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# Characterization of Daunorubicin Resistance in K562 Leukemia Cells Lacking Daunorubicin Reductase Activity

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**Abstract**—*Daunorubicin ($D_1$)-resistant cells have been isolated from daunorubicin reductase-deficient K562 cells, hence, metabolism of $D_1$ to the alcohol metabolite daunorubicinol ($D_2$) will not contribute to the development of resistance. The resistant cell lines were 22–123-fold resistant and were cross-resistant to a variety of drugs. Drug uptake and efflux were altered in the more resistant lines but not in the less resistant cells. Verapamil enhanced $D_1$ cytotoxicity in all resistant lines; it inhibited $D_1$ efflux in the higher resistant line thereby resulting in an increase in the cellular level of $D_1$. However, this was not true for the less resistant line suggesting that verapamil enhancement of $D_1$ toxicity in the less resistant line is probably due to other factors. Additionally, we have been unable to identify a marker glycoprotein in resistant cells. The changes observed in the resistant sublines are moderate and probably drug accumulation differences could not account for the degree of $D_1$ resistance noted, nor could resistance be wholly reversed by calcium antagonist. Other factors may be involved in the development of resistance in these human cells.*

## INTRODUCTION

THE ANTHRACYCLINE antibiotic daunorubicin ($D_1$) is a clinically valuable agent that plays an important role in the treatment of acute leukemias [1]. The development of resistance to $D_1$ represents one of the major obstacles to effective therapy. Mammalian cells selected for their resistance to $D_1$ *in vitro* display cross-resistance to other chemotherapeutic agents including adriamycin, actinomycin D and VP-16 [2–5]. Resistance was mainly attributed to decreased drug retention resulting from increased efflux of drug from resistant cells. Another important characteristic of pleiotropic drug resistant (PDR) cells is the reversal of resistance by calcium channel blockers. It is unclear whether this reversal is a common property of PDR cells; it may be characteristic of only a specific type of PDR and the biochemical basis of the reversal has not been clearly defined; however, it is thought that verapamil acts by inhibiting drug efflux which increases cellular accumulation of drug [6, 7].

Previous reports indicate the lack of correlation between resistance and drug transport and drug levels [8–10]. In addition, we found that certain human cell lines (e.g. ML1) that are resistant to $D_1$ (37–169-fold), did not have any significant alterations in uptake, efflux or drug retention; however, $D_1$ reductase activity was altered in the resistant lines as compared to sensitive cells [10].

In a more recent study we chose a human cell line that does not metabolize $D_1$ via the reductases and developed variously resistant mutants to determine the extent to which drug retention or transport may contribute to the development of resistance.

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## MATERIALS AND METHODS

*Chemicals*

Tritium-labeled $D_1$ (specific activity 2.2 Ci/mmol) was purchased from New England Nuclear, Boston, MA; adriamycin from Adria Laboratories, Columbus, OH; actinomycin D from Merck Sharp & Dohme, VP16 and VM26 from Bristol-Myers, NY; daunorubicin from Ives Laboratories, NY; 4-epiadriamycin from Dr. Melvin Israel, University of Tennessee, Memphis, TN; and mitoxantrone from Dr. Thomas Avery, Nucleic Acid Research Institute, Costa Mesa, CA. All other chemicals were of analytical grade.

*Cell culture*

The K562/I $D_1$-resistant subline of K562 human erythroleukemia cells was developed by growing

the parental K562 cell line in Dulbecco's minimal essential medium (DMEM) medium containing 100 nM $D_1$. The cells were removed to drug-free medium whenever they appeared to be unhealthy and after normal growth resumed, were replaced in medium containing the same concentration of drug. Surviving cells were cloned in medium containing 0.7% agarose (bottom layer) and 0.35% agarose (top layer) and the subsequent colonies transferred to liquid medium containing drug. We have selected many clones that grew well and randomly chose a clone designated K562/I to work with. K562/II cells were developed from K562/I cells by growing them in medium containing 250 nM of $D_1$. K562/III were developed from K562/II by growing the cells in medium containing 500 nM of $D_1$. Cells were used while they were in the logarithmic growth phase ($\approx 0.5$–$0.8 \times 10^6$ cells/ml). The resistant lines have been grown in drug-free medium for 3 months without any reversion indicating that they are stable mutants.

