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COMPARATIVE UPTAKE, RETENTION AND CYTOTOXICITY OF DAUNORUBICIN BY HUMAN MYELOID CELLS*

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Abstract—We studied the cellular uptake and retention of daunorubicin (D_1) in two human leukemic cell lines (ML1 and K562) and myeloblasts from an untreated patient with acute myelogenous leukemia (AML). The rate of uptake and the steady-state level of D_1 were not temperature dependent but increased markedly with increases in the pH of the medium. Also, saturation kinetics were not demonstrable using concentrations of D_1 up to 111 μM. Together, these observations suggest a transport mechanism for D_1 compatible with passive diffusion. Accumulation of D_1 was increased only in cells from the AML patient with addition of sodium azide, whereas drug efflux was not increased significantly in the presence of glucose in MLI or K562 cells. Although the rate of uptake and steady-state levels of D_1 were the same in these cells, metabolism and cytotoxicity of D_1 differed. Our results indicate that ML1 cells can be used as a pharmacologic model for studying the metabolism and resistance of D_1 *in vivo*.

The anthracycline antibiotics are effective antineoplastic agents that show selective toxicity against acute leukemias and certain solid tumors such as breast carcinoma and osteosarcoma [1]. A major limitation to effective use of anthracyclines is the acquisition of resistance by neoplastic cells. In rodent cell systems, several studies have confirmed that the development of daunorubicin (D_1‡) resistance [2–4] is accompanied by a reduced cellular drug uptake [5–7], decreased nuclear binding [8–10] and energy-dependent efflux [11–15]. We have shown previously that, in contrast to rodent cell lines, human cell lines exhibit a high degree of metabolism because of their pH 6.0 D_1 reductase activity [16, 17].

Since chemotherapeutic responses to anthracyclines depends on a number of factors such as uptake, retention and metabolism, and because previous studies have been conducted using rodent cell lines, our purpose has been to clarify the studies of human cells for their use as a more predictive model system of *in vivo* situations. To confirm this impression, we compared the human cell lines to myeloblasts from untreated patients with acute myelogenous leukemia (AML).

MATERIALS AND METHODS

Chemicals. D_1 was obtained from Ives Laboratories, Inc., New York, NY; NADPH, sodium azide and glucose were from the Sigma Chemical Co., St. Louis, MO; [^{3}H]D_1 was purchased from New England Nuclear, Boston, MA (sp. act. 2.2 Ci/mmole); silica gel plates with fluorescent indicator were from the Eastman Kodak Co., Rochester, NY; and C_{18} reverse-phase column was purchased from Waters Associates, Milford, MA.

Cell lines. Cells were maintained in continuous suspension culture: ML1 in RPMI 1640 and K562 in Dulbecco's MEM, each of which was supplemented with 10% fetal bovine serum. Cells were used for studies while they were in the logarithmic growth phase.

Isolation of myeloblasts. Myeloblasts were separated from bone marrow aspirate by diluting 1 vol. of bone marrow sample with 3 vol. of Hanks' Balanced Salt Solution (HBSS). Aliquots (10 ml) were added to 5 ml sodium metrizoate, and components were separated by centrifugation at 1200 rpm for 10 min [16]. The monolayer was removed, washed three times with HBSS, and counted. More than 90% of the cells were myeloblasts. (Experiments using patient myeloblasts were done once using duplicate samples.)

Determination of drug uptake. The conditions for *in vitro* drug uptake studies have been standardized using a cell concentration of 3×10^7 cells/ml in the presence of 0.11 to 111 μM labeled D_1 (1 μCi/ml) as previously published [16]. Generally, cells were suspended in either medium (RPMI 1640 or DMEM), or Gey's balanced salt solution (GBSS) and were incubated with shaking for 30 min at 37°. At specific times, duplicate aliquots (0.1 ml) were removed, transferred to ice-cold tubes containing 300 μl of silicone oil + 400 μl of phosphate-buffered saline (PBS) as the top layer, and centrifuged immediately for 30 sec in an Eppendorf model 3200 centrifuge. The upper layer was removed, tubes were

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‡ Abbreviations: D_1, daunorubicin; D_2, daunorubicinol; GBSS, Gey's Balanced Salt Solution; ML1, myelocytic cell line; K562, erythroleukemia cell line; PBS, phosphate-buffered saline; HBSS, Hanks' Balanced Salt Solution; DMEM, Dulbecco's Minimal Essential Medium; and HPLC, High Pressure Liquid Chromatography.

