Characterization of a K562 Multidrug-resistant Cell Line

Saul Yanovich, Robert E. Hall, and David A. Gewirtz

Departments of Medicine and Pharmacology, Medical College of Virginia, Richmond, Virginia 23298

ABSTRACT

A daunorubicin-resistant variant of the K562 human leukemia cell line (K562-R), which demonstrates cross-resistance to other anthracycline antibiotics and vincristine, was derived from the parent cell line by exposure of logarithmically growing cells to gradually increasing concentrations of daunorubicin in vitro. Cross-resistance to anthracyclines and vincristine, and vinblastine on the growth of K562-S and K562-R cells in suspension was determined by seeding logarithmically growing cells (1 × 10⁶ cells/ml) into 96-well tissue culture plates. Cells were incubated in the presence of drug at 37°C under 5% CO₂ in a humidified 5% CO₂ atmosphere. The 50% inhibitory concentration for a particular agent was defined as the drug concentration which results in a 50% reduction in the cloning efficiency compared to untreated controls. Experiments were performed in triplicate and repeated at least 3 times. Cytotoxicity Studies. The effect of daunorubicin, doxorubicin, vincristine, and vinblastine on the growth of K562-S and K562-R cells in suspension was determined by seeding logarithmically growing cells (1 × 10⁶ cells/ml) into 96-well tissue culture plates. Cells were incubated in the presence of drug at 37°C under 5% CO₂ in a humidified 5% CO₂ atmosphere. The 50% inhibitory concentration for a particular agent was defined as the drug concentration which results in a 50% reduction in cell number at 72 h relative to the untreated control. Experiments were performed in triplicate and repeated at least 3 times. Cell Cycle Analysis. K562-S and K562-R cells (1 × 10⁶ cells/ml) were treated with propidium iodide staining solution consisting of 3.8 mM sodium citrate, 0.1% Triton X-100, RNase B (7000 units/ml), and 0.05 mg propidium iodide/ml for 30 min at room temperature (15). The stained nuclei were analyzed for DNA-propidium fluorescence by using a Coulter Telectronics TPS-1 at a laser setting of 36 mW and an excitation wavelength of 488 nm; resulting DNA distributions were analyzed for the proportion of cells in the G₀, S, and G₂-M phases of the cell cycle as described by Collins et al. (16).

INTRODUCTION

Natural killer cell activity plays an important role in control of the growth and metastatic potential of neoplastic cell (1–3). Previous studies using a human erythroleukemic cell line developed in this laboratory (K562-R) suggested that an important element in MDR (12, 13) was reduced susceptibility to lysis by natural killer cells (4). This association between the development of multidrug resistance and natural killer resistance supports clinical observations of rapid tumor progression in patients who have failed initial chemotherapy.

In the development of multidrug resistance by malignant cells, a MDR gene (mdr1) is frequently overexpressed in animal tumor models and in some human neoplasms as well (5, 6); phenotypically, many resistant tumor cell lines demonstrate increased expression of high-molecular-weight cell membrane glycoproteins (7–9). The MDR phenotype is frequently associated with decreased drug accumulation and/or retention resulting from enhanced drug efflux, presumably via an energy-dependent efflux pump (10, 11). This membrane transport system demonstrates homology with bacterial transport proteins which export large toxic molecules (12–13).

The present studies were designed to define alterations in cell membrane proteins and daunorubicin transport properties in the K562-R leukemic cell line in order to assess the relationship of this cell line to the classical multidrug resistance phenotype.

MATERIALS AND METHODS

Cell Lines. The K562 erythroleukemic cell line, established by Lozzio and Lozzio (14), was grown as a suspension culture in RPMI 1640 medium supplemented with 10% FCS, penicillin, 100 IU/ml, and streptomycin, 50 µg/ml. The K562-R subline was derived from the parent cell line by exposure of logarithmically growing cells to gradually increasing concentrations of daunorubicin in vitro, up to a final concentration of 0.5 µM. K562-R cells were subcloned by limiting dilution and grown in RPMI 1640 supplemented with 10% FCS. K562-R cells were exposed to daunorubicin (0.5 µM) for 1 week of each month. Cells were not exposed to daunorubicin during the week prior to experimental protocols.