*Determination of drug uptake*

The conditions for *in vitro* drug uptake studies have been standardized by using a cell concentration of $3 \times 10^7$ cells/ml in the presence of 2.4 μM tritium-labeled $D_1$ (1 μCi/ml) as previously published [11, 12]. Generally, cells were suspended in either DMEM medium or Geys balanced salt solution (GBSS) as indicated, and incubated with shaking for 30 min at 37°C. 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 s in an Eppendorf Model 3200 centrifuge. The upper layer was removed, the tubes were rinsed three times with PBS, then the oil layer was carefully aspirated. The cell pellet was suspended overnight in 100 μl of tissue solubilizer, neutralized with 34 μl of glacial acetic acid, centrifuged and the radioactivity determined. To determine "zero time" values, drug and cells were added simultaneously and processed as above. Using the oil technique some of the buffer may pass with the cell pellet; to provide a measure of the buffer that passes through the silicone oil, portions of cells incubated for 10 min with [$^{14}$C]inulin were analyzed for radioactivity after centrifugation through oil. This represents 0.15% of the radioactivity associated with the pellet. Additional portions of the cell suspension were incubated with [$^{14}$C]inulin and processed by transfer into empty preweighed microcentrifuge tubes and centrifuged for ≈ 10 s to determine intracellular water. The supernatant was sampled, residual supernatant aspirated and a wet weight determined before the cells were dried at 70° C. Dry weight was determined and the pellets were processed for counting as described above. Intracelluar water was the difference between the wet and dry weights of the cell pellet, less the extracellular volume, as determined from the [$^{14}$C]inulin space as previously reported [13]. Intracellular water was equal in sensitive and resistant cells (≈ 1.3 μl/$10^6$ cells) so that drug concentration can be expressed either in μM or in nmol/cell. Results are presented as the mean of the individual observations of two to four different experiments.

*Determination of drug efflux*

To determine $D_1$ efflux, cells were incubated for 30–40 min at 37°C with 2.4 μM [$^3$H]drug. The cells were centrifuged (100 ***g***) for 10 min and resuspended to their original density ($3 \times 10^7$ cells/ml) in cold GBSS either with or without glucose at 4°C at 100 μl aliquots transferred to an Eppendorf tube containing silicone oil, PBS and processed as above.

*Glycoprotein determination*

This was performed by galactose oxidase labeling, a method that has been used by others to detect changes in glycoprotein patterns [14, 15]. To summarize: cells were washed with PBS and incubated with either galactose oxidase or galactose oxidase and neuraminidase, washed and incubated with $NaB^3H_4$. Cells were washed and prepared for SDS gel electrophoresis [16] by using linear 5–15% acrylamide gradient and 3.5% acrylamide stacking gel. Tritiated components were detected by fluorography [17] and standards having molecular weights between 15,000–200,000 were used.

*Statistical analysis*

A two-tailed *t*-test was performed by using the individual observations from two to four experiments and $P$ values $< 0.05$ are considered to be statistically significant.

## RESULTS

The sensitivity of K562 sensitive (K562/S) and resistant cell lines was examined by *in vitro* exposure to $D_1$ for 72 h. The use of either the clonogenic assay or growth inhibition studies resulted in similar $IC_{50}$ values indicating levels of resistance between 22- and 123-fold (Table 1). The population doubling time for K562/S cells and resistant lines I and II ranged from 14 to 16 h; that of cell line III was ≈ 30 h. The resistant cells were also cross-resistant to a variety of drugs including adriamycin and epiadriamycin. Cross-resistance to other drugs was of the same magnitude as to $D_1$ or higher (VP-16). However, the cells were least cross-resistant to mitoxantrone (Table 2) and increasing the resistance from 23- to 123-fold did not result in a corresponding increase in the cross-resistance to mitoxantrone when compared to othe drugs tested.

*Table 1. $IC_{50}$ values of K562/S and resistant cell lines and their relative resistance* to $D_1$*

| Cell lines | Growth inhibition† $IC_{50}$ value (nm) | Relative resistance | Cloning‡ $IC_{50}$ value (nm) | Relative resistance |
|---|---|---|---|---|
| K562/S | 18 ± 0.5 | 1 | 20 ± 0.7 | 1 |
| K562/I | 398 ± 29 | 23 | 435 ± 7 | 22 |
| K562/II | 838 ± 5 | 48 | 850 ± 14 | 44 |
| K562/III | 2145 ± 78 | 123 | 2200 ± 71 | 113 |

*Relative resistance is the ratio of drug concentration required to inhibit growth by 50% in mutant cells as compared to sensitive cells (K562/S).