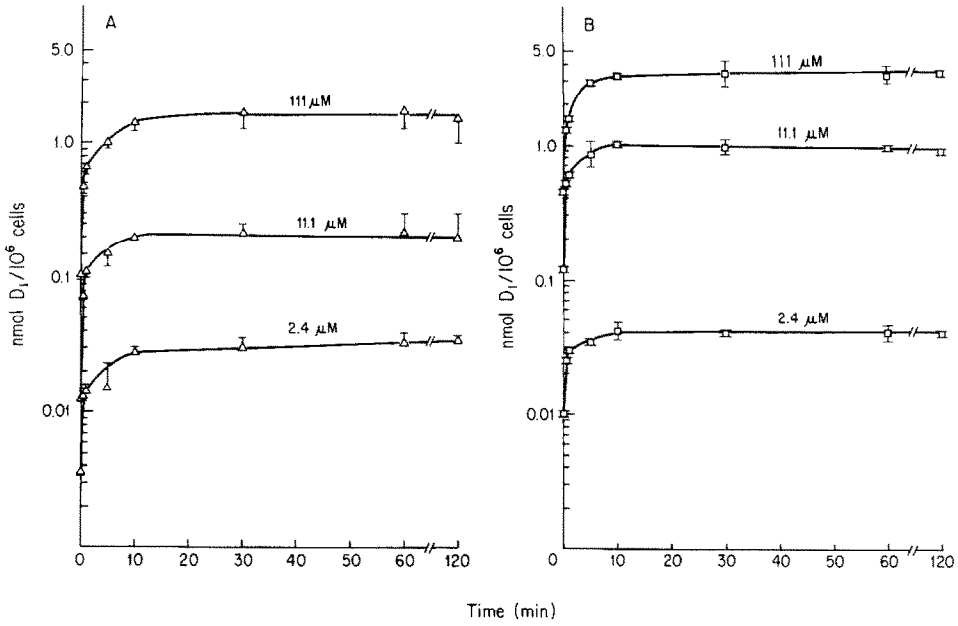

Fig. 1. Uptake of D_1 by (A) ML1 (△--△) and (B) K562 (□--□) at three concentrations, 2.4, 11.1, and 111 μM. Each point represents the mean of two different experiments ± S.E.

rinsed three times with PBS, and the oil was carefully aspirated. The cell pellet was suspended overnight in 100 μl of tissue solubilizer and, then, neutralized with 34 μl of glacial acetic acid and centrifuged; the radioactivity was determined (the radioactivity measured represents parent drug and metabolites). To determine "zero time" values, drug and cells were added simultaneously and processed as above. Results are presented as the mean of the individual observations of two to four serial experiments. Intracellular water was the difference between the wet and dry weights of the cell pellet less the extracellular volume as determined from [^{14}C]inulin.

Determination of drug efflux. To determine D_1 efflux, cells were incubated for 30–40 min at 37° with 2.4 μM [^{3}H]drug. Cells were centrifuged at 4° (100 *g*) for 10 min and resuspended to the original cell density in cold GBSS either with or without glucose at 4°, and 100 μl was transferred to an Eppendorf tube, containing silicone oil, and processed as above.

Metabolism. Cells (3×10^7/ml) were incubated for 0.5 to 6 hr with 2.4 μM D_1 and processed as above

