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Contribution of drug transport and reductases to daunorubicin resistance in human myelocytic cells*

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Summary. We developed three daunorubicin (D_1)-resistant sublines (ML1/I, II, III) from the human myelocytic cell line (ML1). These sublines were 28-, 70- and 162-fold more resistant than sensitive (ML1/S) cells to the cytotoxicity of D_1 and were cross-resistant to adriamycin, epiadriamycin, actinomycin D, VP-16, VM26, and mitoxantrone. Steady-state levels of D_1 in resistant sublines I and II, in the presence or absence of azide, were not significantly different from those of sensitive cells. However, the steady-state level of D_1 in subline III was significantly increased in the presence of sodium azide. D_1 efflux was minimal in ML1/S and resistant cells in the absenced of glucose. Addition of glucose enhanced D_1 efflux only in subline III. Verapamil increased the cellular levels of D_1 and inhibited its efflux from resistant III cells but not from ML1/S cells. Verapamil also greatly enhanced the cytotoxicity of D_1 for sublines I, II, and III. The differences between sensitive and resistant cells in D_1 uptake and retention seemed inadequate to cause 162-fold resistance and suggested other factors may be contributing to the development of resistance. In support of this hypothesis, daunorubicin reductase activity was significantly lower in resistant cells than in ML1/S cells. The greatest decrease in activity occurred at pH 8.5 which represents aldehyde reductases. Currently, we are investigating other possibilities for D_1 metabolism, such as aglycone and free radical formation.

Introduction

The anthracycline antibiotic daunorubicin (D_1) is one of the most effective drugs for the treatment of acute nonlymphoblastic leukemia [35]. A major limitation to the drug's effectiveness is acquired resistance by neoplastic cells. To study the phenomenon of anthracycline resistance in greater detail, several investigators have developed in vitro or in vivo murine cell lines with various degrees of resistance to anthracyclines [8, 10, 11, 20]. These cells expressed multiple drug resistance (MDR) to a wide range of structurally unrelated compounds such as VP-16, *Vinca* alkaloids and actinomycin D [11, 12, 14, 23]. The primary defect in MDR cells is reduced accumulation, which probably results from slower influx, reduced cellular binding and more rapid efflux [9, 13, 20–22, 26, 27, 30]. D_1 efflux in resistant cells is energy-dependent [13, 20]. Another aspect of anthracycline resistance is its attenuation by calcium channel blockers (e.g., verapamil) and calmodulin inhibitors (e.g., trifluoperazine), which increase cellular levels of anthracyclines and thereby enhance cytotoxicity [15, 31, 32, 36].

By contrast, no significant increase in the cellular levels of D_1 were observed in myeloblasts from patients with acute myeloblastic leukemia (AML) in the presence of either azide or verapamil [24]. The authors suggested that there are marked differences between murine and human drug-resistant cells.

We report that drug-resistant human leukemia cell lines more closely mimic the in vivo situation in the way they metabolize daunorubicin than do murine cells [1, 33, 34]. Further, results from our studies of reductase activities suggest that metabolism may be one of many factors contributing to the development of drug resistance in these leukemic cell lines.

Materials and methods

Chemicals. Tritium-labeled D_1 (specific activity 2.2 Ci/mmol) was purchased from New England Nuclear, Boston, Mass; adriamycin from Adria Laboratories, Columbus, Ohio; actionymcin D from Merck, Sharp & Dohme, Rahway, NJ; VP-16 and VM26 from Bristol-Myers, New York, NY; daunorubicin from Ives Laboratories, New York, NY; 4-epiadriamycin from Dr Melvin Israel, University of Tennessee Center for the Health Sciences, Memphis, Tenn; mitoxantrone from Dr Thomas Avery, St Jude Children's Research Hospital, Memphis, Tenn; and verapamil from Knoll Pharmaceutical Company, Whippany, NJ. All other chemicals were of analytical grade.