†$IC_{50}$ values are defined as the concentration of drug inhibiting the 72 h growth of cells (determined by counting the cells with a Coulter counter) to one-half that occurring in drug-free conditions [10].

‡For the clonogenic assay, cells were incubated with different concentrations of drug for 72 h before cloning. Colonies were counted after 10–20 days [10].

*Table 2. Relative sensitivity of resistant sublines to various drugs*

| Drugs | Relative resistance* K562/I | K562/II | K562/III |
|---|---|---|---|
| Daunorubicin | 23 | 48 | 123 |
| Adriamycin | 22 | 42 | 125 |
| Epiadriamycin | 21 | 56 | 121 |
| Actinomycin D | 12 | 26 | 73 |
| VP-16 | 48 | 97 | 188 |
| VM26 | 22 | 51 | 114 |
| Mitoxantrone | 11 | 10 | 20 |

*Relative resistance is the ratio of the concentration of drug required to inhibit growth by 50% in the mutant line as compared to the sensitive cells.

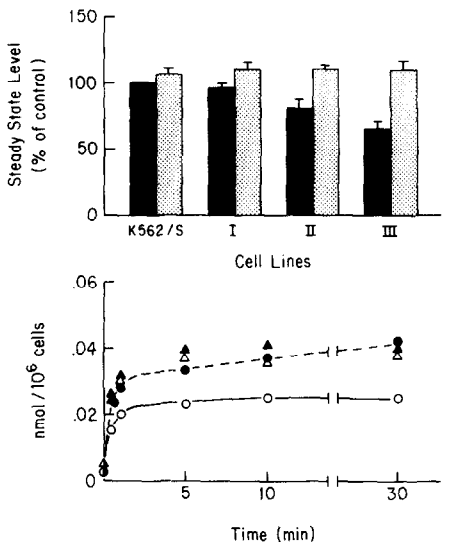


*Fig. 1. $D_1$ uptake of K562/S cells (△, ▲) and K562/III cells (○, ●). The cells were pre-incubated for 10 min (± 10 mM sodium azide) before adding 2.5 μM [$^3$H]$D_1$. ▲, ●: Standard GBSS without glucose and without sodium azide. △, ○: GBSS without glucose and containing 10 mM sodium azide. The inset shows steady-state levels expressed relative to the value of K562/S ± sodium azide after exposure to 2.4 μM $D_1$. Solid bars: without sodium azide. Hatched bars: with sodium azide. Each point represents the mean of four different exposures ± S.D.*

Daunorubicin uptake was compared in all cell lines at a concentration of 2.4 μM $D_1$. Figure 1 illustrates the time course for K562/S and resistant subline III (as a representative). Both the initial uptake rate and steady-state level of $D_1$ in subline II and III cells were significantly reduced in the absence of sodium azide as compared with K562/S cells ($P < 0.001$). In the presence of azide, the steady-state level of $D_1$ (Fig. 1 inset) significantly increased in sublines II and III ($P < 0.001$).

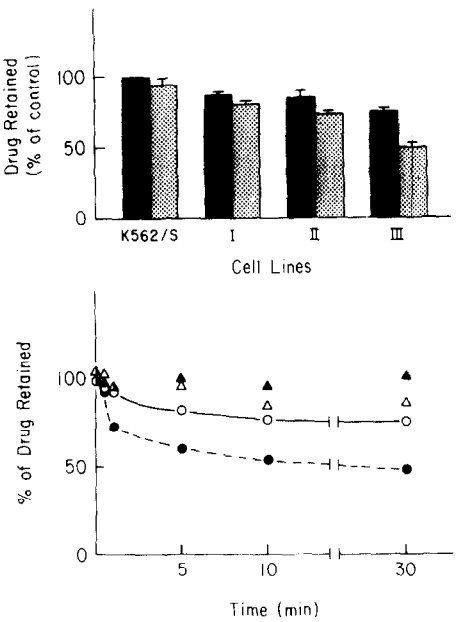


*Fig. 2. Effect of glucose on $D_1$ efflux from K562/S (△, ▲) and K562/III (○, ●) cells. The cell suspension was incubated in GBSS without glucose in the presence in 2.4 μM [$^3$H]$D_1$ for 30 min. Cells were then centrifuged at 4°C, washed once with cold GBSS and resuspended in GBSS without (△, ○) or with (▲, ●), 10 mM glucose for the times indicated. Each point represents the mean of four experiments. The inset shows steady-state levels of $D_1$ after efflux (± 10 mM glucose), expressed relative to the value of K562/S. Solid bars: without glucose; hatched bars: with glucose ± S.D. of four experiments.*