Cell Cycle Analysis. K562-S and K562-R cells (1 × 10⁶ cells/ml) were treated with propidium iodide staining solution consisting of 3.8 mM sodium citrate, 0.1% Triton X-100, RNase B (7000 units/ml), and 0.05 mg propidium iodide/ml for 30 min at room temperature (15). The stained nuclei were analyzed for DNA-propidium fluorescence by using a Coulter Telectronics TPS-1 at a laser setting of 36 mW and an excitation wavelength of 488 nm; resulting DNA distributions were analyzed for the proportion of cells in the G₀, S, and G₂-M phases of the cell cycle as described by Collins et al. (16).

Cross-Resistance Studies. The effect of daunorubicin, doxorubicin, vincristine, and vinblastine on the growth of K562-S and K562-R cells in suspension was determined by seeding logarithmically growing cells (1 × 10⁶ cells/ml) into 96-well tissue culture plates. Cells were incubated in the presence of drug at 37°C under 5% CO₂ in a humidified 5% CO₂ atmosphere. The 50% inhibitory concentration for a particular agent was defined as the drug concentration which results in a 50% reduction in cell number at 72 h relative to the untreated control. Experiments were performed in triplicate and repeated at least 3 times. Cytotoxicity Studies. K562-S and K562-R cells were incubated with daunorubicin, daunorubicin + verapamil, or verapamil, for 2 at 37°C in a humidified 5% CO₂ atmosphere. The cells were subsequently washed and resuspended in drug-free medium at a concentration of 5 × 10⁵ cells/ml. To determine the percentage of survival after drug exposure, cells were then plated (5 × 10⁵ cells/plated) in 35–x 10-mm Petri dishes in RPMI 1640 medium supplemented with 20% FCS, 10 µM 2-mercaptoethanol, and 0.35% agar. After 8 days of incubation in a humidified 5% CO₂ atmosphere, colonies (>50 cells) were enumerated by using a colony-counting unit. Plating efficiency of controls was ~15% for both cell lines. In all cases, the cloning efficiency of the untreated cells was normalized to 100%, and the cloning efficiency of the treated cells was expressed as a percentage of the control survival.

Characterization of Cell Surface Proteins. K562-S and K562-R cell surface proteins were radiolabeled following the methods described by Markwell and Fox (17). Cells in logarithmic growth, washed 3 times with PBS, were transferred to a glass vial previously coated with chicken anti-Fc (Pierce Chemical Co.) and incubated at 4°C for 10 min in 1 ml of PBS with 200 µCi of Na¹²⁵I (Amersham, Arlington Heights, IL). Cells were washed 3 times with cold PBS containing 5 mM NaI, and the cell membrane was disrupted by incubation in lysing buffer (1% Triton X-100, 0.14 M NaCl, 0.01 M Tris, pH 7.8, and 1 mM phenylmethylsulfon fluoride) for 1 h at 4°C. The cell membrane was separated from the nuclei by pelleting the nuclei at 8000 × g. Samples of solubilized¹²⁵I-labeled cell surface proteins (equaled for identical thirilchoelectric acid-pprecipitable counts) were fractionated by electrophoresis on 7.5% acrylamide (Bio-Rad, Richmond, CA) slab gels (18). Autoradiograms of the dried gels were prepared by exposure to X-ray film at -20°C.

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Abbreviations: MDR, multidrug resistance; FCS, fetal calf serum; PBS, phosphate-buffered saline; SDS, lauryl sulfate, sodium salt.
DR11, in pUC19 (ampicillin resistant). Blots were washed with a final stringency of 0.5x standard sodium citrate with 0.2% SDS at 60°C, followed by autoradiography.

**Immunofluorescence Assay**. In addition to G8-2, four other antibodies, which recognize different antigenic determinants in a variety of normal and leukemic cells and leukemic cell lines, were used to study the expression of surface markers. These monoclonal antibodies were purchased from Coulter Immunology (Hialeah, FL) and include 12, an HLA-DR clone based on the degree of reactivity of K562-R cells and absence of reactivity of K562-S cells, using the indirect immunofluorescence assay. Intensity of fluorescence was determined for 10,000 cells in each population and was compared with the fluorescence of a control nonreactive immunoglobulin (normal mouse IgG).