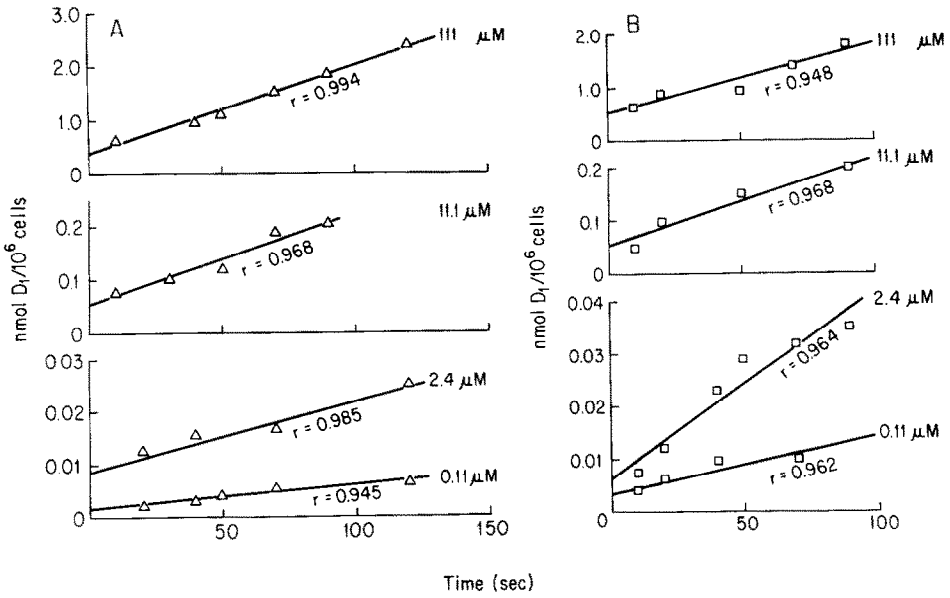

Fig. 2. Initial uptake of D_1 between 5 and 120 sec for four D_1 concentrations, 0.11, 2.4, 11.1 and 111 μM, using cells of ML1 (A) and K562 (B).

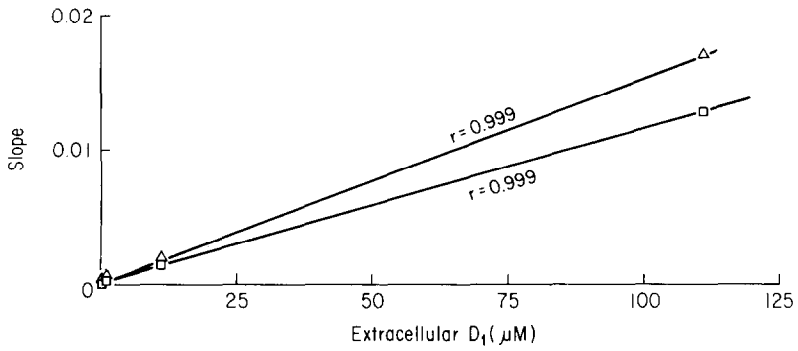

Fig. 3. Initial rate of uptake (slope) of D_1 as a function of the extracellular concentration of D_1 (0.11, 2.4, 11.1 and 111 μM). Key: (△--△) ML1 cells and (□--□) K562 cells.

(determination of drug uptake) except that the cell pellet was washed twice, and D_1 and metabolites were extracted from cells by chloroform–isoamyl alcohol (24:1, v/v). After centrifugation, the organic phase was evaporated to dryness in a water-bath at 40° under an air system. During evaporation the sides of the tube were washed with cloroform to increase recovery (≈96%). The residue was dissolved in methanol, and 50–100 μl samples were used for chromatography. D_1 and metabolites were determined by the HPLC-electrochemical method [18]. An analytical column, a 30 cm × 3.9 mm μBonapak C_{18} 10 μM (Waters Associates, Milford, MA), was used. The mobile phase consisted of acetonitrile–water–acetic acid (35:64:1, by vol.), adjusted to pH 4.0 with 20% sodium acetate.

Daunorubicin reductase activity. The enzyme was prepared from ML1 and K562 cells, and enzyme activity measurements were performed as described previously [16, 17]. The separation and quantitation of D_1 and daunorubicinol (D_2) were performed by thin-layer chromatography and fluorescence measurement [16, 17].

Statistical analysis. A two-tailed *t*-test was performed using the individual observations; a P value of less than 0.05 was considered to be statistically significant.