The time courses of $D_1$ efflux in K562/S and resistant subline III (as a representative) are shown in Fig. 2.

When both sensitive and resistant cells were pre-loaded with $D_1$ (30 min), and resuspended in drug

*Table 3. Verapamil enhancement of $D_1$ cytotoxicity in K562/S and resistant sublines*

| Cell lines | $IC_{50}$ value (nm) of $D_1$ Control | + Verapamil |
|---|---|---|
| K562/S | 18 ± 0.5 | 15 ± 0.35 |
| K562/I | 398 ± 29 | 72 ± 5 |
| K562/II | 838 ± 5 | 86 ± 6 |
| K562/III | 2145 ± 78 | 220 ± 28 |

*The $IC_{50}$ values of $D_1$ were obtained from growth-inhibition studies (72 h) in the presence or absence of 10 μM verapamil. Values represent the mean ± S.D. of three experiments.

free GBSS without glucose, no significant efflux of $D_1$ was observed in K562/S or resistant subline I cells ($P > 0.05$). However, $D_1$-efflux from resistant subline III (Fig. 2) was considered statistically significant ($P < 0.005$). Addition of glucose did induce apparent additional efflux in resistant cells and this efflux was found to be statistically significant only in resistant subline III (Fig. 2 inset). These results suggest that $D_1$-efflux may be energy-dependent or mediated only in the more resistant line.

Verapamil (10 μM) enhanced the cytotoxicity of $D_1$ in all resistant cell lines. However, when verapamil was added to K562/S cells, no significant change in the $IC_{50}$ value for $D_1$ was observed (Table 3). Although verapamil decreased the $IC_{50}$ values markedly in all resistant cells, a complete reversal did not occur and we also found that higher concentrations of verapamil were more toxic to resistant cells than to sensitive cells (data not shown). The enhanced cytotoxicity of $D_1$ in the presence of verapamil has been associated with increased accumulation and retention of anthracyclines in some models [6, 7]. Accordingly, we examined the effect of verapamil on uptake (Fig. 3) and efflux (Fig. 4) of $D_1$ in sensitive and resistant cells. Verapamil significantly increased steady state level of $D_1$ in resistant subline III (as a representative) when compared to sensitive cells ($P < 0.05$).

Figure 3 shows the results with all cell lines. This increase in the steady state level of $D_1$ is probably due to inhibition of $D_1$ efflux in resistant cell (see Fig. 4, resistant line III as representative). This was true also for resistant sublines II and III (Fig. 4).

The galactose oxidase-boro [$^3$H]hydride labeling pattern of sensitive and resistant cell membranes failed to show specific labeling of gp170 (Fig. 5). However, lower molecular weight proteins (11.5, 56 and 65 kd) were specifically labeled in resistant cells. The 56 and 65 kd proteins were also found in ML1 $D_1$-resistant cell lines (data not shown). Conversely, gp170 has been found (with monoclonal antibodies) on multidrug-resistant cells in which the glycoproteins was not labeled by the galactose oxidase borohydride method. A conclusive statement concerning the absence of gp170 in these cell lines must await the use of specific antibodies.