**Preparation of Monoclonal Antibody to K562-R Cells**. The monoclonal antibody, G8-2, was obtained by injecting to BALB/c mice i.p. of K562-R cells every week for 3 weeks; 3 days after the final injection, spleen cells were harvested and fused with NS-1 myeloma cells using 50% polyethylene glycol (23). Hybrid cells were selected in hypoxanthine-amethopterin-thymine medium. The initial screening procedure was performed by using an indirect immunofluorescent assay (see above) and cells were examined for reactivity under a fluorescent microscope. Expansion of the positive clones and subcloning were performed by using standard techniques (24). The selection of the final clone was based on the degree of reactivity of K562-R cells and absence of reactivity of K562-S cells, using the indirect immunofluorescence assay.

**Uptake and Retention of Daunorubicin**. K562-S and K562-R cells, at a concentration of 2 x 10⁶ cells/ml, were incubated with 1 μM daunorubicin in dextrose-PBS with 20 μM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4) for 2 h in a shaking water bath at 37°C. One-ml samples of cells were briefly centrifuged in microfuge tubes and pelleted (500 x g for 20 s); in order to minimize manipulation of the cells and to prevent leakage of intracellular daunorubicin, medium (containing drug) was thoroughly and completely removed from the pellet without pellet wash. Cells were resuspended in dextrose-PBS drug-free medium at 37°C and drug retention was monitored over 2-20 min. Quantitation of intracellular drug levels follows extraction of the drug in chloroform:methanol (2:1), followed by probe sonication. Cell fragments were removed, the organic layer was evaporated to dryness under nitrogen, and the extract was dissolved in 50 μl of methanol. Following isocratic elution from a prepacked 10-μm Bondapack phenyl column (3.9 mm x 30 cm) (Waters Associates, Inc.), using a mobile phase of 29% acetonitrile in 0.1 ammonium formate buffer at pH 4.0 and a flow rate of 2 ml/min (25), fluorescence was monitored with a Gilson Model 121 fluorometer, with an excitation wavelength of 470 nm and an emission wavelength of 585 nm. Peak areas were computed with a Shimadzu Model C-R3A integrator and quantitated by comparison to daunorubicin standards of known concentration. This assay was utilized because of its high sensitivity and capacity to quantitate drug levels in small volumes of cell extracts. No metabolites of daunorubicin were formed in the K562 cell lines.

**RESULTS**

**Morphology and Growth Characteristics of K562-S and K562-R Cell Lines**. The K562-S and K562-R cells have similar plastic morphology as determined by Wright stain, and demonstrate no erythroid differentiation as defined by negative benzidine staining (20). Growth characteristics of the two cell lines were similar, with a doubling time of 21 h for the K562-S and 23 h for the K562-R subline. Cell cycle analysis (Table 1) indicates that a similar proportion of cells were in G1, S, and G2-M phases in sensitive and resistant cells. Both cell lines were capable of forming colonies in soft agar in the absence of exogenous colony-stimulating factor(s), with a similar plating efficiency (10-15%).

**Analysis of Cross-Resistance of K562-R Cells to Chemotherapeutic Agents**. Table 2 demonstrates that K562-R cells are 15-fold more resistant to daunorubicin than K562-S cells; daunorubicin concentrations resulting in 50% inhibition of growth at 72 h were 815 nM and 55 nM for K562-R and K562-S cells, respectively. The K562-R cells also exhibit cross-resistance to Adriamycin, vincristine, and vinblastine (24-, 14-, and 9-fold, respectively, as compared to the K562-S cells). Effects of Verapamil on Daunorubicin Cytotoxicity. Table 3 illustrates the effects of verapamil on the cytotoxicity of daunorubicin in sensitive and resistant K562 cells. No changes in the number of colonies were observed when K562-R cells were incubated with 1.5 μg/ml of daunorubicin, but when verapamil (at nontoxic concentrations) was added to the incubation medium, a greater proportion of drug-resistant cells were able to form colonies.