Cell culture. The ML1/S myeloblastic leukemia cell line was established in 1978 from peripheral blood of a 24-year-old patient with acute myeloblastic leukemia [28]. ML1/S and resistant sublines were grown in RPMI 1640 medium containing 10% fetal bovine serum. The D_1-resistant subline was developed by growing the parental ML1/S cell line in medium containing 100 n*M* D_1. The cells were removed to drug-free medium whenever they appeared to be unhealthy, then were replaced (within a

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Abbreviations used: D_1, daunorubicin; D_2, daunorubicinol; GBSS, Gey's balanced salt solution; PBS, phosphate-buffered saline; I, II, III, ML1/I-, ML1/II-, ML1/III-resistant sublines; ML1/S, sensitive cell

week) in medium containing the same concentration of drug. Surviving cells were cloned by SeaPlaque two-layer agarose technique (the bottom layer is 0.7% and the top layer, 0.35%), and the colonies appearing in the agarose were transferred to liquid medium with a corresponding concentration of drug. We have selected many clones and randomly chose ML1/I to work with. Resistant line ML1/II was developed from ML1/I by growing the cells in medium containing 250 n*M* D_1. Resistant line ML1/III was developed from ML1/II by growing the cells in 500 n*M* D_1. Cells were processed and cloned as described for resistant line I. The cells were maintained in culture with D_1 at a subinhibitory concentration. The resistant sublines remained stable during growth in the absence of drug for at least 4 months. D_1 was removed 24 h before any experimentation with resistant sublines. Cells were used while they were in the logarithmic growth phase ($\approx 0.5-0.9 \times 10^6$ cells/ml). The IC_{50} value is defined as the concentration of drug inhibiting the growth of cells to such an extent that their growth is reduced to one-half that observed under drug-free conditions. For the clonogenic assays ≈ 100 cells were added per petri dish, and duplicate plates containing colonies were counted after 10–15 days. For drug treatment, when possible drugs were dissolved in water. Water-insoluble drugs, such as VP-16, were dissolved in ethanol, VM26 in DMSO, and epiadriamycin in DMSO. The highest concentrations used for the vehicle were 1%, 9.5%, and 2%, respectively. Whenever a drug was solubilized in a solvent other than water, a control flask containing cells and the solvent was used as a control. No more than 10% difference was observed between control flasks containing or lacking the solvent vehicle.

Determination of drug uptake. The conditions for in vitro drug uptake studies have been standardized by using a cell concentration of 30×10^6 cells/ml in the presence of 2.4 μ*M* labeled D_1 (1 μCi/ml) as previously published [33, 34]. Cells were suspended in either RPMI 1640 medium or GBSS, and incubated in shaking water bath for 30 min at 37 °C. At specific times, duplicate aliquots (0.1 ml) were removed, transferred to ice-cold tubes containing 300 μl silicone oil +400 μl phosphate-buffered saline (PBS) as the top layer, and centrifuged immediately for 30 s in an Eppendorf Model 3200 centrifuge. The upper layer was removed and tubes rinsed three times with cold PBS, after which the oil was carefully aspirated. Cell pellets were suspended overnight in 100 μl tissue solubilizer, neutralized with 34 μl glacial acetic acid, and centrifuged. Radioactivity in the supernatant fluid represents D_1 and metabolites. To determine "zero time" values, drug and cells were added simultaneously and processed as above. Results are presented as means of the individual observations recorded in four different experiments.

Determination of drug efflux. To determine D_1 efflux, cells (30×10^6 cells/ml) were incubated for 30 min at 37 °C with 2.4 μ*M* [^{3}H]drug. The cells were centrifuged (100 *g*) for 10 min at 4 °C, washed once, and resuspended to their original density in cold GBSS with and without glucose, then incubated at 37 °C. After incubation for various intervals, 100 μl was transferred to an Eppendorf tube containing silicone oil and PBS, and processed as above.

D_1 reductase activity. Daunorubicin reductase was prepared from ML1/S and resistant sublines and assayed as described previously [1–5]. The separation and quantitation of D_1 and daunorubicinol (D_2) were performed by thin-layer chromatography and fluorescence measurement [1, 33].

Statistical analysis. A two-tailed *t*-test was performed using the individual observations. *P* values lower than 0.05 are considered to be statistically significant.

Results

The IC_{50} values for ML1/S and resistant lines were determined as shown in Table 1. The use of either the clonogenic assay or growth-inhibition studies resulted in similar IC_{50} values indicating low, intermediate, and high levels of resistance to D_1. The population doubling times for ML1/S cells and sublines I, II, and III were 20, 22, 24, and 30 h, respectively.