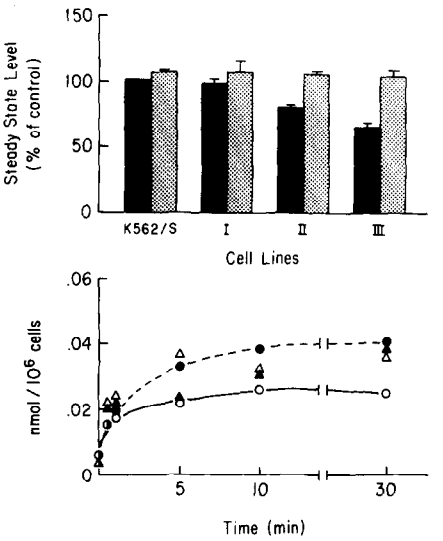


*Fig. 3. $D_1$ uptake by K562/S cells (△, ▲) and resistant K562/III cells (○, ●) in the absence and presence of verapamil. The cell suspension was preincubated in DMEM medium with or without 10 μM verapamil for 10 min before adding 2.4 μM [$^3$H]$D_1$. △, ○ Standard medium; ▲, ● standard medium containing 10 μM verapamil. Each point represents the mean of two to four experiments. The inset shows steady-state levels of $D_1$ expressed relative to the value of K562/S ± verapamil after exposure to 2.4 μM. Solid bars: without verapamil. Hatched bars: with verapamil.*

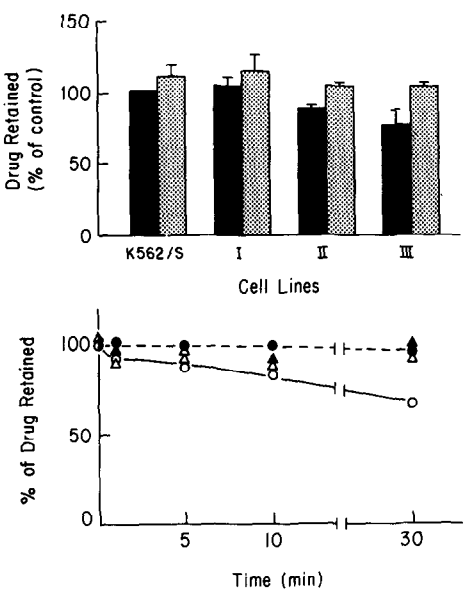


*Fig. 4. Effect of verapamil on $D_1$ efflux from K562/S (△, ▲) and resistant K562/III (○, ●) cells. Cells were resuspended in drug-free standard medium after uptake for 30 min (△, ○) or standard medium containing 10 μM verapamil (▲, ●). Each point represents the mean of four experiments. The inset shows steady-state levels of $D_1$ after efflux (± verapamil), expressed relative to the value of K562/S. Solid bars: without verapamil; hatched bars: with verapamil ± S.D. of two to four experiments.*

