 6.0 and pH 8.5 (Fig. 4).

The in vitro uptake of daunorubicin by KGl, MLl, K562, and patients cell at two different drug concentrations (11.15 μ*M* and 111.5 μ*M*) is shown in Fig. 5. At each concentration there was rapid influx of drug during the first 5 min and a slower accumulation of drug to the steady-state level by 30 min. Intracellular levels of daunorubicin were 20–40 times higher than the outside concentration after 30 min of exposure to the drug. Figure 6 shows that the intracellular level of daunorubicin in MLl cells appeared to be dependent on the outside concentration of the drug and time of exposure; a similar concentration-dependent profile was seen in KGl cells (data not shown). Table 1 shows the subcellular distribution of daunorubicin in KGl, MLl, and K562 cells at the steady-state level. Eighty-four percent of the radioactivity measured was associated with daunorubicin and daunorubicinol. About two-thirds of the radioactivity was associated with nuclei, 13%–21% was found in the cytoplasmic supernatant, and a small portion was associated with the mitochondrial and microsomal fractions. All three cell lines showed a similar distribution of radioactivity in the subcellular fractions.

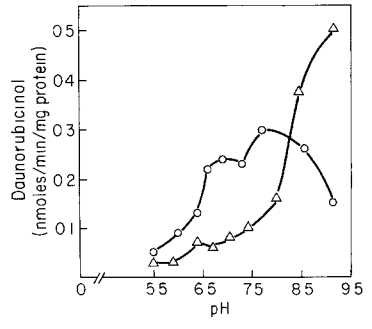

Fig. 2. Activity pH profile of daunorubicin reductase from myeloblasts of two patients, KLM (O – O) and MNM (Δ – Δ), with AML. Buffers used were the same as in Fig. 1

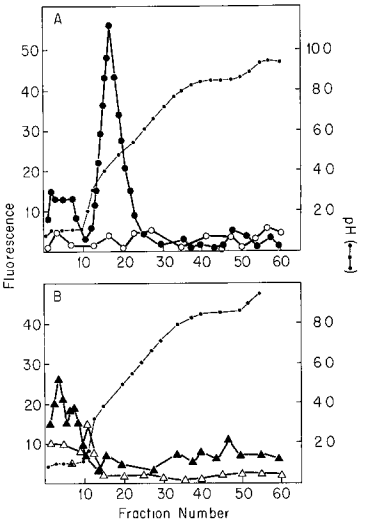

Fig. 3A, B. Isoelectric focusing of cell homogenates over a pH range of 3.5–10. **A** KGl cell homogenate. Daunorubicin reductase activity at pH 6.0 (O – O) and activity at pH 8.5 (● – ●); **B** MLl cell homogenate. Daunorubicin reductase activity at pH 6.0 (Δ – Δ) and activity at pH 8.5 (▲ – ▲)

Discussion

To realize the full potential of daunorubicin as a chemotherapeutic agent against leukemia it is essential to identify and understand the enzymatic mechanisms of its metabolism. Variations in or the lack of either class of daunorubicin reductase in leukemic cells could influence overall metabolism of the drug, and hence its therapeutic efficacy. Indeed, in this study we have shown that while uptake of daunorubicin by cells of different human myeloid cell lines and patients' myeloblasts is very similar, reductase levels and metabolism of daunorubicin are extremely variable. In additon, the IC_{50}'s (72-h growth inhibition assay) of daunorubicin in the three cell lines, MLl, KGl, and

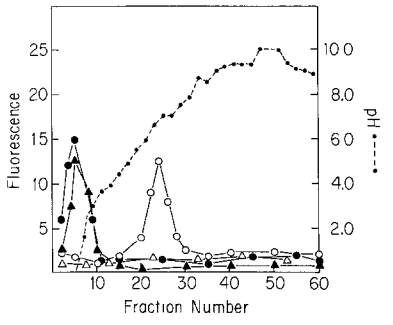

Fig. 4. Isoelectric focusing of cell homogenates from patient myeloblasts over a pH range of 3.5–10. Daunorubicin reductase activity of KLM at pH 6.0 (O – O) and activity at pH 8.5 (● – ●). Daunorubicin reductase activity of MNM at pH 6.0 (Δ – Δ) and activity at pH 8.5 (▲ – ▲)