Table 2 shows that the resistant sublines were cross-resistant to adriamycin, epiadriamycin, actinomycin D, VP-16, VM26, and mitoxantrone. Although resistance developed to D_1, the degree of resistance to adriamycin, actinomycin D, and VP-16 was greater than that to D_1. Epiadriamycin, which differs in structure from adriamycin only by inversion of the 4′-OH group was less cytotoxic than adriamycin. Similar results were observed when epiadriamycin was used against HL60-sensitive and daunorubicin-resistant cells (data not shown). Cross-resistance to mitoxantrone in these cells and other human drug-resistant leukemia cells was always less than the other compounds tested. As resistance to D_1 increased from 28-fold to 162-fold, cross-resistance also increased for all compounds.

Table 1. Relative resistance[a] and IC_{50} values of ML1/S and resistant cell lines

Cell line	Growth inhibition		Cloning	
	IC_{50} value (μ*M*)	Relative resistance	IC_{50} value (μ*M*)	Relative resistance
ML1/S	0.010 ± 0.004	1.0	0.008 ± 0.001	1.0
ML1/I	0.282 ± 0.039	28.2	0.302 ± 0.046	37.8
ML1/II	0.700 ± 0.163	70.0	0.675 ± 0.001	84.4
ML1/III	1.623 ± 0.073	162.3	1.350 ± 0.070	168.8

[a] Relative resistance is the ratio of the concentration of drug required to inhibit 50% of growth (72-h exposure) in the mutant line to that required in the sensitive line. For the clonogenic assay, colonies were counted after 10–20 days

Table 2. Sensitivity of ML1/S and resistant sublines to various drugs

Drug	IC_{50} value[a] (μM)				RR		
	ML1/S	ML1/I	ML1/II	ML1/III	ML1/I	ML1/II	ML1/III
Daunorubicin	0.0100±0.000	0.282 ±0.039	0.700±0.163	1.623±0.073	28.2	70.0	162.3
Adriamycin	0.0035±0.000	0.1245±0.013	0.480±0.020	0.840±0.028	35.6	137.1	240.0
Epiadriamycin	0.1130±0.024	2.270 ±0.325	5.050±0.919	8.300±0.141	20.0	44.7	73.5
Actinomycin D	0.0038±0.001	0.251 ±0.003	0.342±0.011	0.785±0.035	65.8	90.0	206.6
VP-16	0.1055±0.001	4.800 ±0.028	19.800±5.374	52.000±9.192	45.5	187.7	492.3
VM26	0.0180±0.001	0.317 ±0.084	1.068±0.060	2.370±0.269	17.6	59.3	131.7
Mitoxantrone	0.0074±0.004	0.043 ±0.006	0.145±0.007	0.444±0.006	5.8	19.6	60.0

[a] The IC_{50} values were obtained from 72-h growth-inhibition studies

In vitro cellular uptake of D_1. We have previously shown that the intracellular concentrations of D_1 in ML1/S and resistant cell lines was directly proportional to the drug concentration in the medium [34]. The initial rate of uptake and the steady-state level showed a linear relationship with external concentration of drug when the concentration of D_1 was increased from 0.11 to 111 μM, suggesting a transport mechanism compatible with simple diffusion [34].

The time-course of [^{3}H]D_1 uptake was compared in all cell lines at a concentration of 2.4 μM D_1. Figure 1 illustrates the time course for ML1/S and resistant subline III cells (as a representative). Both the initial uptake rate and the steady-state level of D_1 in subline III cells were significantly lower in the absence of sodium azide than in ML1/S cells ($P<0.05$). In the presence of sodium azide, the initial uptake and steady-state level of D_1 significantly increased in subline III cells ($P<0.05$). There were no significant differences in the initial rate (not shown) or steady-state levels in the two other resistant sublines either with or without sodium azide ($P>0.05$) (Fig. 1 inset).

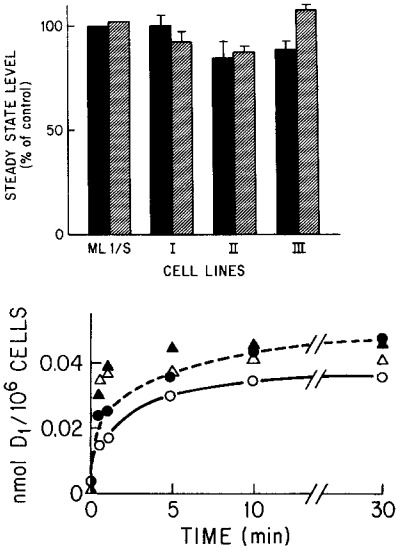

Fig. 1. Uptake of D_1 by ML1/S (△, ▲) and resistant line (III) (○, ●). The cells were preincubated for 10 min (±10 mM sodium azide) then 2.4 μM [^{3}H]-D_1 was added. △, ○, standard GBSS without glucose; ▲, ●, GBSS without glucose and containing 10 mM sodium azide. The SD did not exceed 10%. *Inset* shows the steady-state level expressed relative to the value of ML1/S ± sodium azide after exposure to 2.4 μM D_1. *Solid bars*, absence of sodium azide; *hatched bars*, presence of sodium azide. Each *point* represents the mean from four different experiments ±SD