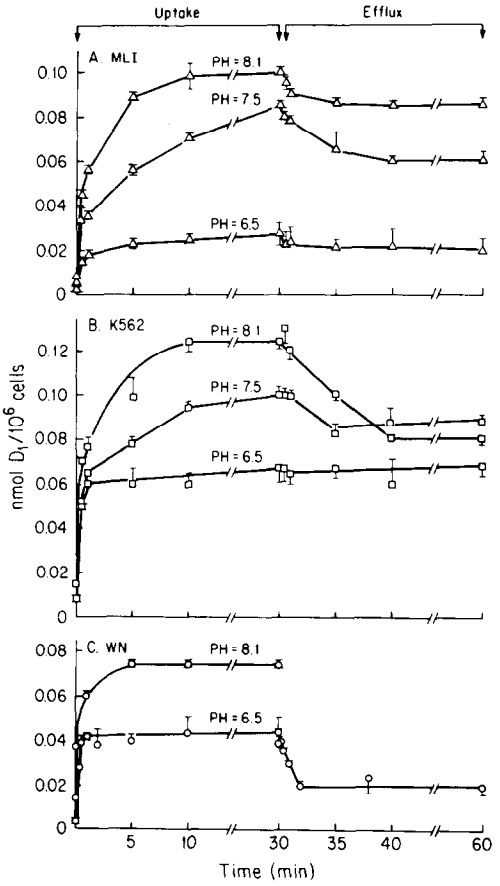

Fig. 4. Effect of the pH of the medium on time course of uptake and efflux of D_1. Cells were preincubated in standard medium with pH adjusted to specified values and then 2.4 μM [^{3}H]D_1 was added. Key: ML1 (△--△) (A), K562 (□--□) (B), and patient myeloblast (○--○) (C). Each point represents the mean of duplicate samples from at least one to three independent experiments.

RESULTS

Uptake of D_1 at various concentrations by human cell lines. When the ML1 and K562 cells were exposed to different concentrations of D_1 (2.4 to 111 μM), there was a rapid influx of drug during the first 5 min, followed by a slower accumulation to a steady-state level by 10 min (Fig. 1, A and B). The intracellular concentration of D_1 was dependent on the drug concentration in the medium and the duration of exposure (Fig. 1, A and B). At steady state, the intracellular levels of D_1 exceeded the concentration in the medium by 10- to 25-fold for ML1 and by 10- to 28-fold for K562, depending on the extracellular concentration. Figure 2 shows that the initial uptake of D_1 was linear in both MLI and K562 between 5 and 120 sec for the D_1 concentrations used. Straight lines with a correlation coefficient between 0.94 and 0.99 were obtained. When the rate (slope) was plotted against the extracellular concentration of D_1 (Fig. 3), linear kinetics were observed with no saturation at a D_1 concentration up to 111 μM in both ML1 and K562 cells.

Effect of pH on uptake and efflux of D_1. An important parameter in the transport of D_1 across the cell membrane is the extent of ionization. Usually, ionized molecules penetrate the membrane poorly

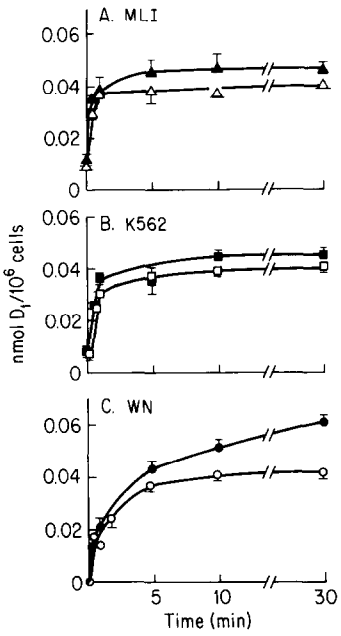

Fig. 5. Uptake of D_1 by ML1 (△, ▲) (A), K562 (□, ■) (B) and myeloblasts of patient (○, ●) (C) in the absence and presence of sodium azide. Cells were preincubated in glucose-free GBSS (△, □, ○) or glucose-free GBSS with 10 mM sodium azide (▲, ■, ●) for 10 min, and then [^{3}H]-D_1 was added. Each point represents the mean of duplicate samples from at least three independent experiments ± S.D.