**Table 1 Cell cycle analysis**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>G1</th>
<th>G2-M</th>
</tr>
</thead>
<tbody>
<tr>
<td>K562-S</td>
<td>46.6 ± 3.6</td>
<td>38.9 ± 3.0</td>
</tr>
<tr>
<td>K562-R</td>
<td>47.0 ± 2.5</td>
<td>36.1 ± 2.1</td>
</tr>
</tbody>
</table>

*Mean ± SE of 3 separate experiments.

**Table 2 Relative sensitivity of K562-R and K562-S leukemic cells to antineoplastic agents**

<table>
<thead>
<tr>
<th>Drugs</th>
<th>IC₅₀ (nM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>K562-S</td>
<td>85 ± 40</td>
</tr>
<tr>
<td>K562-R</td>
<td>35 ± 9</td>
</tr>
</tbody>
</table>

*Mean ± SE of 3 experiments.

**Table 3 Effect of a 2-h treatment with daunorubicin or daunorubicin + verapamil on colony-forming ability of K562-S and K562-R cells**

<table>
<thead>
<tr>
<th>Daunorubicin concentration (μg/ml)</th>
<th>Verapamil concentration (μg/ml)</th>
<th>Survival (% of control)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>K562-S</td>
<td>K562-R</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100 ± 9</td>
<td>100 ± 7</td>
</tr>
<tr>
<td>0.05</td>
<td>59 ± 6</td>
<td>37 ± 8</td>
</tr>
<tr>
<td>0.1</td>
<td>11 ± 4</td>
<td>8 ± 3</td>
</tr>
<tr>
<td>0.5</td>
<td>94 ± 8</td>
<td>89 ± 9</td>
</tr>
<tr>
<td>1.0</td>
<td></td>
<td>100 ± 7</td>
</tr>
<tr>
<td>1.5</td>
<td></td>
<td>100 ± 6</td>
</tr>
<tr>
<td>0.05</td>
<td>54 ± 5</td>
<td>39 ± 4</td>
</tr>
<tr>
<td>0.1</td>
<td></td>
<td>31 ± 7</td>
</tr>
<tr>
<td>0.25</td>
<td></td>
<td>10 ± 4</td>
</tr>
<tr>
<td>0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td></td>
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</tbody>
</table>

*Mean ± SE from triplicate experiments.
K562 DRUG-RESISTANT CELL LINE

dium containing 0.5 μg/ml of daunorubicin, the numbers of colonies decreased significantly. Similarly, verapamil reversed the resistance to vinblastine and vincristine in the K562-R cells (data not included). In the case of K562-S cells, the addition of verapamil had no effect on the number of colonies formed.

Analysis of Cell Surface Proteins. Surface membrane proteins isolated from K562-S and K562-R cells previously labeled with 123I were compared by using SDS-polyacrylamide gel electrophoresis. Autoradiograms presented in Fig. 1 demonstrate that the K562-R cell line expresses five distinct protein bands with molecular weights of 56,000, 67,000, 75,000, 116,000, and 210,000, respectively, proteins not observed in the K562-S cells. Expression of a Mr 170,000 protein (P-glycoprotein) was not observed in at least 10 different experiments, utilizing varied conditions and solubilization procedures (data not included).

Expression of a 4.5-Kilobase mRNA in the K562-R Cell Line. In order to determine whether the K562-R cell line expresses a 4.5-kilobase mRNA associated with the genomic sequences for MDR1, transcription analysis was performed with the 32P-labeled complementary DNA subclone pcDR1.3. It is clear from Fig. 2 that high levels of the 4.5-kilobase mRNA are expressed only in K562-R and not in K562-S cells.

Detection of Intracytoplasmic P-Glycoprotein. Indirect immunofluorescence microscopic analysis of K562-S and K562-R cells for P-glycoprotein demonstrated that approximately 45% of K562-R cells react with the C219 antibody as defined by the presence of intracellular fluorescence (data not shown); K562-S cells failed to react with the antibody.