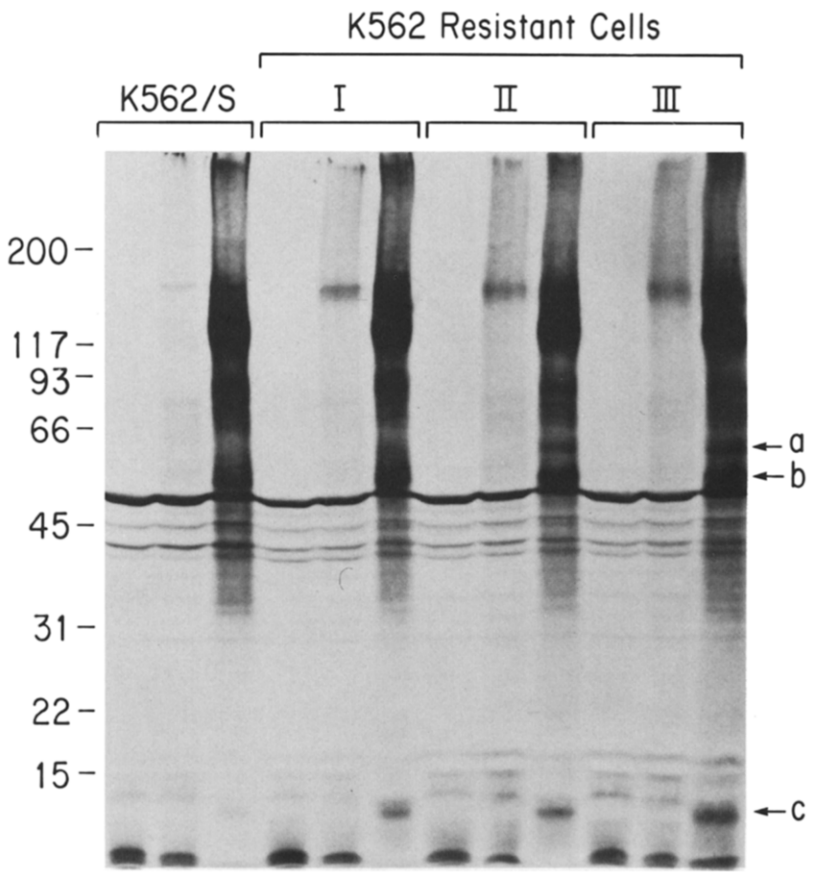


*Fig. 5. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of* $NaB^3H_4$ *labeled cell extracts of sensitive and resistant cells. Each cell line was run in three lanes. First lane in each cell line is control (without enzyme treatment), second lane, cells were treated with galactose oxidase and third lane, cells were treated with galactose oxidase and neuroaminidase. Arrows: a = gp65; b = gp56; c = gp11.5 kd.*

## DISCUSSION

Acquired *in vitro* resistance to anthracyclines by murine cells is thought to be based on decreased drug retention as a result of enhanced efflux of drug from the resistant cells [18–22]. In previous publications we have presented evidence to indicate that some human cell lines (HL60 and ML1) expressing different degrees of resistance did not exhibit significant alteration in drug uptake or efflux to justify the high level of resistance observed. However, in ML1 cells we found significant alterations in aldehyde and ketone $D_1$ reductases which metabolize $D_1$ to the alcohol metabolite $D_2$ [10]. We thought that altered metabolism might be another mechanism by which these human tumor cells develop resistance, especially in those cell lines that did not express any defects in uptake or efflux. The pH profile for $D_1$ reductase using ML1 cell line as a control; a cell line that has been shown to metabolize $D_1$ to $D_2$ and contains both aldehyde and ketone reductase activities [23] and K562 cell line indicated that K562 cells did not metabolize $D_1$ at all [23]. These results were confirmed previously by HPLC [12]. When $D_1$ was incubated with intact cells for periods of 0.5–6 h, $D_2$ was observed only in ML1 cells and not in K562 cells [12]. Also no $D_1$ reductase activity was detected in the resistant cell lines. These results indicate clearly that K562/S and resistant cells are deficient in $D_1$ reductases.

For the study we are reporting, however, we chose K562 cells because they are deficient in $D_1$ reductases, expecting that alterations in uptake and efflux may be more pronounced when compared to resistant cells in which metabolism may influence resistance. Our results indicate that there were alterations in drug uptake and retention (only in the more resistant sublines) but these alterations probably are not sufficient to account for 123-fold resistance. In addition, there was no correlation between the level of resistance and drug uptake or retention. Moreover, the less resistant line, K562/I did not show any alterations in either uptake or efflux, suggesting that the mechanisms involved in the development of low resistance may be different from those of a high degree of resistance.

Other investigators have identified several calcium antagonists and calmodulin inhibitors that enhance $D_1$ cytotoxicity in resistant cells [6, 7]. The enhanced cytotoxicity of $D_1$ has been associated with increased cellular accumulation and retention of $D_1$ [6, 7]. In our studies, verapamil inhibited $D_1$ efflux, thereby increasing the cellular levels of $D_1$ in resistant sublines II and III. However, verapamil greatly enhanced the $D_1$ cytotoxicity in the less resistant line K562/I without significantly affecting drug retention or accumulation, which suggests that verapamil enhancement of $D_1$ cytotoxicity may be mediated by other mechanisms in addition to increased cellular accumulation of drug.

Many studies have shown that gp170 was overexpressed in resistant cells as compared to sensitive cells. We were unable to show overproduction of gp170 by galactose oxidase labeling. However, other proteins were labelled in resistant cells. Whether or not these proteins can be identified in other resistant cells is unknown. We have been unable to identify a consistent "marker" protein in any of the resistant cell lines studied.

In summary, this study shows that resistant sublines derived from K562, a cell line that is deficient in metabolism of $D_1$ to $D_2$ via the reductases, did not exhibit the typical phenotypic alteration that accompanies the development of resistance (e.g. altered glycoprotein, efflux, uptake). Although there were changes in drug accumulation, these changes did not correlate with the degree of resistance and probably are insufficient to account for the high degree of resistance (123-fold). Our data, as well as that of others [24, 25], suggest that additional factors may be involved in the development of $D_1$ resistance in these cell lines. Currently, we are examining single, double, and protein-associated DNA strand breaks and their relationship to cytotoxicity and degree of resistance.

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