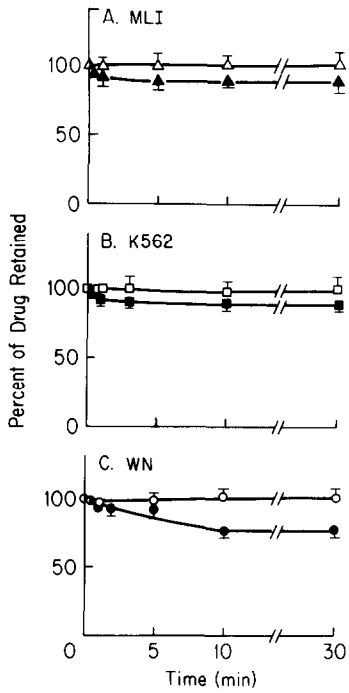

Fig. 6. Effect of glucose on efflux of D_1 from ML1 (△, ▲) (A), K562 (□, ■) (B) and patient myeloblasts (○, ●) (C). Cells were preloaded in glucose-free GBSS in the presence of 2.4 μM [^{3}H]D_1 for 30 min. The cells were then centrifuged at 4° and washed twice with cold GBSS and resuspended in GBSS in the absence (△, □, ○) or presence of 10 mM glucose (▲, ■, ●). Samples were withdrawn at the time indicated. Each point represents the mean of duplicate samples from at least one to three independent experiments ± S.D.

by simply diffusion. To investigate the influence of D_1-dissociation in cellular uptake, the uptake was studied at different pH levels (6.5 to 8.1). Uptake was affected markedly by the pH of the medium in both ML1 and K562 cells (Fig. 4 A and B). Also, the initial rate (10–120 sec) was increased by increasing the pH from 6.5 to 8.1 (data not shown). Higher uptake was observed at higher pH which was expected based on the pK_a of D_1 (≈8.2); hence, increasing pH resulted in fewer charged molecules. This profile was also observed with myeloblasts of the AML patient. Uptake was significantly higher at pH 8.1 as compared with pH 6.5 (Fig. 4C). D_1 efflux from ML1, K562 and patient's cells was also studied (Fig. 4 A, B and C). Biphasic curves were obtained with initial rapid loss of D_1 (≈2–5 min) followed by a constant rate of release for at least 10 min. The efflux seemed to be the same at lower pH values but was increased at higher pH. Not more than 29% of D_1 was effluxed from ML1 cells, 35% from K562 cells, and 50% from patient's myeloblasts.

Effect of metabolic inhibitor on uptake and efflux. In the presence of sodium azide, the steady-state level was increased slightly (15%) in ML1 (Fig. 5A) and significantly ($P < 0.005$) in patient cells (Fig. 5C). However, no significant increase was observed with K562 cells (Fig. 5B). Efflux was increased significantly in the presence of glucose ($P < 0.005$) in patient cells, suggesting an energy-dependent (or mediated) efflux of D_1 from these cells (Fig. 6C). Efflux of D_1 in K562 and ML1 cells was not significantly different in the presence or absence of glucose (Fig. 6A and B). We found that 70–80% of the labeled D_1 and metabolites were associated with nuclei when cells are exposed to 2.4 μM D_1 for 30 min, and less than 10% of D_1 is found in the cytoplasm, microsomes and mitochondria [16].

Although uptake, efflux and retention of D_1 were similar in patient's cells and in ML1 and K562 human

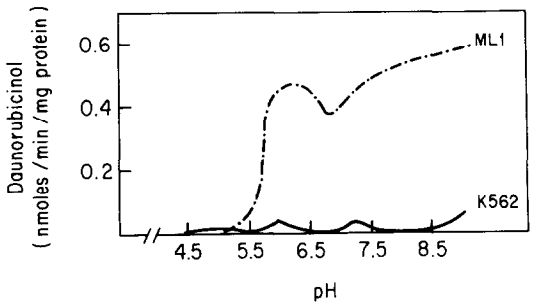

Fig. 7. Activity–pH profile of D_1 reductase from ML1 (–·–·–) and K562 (——). Buffers used were citrate phosphate (pH 4.5, 5.0, 5.5 and 6.0), potassium phosphate (pH 6.0, 6.6, 7.0 and 7.5), and Tris–HCl (pH 7.5, 8.0, 8.5 and 8.8). Assays were run immediately after enzyme preparation as described under Materials and Methods.