D_1 efflux. The time course of D_1 efflux in ML1/S and subline III cells are illustrated in Fig. 2. When both sensitive and resistant cells were preloaded with D_1 (30 min) and resuspended in drug-free GBSS without glucose no efflux occurred over a period of 30 min in either sensitive or resistant cells. Addition of glucose did not induce additional D_1 efflux from sensitive cells but only in resistant cells (subline III) ($P<0.05$). The inset to Fig. 2 shows D_1 retention with and without glucose in all cell lines. Both ML1/S and resistant sublines I and II had equivalent intracellular concentrations of D_1 (30 min) in the presence and absence of glucose. However, in the presence of glucose there was a trend toward lower intracellular levels of D_1 in sublines I and II, although the D_1 concentration was significantly reduced in III, suggesting that efflux in the more resistant line may be energy-mediated.

Effect of verapamil on D_1 uptake. The time-course of [^{3}H]D_1 uptake was studied in both ML1/S cells and resistant subline III (as a representative) at a concentration of 2.4 μM D_1 with and without 10 μM verapamil (Fig. 3). In the presence of verapamil, there was no significant enhancement of cellular D_1 accumulation in ML1/S cells ($P>0.05$). However, significant increases in the steady-state levels of D_1 were observed after the addition of verapamil in resistant cells (Fig. 3, inset).

Effect of verapamil on D_1 efflux. Figure 4 shows that when cells were preloaded with D_1 (30 min) in the presence of verapamil and then resuspended in D_1-free medium containing verapamil, D_1 efflux was significantly inhibited in resistant subline III ($P<0.05$). When D_1 retention (30 min efflux) was measured (Fig. 4 inset) we found that the D_1 concentration was significantly increased only in resistant cell line III ($P<0.05$) in the presence of verapamil.

Enhanced cytotoxicity of D_1 by Verapamil. In the presence of 10 μM verapamil the IC_{50} values decreased markedly for all resistant cell lines. However, when verpamil was added to ML1/S cells no significant change in the IC_{50} value for D_1 was noted (Table 3). The addition of 5 μM verapamil enhanced D_1 toxicity somewhat but was less effective than 10 μM verapamil. Verapamil alone (10 μM) inhibited the cell growth of resistant sublines by 15%–20%, but had no effect on ML1/S cells. Resistant cells apparent-

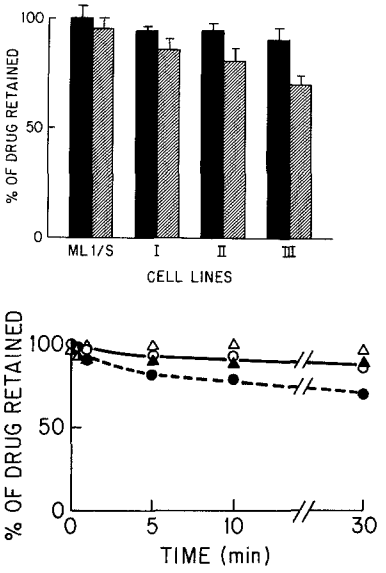

Fig. 2. Effect of glucose on D_1 efflux from ML1/S and resistant cells (III). The cell suspension was incubated in GBSS without glucose in the presence of 2.4 μM [^{3}H]D_1 for 30 min. The cells were then centrifuged at 4 °C and washed once with cold GBSS and resuspended in GBSS in the absence (△, ○) or presence (▲, ●) of 10 m*M* glucose. Samples were withdrawn at the time indicated. △, ▲ ML1/S; ○, ● ML1/III; each *point* represents the mean of four experiments. The SD did not exceed 10%. *Inset* shows the steady-state level of D_1 after efflux (± 10 m*M* glucose), expressed relative to the value of ML1/S. *Solid bars,* absence of glucose; *hatched bars,* presence of glucose. Observations recorded are means from four different experiments ± SD