Reactivity of K562-S and K562-R Cells with Monoclonal Antibodies. Binding of the G8-2, I2, Mo1, MY7, and MY9 monoclonal antibodies to K562 cells was analyzed with the fluorescence-activated cell sorter. K562-S and K562-R cells, incubated with culture supernatant containing monoclonal antibody, or with normal mouse IgG, were incubated with fluorescein-labeled Fab fragments from affinity-purified goat antimouse immunoglobulin. The two cell lines exhibit significant differences in their reactivity with some of the antibodies. The G8-2 antibody reacts only with the K562-R cell line (Fig. 3), while both cell lines are I2 negative. The K562-S cell line has fewer cells positive for the myeloid markers than the K562-R cells (Table 4).

![Fig. 1. Autoradiogram of 123I-labeled cell surface proteins from K562-S and K562-R cells. Electrophoresis was performed on 7.5% acrylamide slab gel in 0.1% SDS. An equal amount of radiolabel was loaded on both lanes. Migration of molecular weight standards was as indicated (molecular weight × 10^4).](image1)

![Fig. 2. Blot hybridization. Total RNA from K562-S and K562-R cell lines were hybridized with the 32P-labeled pcDR1.3 probe, as described in "Materials and Methods." kb, kilobase.](image2)
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Fig. 3. Fluorescence-activated cell sorter profile of the reactivity of the G8-2 monoclonal antibody with K562-S and K562-R cells.

Fig. 4. A, intracellular accumulation and retention of daunorubicin in K562-S and K562-R cells. Cells were initially incubated with daunorubicin (0.5 μg/ml) in dextrose-PBS in a shaking water bath at 37°C; after 2 h, the daunorubicin-containing medium was removed and the cells were resuspended in dextrose-PBS at 37°C in a shaking water bath for an additional 30 min. Samples from each cell line were obtained for high-performance liquid chromatography analysis after the initial 2-h incubation with daunorubicin (initial point), and at different times following incubation in drug-free medium. B, percentage of intracellular daunorubicin (initial point), and at different times following incubation in drug-free medium.

Table 4 Reactivity of monoclonal antibodies with K562-S and K562-R cells

<table>
<thead>
<tr>
<th>Antibody</th>
<th>K562-S</th>
<th>K562-R</th>
</tr>
</thead>
<tbody>
<tr>
<td>I2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Mo1</td>
<td>46 ± 5</td>
<td>65 ± 7</td>
</tr>
<tr>
<td>My7</td>
<td>4 ± 2</td>
<td>46 ± 4</td>
</tr>
<tr>
<td>My9</td>
<td>15 ± 4</td>
<td>40 ± 5</td>
</tr>
<tr>
<td>G8-2</td>
<td>1</td>
<td>82 ± 9</td>
</tr>
</tbody>
</table>

* Mean ± SE of 3 experiments.

Intracellular Accumulation and Retention of Daunorubicin.

Studies of intracellular accumulation and retention of daunorubicin in the K562-S and the K562-R line by using high-performance liquid chromatography indicated that no daunorubicin catabolites were formed in either cell line. A steady state level of intracellular daunorubicin was achieved in the K562-S and K562-R cell lines after approximately 1 h of incubation (data not shown). Accumulation of daunorubicin (1 μM) after 2 h of incubation is 4-fold greater in sensitive than resistant cells (Fig. 4A). Retention of daunorubicin in sensitive and resistant cells, after incubation for 10 min in drug-free medium, accounts for 52.4 and 17.2% of initial drug levels, respectively (Fig. 4B). Prolongation of incubation time results in a slight decline in intracellular drug level in both cell lines (47.4 versus 15.2% of drug retained in sensitive versus resistant line at 20 min). An initial rapid loss of drug in both cell lines suggests the presence of both slow and rapidly exchanging drug pools, the latter being more prominent in the resistant cell line.

Discussion

A daunorubicin-resistant subline of the K562 human promyelocytic leukemia cell line, initially described by Lozzio and Lozzio (14), has been isolated in vitro by sub culturing the parent line in gradually increasing concentrations of daunorubicin. K562-S and K562-R cells share a variety of biological characteristics. Both cell lines demonstrate similar morphology, with no erythroid differentiation as defined by negative benzidine staining (26). As previously described for other drug-resistant murine and human cell lines, (7–9), the K562-R cell line expresses cross-resistance to other anthracyclines, vincristine and vinblastine.