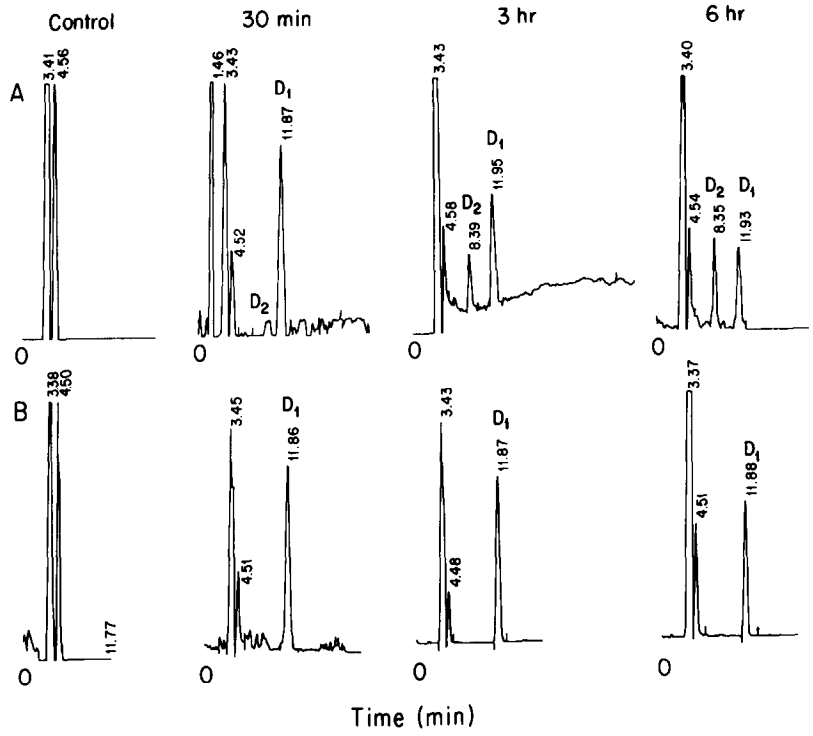

Fig. 8. Separation of D_1 and metabolite D_2 in ML1 (A) and K562 (B) by HPLC. The cells were incubated with 2.4 μM D_1 for periods of 0–6 hr. D_1 and D_2 were extracted as described in Materials and Methods. The HPLC column is a μBondapak C_{18} (30 cm × 3.9 mm), and the solvent contained acetonitrile–water–acetic acid (35:64:1) adjusted to pH 4.0. The mobile phase flow rate was 1.0 ml/min, and the retention times of D_1 and D_2 were 11.9 and 8.35 min respectively.

cell lines, metabolism of D_1 differed. The pH profile showed that whereas ML1 cells probably have the two reductases, the pH 6.0 and the pH 8.5 reductase activity, the K562 cells did not metabolize D_1 at all (Fig. 7). These results were confirmed by HPLC (Fig. 8). It is clear that, when D_1 was incubated with intact cells for periods of 0.5 to 6 hr, D_2 was observed in ML1 cells (Fig. 8A) but not in K562 cells (Fig. 8B). D_2 was detected in these cells within the first 30 min (Fig. 8A). A very strong correlation was noted between D_1 reductase activity and the formation of D_2. Since ML1 cells have both the pH 6.0 and pH 8.5 D_1 reductases, they metabolized D_1 to D_2 more than did K562 cells. No D_2 formation occurred when K562 cells were used, confirming the absence of reductase activity observed *in vitro* in these cells. Interestingly, D_2 concentration exceeded that of the parent drug after 3–6 hr of incubation with ML1 cells. These results suggest that conversion of $D_1 \rightarrow D_2$ is favoured *in vivo*. No other metabolites were detected by either HPLC analysis or fluorescence assay. Seemingly, the major pathway by which D_1 is metabolized in these human cells is via the reductases.

Table 1. Relationship between IC_{50} values (24-hr exposure) and intracellular concentration of D_1

Cell Line	IC_{50}* (μM)	Intracellular D_1† (μM)
ML1	11.1 ± 0	101.0 ± 2.4
K562	44.4 ± 0.25	299.0 ± 3.81

* The IC_{50} value is defined as the concentration of D_1 that inhibited proliferation rates by 50% over 24 hr of exposure.