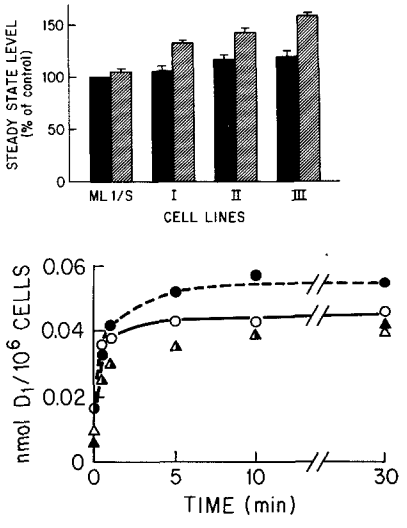

Fig. 3. Uptake of D_1 by ML1/s (△| ▲) and resistant line (III) (○, ●) in the presence and absence of verapamil. The cell suspension was preincubated in 1640 RPMI medium containing 10 μ*M* verapamil for 10 min. This was followed by the addition of 2.4 μ*M* [^{3}H]D_1. △, ○, standard medium; ▲, ●, standard medium containing 10 μ*M* verapamil. Each *point* represents the mean from two to four different experiments. The SD did not exceed 10%. *Inset* shows the steady-state level (± verapamil) expressed relative to the value of ML1/S after exposure to 2.4 μ*M* D_1 in the absence of verapamil. *Solid bars,* absence of verapamil; *hatched bars,* presence of verapamil

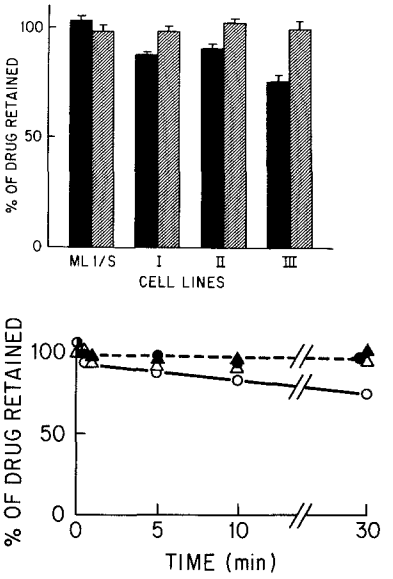

Fig. 4. Effect of verapamil on D_1 efflux from ML1/S and resistant cells (III). Conditions are similar to those of Fig. 2 except cells were resuspended in drug-free standard medium after 30 min (△, ○) or in standard medium containing 10 μ*M* verapamil (▲, ●). Each *point* represents the mean of four different experiments. The SD did not exceed 10%. *Inset* shows the steady-state level of D_1 ± verapamil) after efflux, expressed relative to the value of ML1/S. *Solid bars,* absence of verapamil; *hatched bars,* presence of verpamil. Observations recorded are means from two to four different experiments ± SD

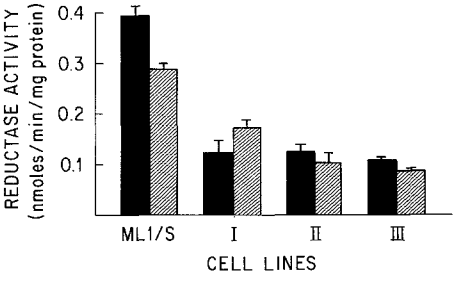

Fig. 5. Levels of reductase activity in ML1/S and D_1-resistant sublines. *Solid bars,* D_1 reductase activity at pH 6.0; *hatched bars,* D_1 reductase activity at pH 8.5 Observations recorded are means from three different experiments ± SD

Table 3. Verapamil enhancement of D_1 cytotoxicity in ML1/S and resistant sublines

Cell line	ICt_{50}[a] value (μ*M*) of D_1	
	Control	+ Verapamil (10 μ*M*)
ML1/S	0.010 ± 0.000	0.0089 ± 0.0001
ML1/I	0.282 ± 0.039	0.023 ± 0.0036
ML1/II	0.700 ± 0.163	0.050 ± 0.0028
ML1/III	1.623 ± 0.073	0.146 ± 0.0042

[a] The IC_{50} values of D_1 were obtained from growth-inhibition studies (72 h) in the presence or absence of 10 μ*M* verapamil. Values shown are means ± SD from three experiments

ly are more sensitive to verapamil concentrations that have no effect on sensitive cells.