Recent studies have characterized specific membrane abnormalities in cell lines selected for MDR in vitro. The most well-documented biochemical change is the overexpression of a high-molecular-weight cell membrane glycoprotein (P-glycoprotein) which has been demonstrated in a variety of hamster, mouse, and human MDR cell lines (5–9). The molecular weight and immunological cross-reactivity of the P-glycoprotein is similar in different resistant cell lines (5, 6, 9, 27–30). The level of expression of the P-glycoprotein appears to be related to the degree of drug resistance; experimental evidence suggests that this glycoprotein may play a role in drug binding and reduced drug accumulation (31, 32). Photaffinity labeling experiments with radiolabeled vinblastine analogues have demonstrated direct binding of vinblastine to at least two M, 150,000–180,000 membrane proteins (33, 34).

Using iodination and SDS-polyacrylamide gel electrophoresis analysis of the K562-R plasma membranes, we demonstrate overexpression of at least 5 different proteins not detected in the sensitive cell line, ranging in molecular weight between 50,000 and 210,000; we do not detect the presence of the P-glycoprotein. However, using the complementary DNA clone, pcDR1.3 (21, 22), we were able to demonstrate significant overexpression of a 4.5-kilobase mRNA. Previous studies have shown that the mdrl gene encodes this 4.5-kilobase mRNA (whose level of expression correlates with the level of resistance), and that the P-glycoprotein is the product of the mdrl gene (11, 27). Since previous investigators (33, 34) have shown that calcium channel blockers may reverse MDR by inhibition of drug binding to P-glycoprotein, our findings of reversal of drug resistance by verapamil provide indirect support for the presence of overexpression of the P-glycoprotein in the K562-R cells. Furthermore, the K562-R cells also react with the monoclonal antibody C219 which is directed against the P-glycoprotein. This observation, and the reversal of drug resistance by verapamil, a calcium channel blocker, which may counter MDR by inhibition of the P-glycoprotein, indicate that the K562-R cell line has a phenotype similar to that of other MDR lines, and that the apparent lack of expression of the P-glycoprotein in the SDS-polyacrylamide gel electrophoresis assay may be due to its limited sensitivity.

Although a number of explanations have been advanced to account for the development of resistance to the anthracycline antibiotics, resistance has generally been associated with decreased intracellular drug retention (6, 10, 11). The present studies demonstrate a significant decrease in the intracellular accumulation and retention of daunorubicin in the K562-R cell line as compared to the K562-S cells.

The most striking findings in these transport studies is the very rapid loss of drug from both cell lines within the first few
minutes of incubation in drug-free medium with a much greater loss observed in the resistant cells. This observation is consistent with the concept that drug resistance in MDR is in part a readily exchangeable, loosely bound pool of drug which may be inaccessible in the resistant cell lines, it appears that differences in active outward efflux alone may not be sufficient to explain differences in drug retention. We and other investigators (37-40) have suggested the possibility that this early loss could also be explained, in part, by the existence of two intracellular drug compartments, a readily exchangeable, loosely bound pool of drug which may be larger in the resistant cells and a more tightly bound pool with tightly bound drug. Recent data from Hindenburg et al. (41) support this hypothesis, as this group has presented evidence for alterations in the intercompartmental distribution of daunorubicin and doxorubicin in an HL-60 anthracycline-resistant cells, which may account for decreased net accumulation and retention of these agents.

One of the most important differences between the K562-S and K562-R cell lines was demonstrated by their reactivity with the G8-2 antibody. This antibody is an IgG3 which recognizes a M, 67,000 protein expressed in the K562-R cell line but not in the K562-S cell line. Differences were also noted in the number of K562-R and K562-S cells expressing the myeloid markers MY7 and MY9. These findings suggest that the resistant cell lines may have a different degree of cell membrane differentiation as compared to the wild-type parent cell lines. In summary, this study has demonstrated significant differences in daunorubicin transport and retention in a K562-R cell line that expresses a number of surface membrane receptors and cell membrane proteins which differ from the sensitive parent line. The role and function of the different membrane proteins expressed in the K562 resistant cell line, and the possible use of the G8-2 antibody as a probe in the recognition of other cell lines expressing MDR and in the modification of drug transport, are being investigated.

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