† The intracellular concentration of D_1 and metabolites after a 24-hr exposure of cells to IC_{50} concentration of D_1, 11.1 μM for ML1 and 44.4 μM for K562.

When ML1 and K562 cells were incubated with D_1 at the IC_{50} concentration (24-hr exposure), the intracellular concentrations of D_1 and metabolites were different (Table 1). Although the intracellular D_1 concentration in K562 cells was 299 μM (≈3 times that of ML1), K562 cells were more resistant than ML1 cells to the cytotoxic effects of D_1.

DISCUSSION

Chemotherapeutic response to D_1 depends on several dynamic factors such as uptake, efflux, retention, and metabolism. We are interested in understanding the influence of these factors in response to D_1 therapy and are examining them in human myeloid cells. Three modes of action of anthracycline antibiotics have been postulated: binding and intercalation to DNA, resulting in inhibition of the template activity of both DNA and RNA polymerase [19–22]; direct membrane effect (when adriamycin was covalently linked to agarose beads, it was cytotoxic by interaction only at the cell surface [23]); and free radical activation. In leukemic patients, D_1 is rapidly reduced to its metabolite D_2 within 1 hr of

treatment [24]. This reduction is mediated by at least two classes of enzymes, the aldehyde (pH 8.5) D_1 reductase and the ketone (pH 6.0) D_1 reductase [25–27]. Variations in either class of reductase could influence overall metabolism of D_1 and, hence, chemotherapeutic response [28–31]. We have shown previously that rodent (rat or mouse) cell lines do not metabolize the drug [16]; however, *in vivo* anthracyclines are subject to extensive metabolism [28–30]. The use of human, rather than rodent, cell lines to test these variables should increase our understanding of the *in vivo* situation. We have studied two myeloid cell lines, ML1 and K562, and compared them with myeloblasts from AML patients. Our results indicate that, although uptake was similar in ML1 and K562 cells, metabolism differed. K562 cells do not metabolize D_1; however, ML1 and HL60 cells do [16]. We found that D_1 uptake in human leukemic cell lines is pH dependent, suggesting that the drug is transported mainly in the unionized form and probably by simple diffusion. This was strengthened by our data showing a non-saturable uptake of D_1 when the concentration was increased to 111 μM. D_1 efflux from both K562 and ML1 cells was not significant under physiologic pH, indicating that D_1 binding to intracellular targets may be irreversible since loss of D_1 was not observed for periods of up to 30 min. Efflux was not increased significantly in ML1 cells in the presence of glucose suggesting that efflux is not energy-dependent (or mediated). However, patient's myeloblasts seemed to efflux more D_1 ($\approx$22%) than the other cell lines. This became more evident when azide resulted in an increase in the steady-state level ($P < 0.005$), presumably due to the inhibition of efflux. When we studied the IC_{50} of D_1 (72-hr growth inhibition) for ML1 and K562 cells, we found it to be 10 and 19 nM respectively [16]. Also, IC_{50} determinations based on 24-hr growth inhibition studies (Table 1) indicate that K562 cells are more resistant to D_1 than are ML1 cells. This may be attributed to a number of factors; K562 cells may metabolize the drug more to the inactive aglycones. However, as there were no detectable aglycones up to 6 hr of incubation, it is possible that aglycones are formed slowly and may be detected after 24 hr. It is also possible that D_1 is inactivated to nonfluorescent metabolites in K562 cells. Possibly, ML1 cells may be more sensitive to D_2 than K562 and, since K562 does not metabolize D_1, it requires much more D_1 to cause the same toxicity. Other mechanisms may be involved such as differences in topoisomerase activity in the two cell lines.

The IC_{50} for patient myeloblasts was $\approx$2.6 μM; possibly patient's cells are resistant to D_1 which may explain the increased efflux of D_1 compared with the other cell lines. We are interested in studying the mechanisms of resistance to D_1 in a variety of human cell lines (e.g. HL60, K562, ML1) that adequately reflect the heterogeneity of blast cells in AML patients. Recently, we have successfully obtained low and high levels of resistant cell lines from HL60, K562 and ML1. Our early findings with HL60 cells have been reported, and we plan to use the resistant cell lines from ML1 and K562 to compare uptake, retention and metabolism of D_1 and D_2 to those of resistant cells.

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