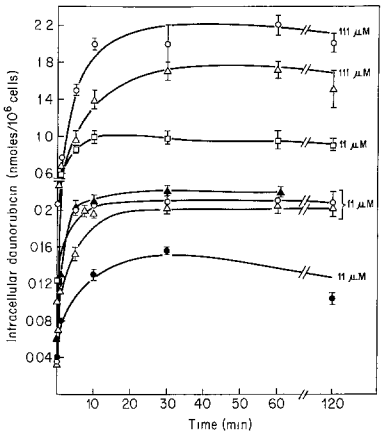

Fig. 5. Uptake of daunorubicin by cells of KGl (○–○), MLl (△–△–△), and K562 (□–□–□), and myeloblasts of patients MNM (▲–▲) and KLM (●–●) at concentrations of 11.15 μ*M*. Each *point* represents the mean of duplicate samples from at least three independent experiments; SEMs (indicated by *bars)* did not exceed 10%

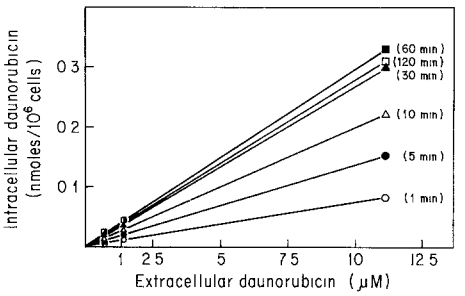

Fig. 6. Relationship of intracellular to extracellular daunorubicin concentration in MLl cells with time over an extracellular concentration range of 0.11–11.1 μ*M*. (○–○, ●–●, △–△, ▲–▲, ■–■, □–□ represent curves at 1, 5, 10, 30, 60, and 120 min respectively)

Table 1. Subcellular distribution of [^{3}H]daunorubicin equivalent in KGl, MLl and K562 cells after exposure to drug (0.1 μg/ml) for 1 h in vitro (uptake)

Cell Line	Radio activity (dpm × 10^3)				
	Whole cells	Nuclear fraction	Mitochondrial fraction	Microsomal fraction	Cytoplasmic supernatant
KG I	1065±5[a]	667±10 (67)[b]	22±2 (2)	18±1 (2)	210± 3 (21)
ML I	1130±3	806±18 (71)	53±4 (5)	32±3 (3)	184± 5 (16)
K562	1186±2	984± 9 (83)	41±1 (3)	24±3 (2)	160±10 (13)

[a] DPMs represent daunorubicin and metabolites (mean ± SD)
[b] Figures in parentheses give percentages of total

K562, were 10±0.5, 13±1.0, and 17.0±1.0 n*M*, respectively (unpublished work). This suggests that K562 cells may be more resistant to daunorubicin because of the lack of reductases. It is also possible that the efficacy of daunorubicin as an antileukemia drug will vary from patient to patient depending on the reductase levels in their leukemic blasts.

In our future studies, it will be important to use a variety of human cell lines that adequately reflect the heterogeneity of blast cells from one patients to another with acute leukemia. It will then be possible to study uptake, metabolism, retention and efflux of both daunorubicin and daunorubicinol in vitro with models that will provide information that is applicable to the in vivo situation in humans. A cell line such as K562, which shows almost no daunorubicin reductase activity, is inadequate as a model for the study of daunorubicin metabolism, but it could be used for comparison with cell lines exhibiting some degree of metabolism.

We have examined some mouse leukemia cells (P388 and L1210) and found them to have very low levels of metabolism. P388 was very similar to K562 and L1210 had no pH 6.0 reductase activity and only 16%–25% of the pH 8.5 activity observed with KGl and MLl, respectively (unpublished research). Accordingly, rodent cell lines, which do not appreciably metabolize daunorubicin and apparently lack the pH 6.0 ketone reductase, are simply not enough analogous, to human cells to make them appropriate models.

In our current studies, we are interested in the apparent relationship between the state of maturity of the cell lines we have examined and the level of daunorubicin reductase activity. Although we have only looked at three cell lines, the most highly differentiated, MLl, had the highest reductase activity and both the pH 6.0 and pH 8.5 reductases; the next, KGl, had an intermediate level of reductase activity and only the pH 8.5 reductase; and the least differentiated, K562, had almost no reductase activity. Whether inducing K562 to differentiate will induce an increase in daunorubicin reductase activity remains to be tested. In addition, we are developing drug-resistant cell lines from K562, KGl, and MLl to compare daunorubicin uptake, metabolism, retention, and efflux in sensitive and resistant cells. We are particularly interested in looking for any changes in the reductase isozymic patterns that may accompany the acquisition of resistance to daunorubicin.

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