D_1 reductase activity. The differences in the reductase activity (pH 8.5) between sensitive cells and cell line I and between cell lines I and II were considered statistically significant ($P<0.05$). Activity at pH 8.5 was reduced by 49%, 66%, and 68% in sublines I, II, and III compared with sensitive cells (Fig. 5). However, the difference in activity between lines II and III was not significantly different. D_1 reductase activity (pH 6.0) was decreased significantly in resistant subline I compared with ML1/S cells (Fig. 5). Enzymatic activity at pH 6.0 was reduced by 66%, 68%, and 70% in sublines I, II, and III. It is possible that the lower values of D_1 reductases may contribute to resistance in the less resistant line but that other factors (e.g., efflux, uptake) contribute in the more resistant line.

Discussion

Cell lines having various levels of resistance to D_1 ranging from low to high were selected with the expectation that sublines with low levels of resistance may mimic the in vivo situation more closely than do highly resistant cell lines. The isolated D_1-resistant sublines were cross-resistant to a variety of anthracyclines as well as compounds with diverse structures and pharmacologic actions. These cell lines appear to be among a group of mutant cells that are characterized by a wide range of cross-resistance [6, 7, 11, 12, 16, 19] and reflect a clinical situation in which malignant cells develop multiple drug resistance.

Acquired in vitro resistance to anthracyclines by murine cells is associated with decreased cellular accumulation of drug [13, 25, 27–30]. Increased drug efflux has also been proposed as a mechanism for drug resistance [13, 20, 22]. We present evidence indicating that uptake was not significantly altered in D_1-resistant sublines compared with sensitive cells ($P>0.05$). Moreover, no significant alteration in uptake or steady-state level was noted in these resistant cell lines, which varied widely in their degrees of resistance.

Although the changes observed, especially in resistant subline III, such as increased efflux in the presence of glucose, suggest that efflux is a mechanism that may contribute to 2- to 3-fold resistance, it would not account for 162-fold resistance. In addition, the altered uptake of D_1 in resistant line III was only observed when the experiments were performed in GBSS. When uptake measurements were made using media rather than GBSS (verapamil experiments, Fig. 3) no significant difference in uptake was observed in sensitive or resistant cells.

Verpamil inhibited the D_1 efflux and thereby increased the cellular levels of D_1 in the resistant sublines. However, verapamil greatly enhanced the cytotoxicity of D_1 in all the resistant lines. By contrast, verapamil did not enhance the cytotoxicity of the anthracycline in ML1/S cells.

Studies of myeloblasts from patients with AML having various responses to anthracycline therapy showed that neither sodium azide nor verapamil enhanced D_1 uptake [24]. These results differ from those reported for murine cells. In addition, there was no correlation between drug responsiveness in these patients and in vitro cellular D_1 accumulation.

D_1 metabolism via the reductases has been shown to be species-dependent [1, 2]. Ketone reductase activity was found only in liver preparations derived from humans or rabbits, while rat and mouse preparations were devoid of this activity. In addition, D_1 is metabolized extensively in vivo [5, 37], however, murine leukemia cells (P388 and L1210) do not appreciably metabolize D_1 [1]. Therefore, metabolism of D_1 may be important in drug-resistant human cells but not in murine cells. Accordingly, we selected human myeloid cell lines such as ML1, KG1 and HL60 which have different degrees of metabolism [1, 33] to study the contribution of drug metabolism to drug resistance. Other investigators have reported an association between increased reductase activity and a more favorable response to drug in patients with leukemia [17, 18].

Our results indicate clearly that D_1 metabolism via reductases is altered in resistant human cells. Both aldehyde and ketone reductase activity were reduced in D_1-resistant cells, especially ML1/I and ML1/II. However, reductase activity was not significantly different in resistant cell lines II and III.

These results suggest that resistance to D_1 is multifactorial, and it is possible that the factors contributing to a lower level of resistance are different from those involved in higher degrees of resistance.

In summary, our results indicate the lack of correlation between drug resistance in ML1 cells and drug levels or increased efflux. We suggest that modes of drug resistance in ML1 human cells may be unrelated to transport mechanism. Our results, as well as those of others, suggest that factors such as D_1 metabolism via the reductases or aglycones binding to a specific target or cellular protein and/or free radical formation may be among the factors contributing to resistance in leukemic cells. Currently, we are investigating other aspects of D_1 metabolism, such as aglycone and free radical formation.

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