

Sensitivity of K562 Human Chronic Myelogenous Leukemia Blast Cells Transfected with a Human Multidrug Resistance cDNA to Cytotoxic Drugs and Differentiating Agents

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Abstract

The blast crisis of chronic myelogenous leukemia (CML) is refractory to most forms of cancer chemotherapy, but may be amenable to drugs that differentiate rather than kill leukemic cells. One mechanism implicated in resistance to cytotoxic drugs is overexpression of P-glycoprotein, the *MDR1* gene product. While several classes of drugs sensitize multidrug-resistant (MDR) cells by interfering with the function of P-glycoprotein in vitro, few sensitizers have been effective in vivo. We have developed a preclinical model of MDR/CML uncomplicated by other mechanisms of drug resistance to evaluate the effects of *MDR1* overexpression on cytotoxic and differentiation therapy and the ability of sensitizers to restore chemosensitivity in this disease. The CML-derived cell line K562 was transfected with a human *MDR1* cDNA from the pHaMDR1/A expression vector and selected with vinblastine. Resistant K562 clones were 20–30-fold resistant to vinblastine, were cross-resistant to doxorubicin and etoposide, and remained sensitive to cytosine arabinoside, 6-thioguanine, hydroxyurea, and mechlorethamine. Resistance was associated with decreased cellular accumulation of cytotoxic drug and was reversed by cyclosporin A and *trans*-flupenthixol. The MDR phenotype did not adversely affect the ability of K562 cells to produce fetal hemoglobin in response to hemin, and was associated with increased responsiveness of cells to differentiate with cytosine arabinoside. Upon differentiation, the resistant clones increased *MDR1* mRNA and P-glycoprotein. These studies suggest that the overexpression of the *MDR1* gene in CML may not adversely affect the ability to undergo erythroid differentiation and that these resistant K562 cell lines are good models for studying drug resistance mediated by P-glycoprotein in CML. (*J. Clin. Invest.* 1993. 91:2207–2215.) **Key words:** multidrug resistance • differentiation • cyclosporin A • *trans*-flupenthixol

Introduction

The blast crisis of chronic myelogenous leukemia is resistant to treatment with standard cancer chemotherapy (1). Cross-resistance is seen to drugs from a variety of pharmacological classes, including natural products such as anthracyclines and vinca alkaloids, a situation reminiscent of the laboratory phenomenon of multidrug resistance.

Multidrug resistance (MDR)¹ is characterized by cellular resistance to several classes of chemotherapeutic drugs and is attributable, at least in part, to the overexpression of P-glycoprotein (reviewed in reference 2). *MDR1* is a member of a family of genes that include *MDR1* and *MDR2* (also referred to as *MDR3*) in humans, and appears to be the only human gene expressing a functional protein (3, 4). P-glycoprotein has structural and functional characteristics of an energy-dependent membrane transporter, and is believed to produce drug resistance through the active efflux of drugs from tumor cells (5).

Considerable attention has been given to identifying drugs that block the function of P-glycoprotein (reviewed in reference 6). Verapamil (7), trifluoperazine (7, 8), *trans*-flupenthixol (9, 10), tamoxifen (11), and cyclosporin A (12, 13) are examples of compounds that sensitize multidrug-resistant cells to chemotherapeutic drugs in vitro. However, few drugs have activity in vivo (6). There are several possible explanations for this lack of in vivo effectiveness, including the pharmacokinetics and pharmacodynamics of the sensitizers, or the presence of alternative mechanisms of drug resistance (14). In fact, the latter has been demonstrated in several cell lines selected for resistance by continuous exposure to drugs and may account for the inability of most modifiers to completely restore sensitivity of the resistant cells to that of the sensitive parental lines (6).

Recently, P-glycoprotein has been detected in a high percentage of patients in chronic myelogenous leukemia (CML) blast crisis (15, 16) and has been suggested as a possible cause of the refractoriness of this disease to treatment. To develop a model for identifying effective drugs against CML blast cells expressing P-glycoprotein and to study the effect of *MDR1* gene expression on cellular differentiation, we have transfected K562 cells, a human CML blast cell line (17), with a human *MDR1* cDNA (18). These studies demonstrate the cross-resistance pattern of the MDR clones, identify chemotherapeutic drugs that are not affected by P-glycoprotein in K562 cells, and highlight drugs that can sensitize these cells to chemotherapeutic

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1. *Abbreviations used in this paper:* CML, chronic myelogenous leukemia; MDR, multidrug resistance; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide.

tic agents. Furthermore, we found that the expression of the *MDR1* gene did not affect the ability of K562 cells to undergo erythroid differentiation in response to hemin or cytosine arabinoside, and that differentiation was associated with increased expression of P-glycoprotein.

Methods

Cell culture. K562 human CML blast cells were maintained in logarithmic growth in RPMI 1640 medium containing penicillin (100 U/ml) and streptomycin (100 µg/ml) supplemented with 10% FCS. They were checked routinely and found to be free of contamination by mycoplasma or fungi. Cells lines were discarded after 6 mo of continuous passage, and new lines were started from frozen stocks.

Liposome-mediated transfection of K562 cells with purified plasmid DNA (pHaMDR1/A). Plasmid DNA was purified from DH 5 α harboring the pHaMDR1/A cDNA (map shown in reference 18) after amplification with chloramphenicol (19). The DNA was banded in cesium chloride/ethidium bromide gradients. The plasmid DNA was prepared by phenol/chloroform extraction (19).

1×10^7 K562 cells were washed twice with serum-free medium and transfected with 10 µg of purified plasmid DNA using 100 µg of lipofectin reagent that was prepared immediately before use (20). The suspension cultures were diluted with 30 ml of RPMI 1640 medium supplemented with 10% FBS. The viability of the cells was monitored daily by trypan blue exclusion and dead cells were removed using lymphocyte separation medium. Viable cells were cloned in 0.12% agar for 14 d in the presence of 10 nM vinblastine. A dozen clones were picked and expanded in liquid culture. Three clones labeled K562/Vbl₁, K562/Vbl₂, and K562/Vbl₃ were randomly chosen for further study.

DNA isolation and Southern blot analysis. Genomic DNA from cultured cells was prepared by phenol-chloroform extraction (19). After digestion with HindIII, 15 µg of DNA from each cell line were electrophoresed in 0.8% agarose gels and transferred to nitrocellulose filters. The filters were prehybridized overnight at 42°C in 50% formamide, 5 \times Denhardt's reagent, 5 \times SSC (1 \times SSC contains 0.15 M NaCl and 0.015 M sodium citrate), 1% SDS, and 100 µg/ml denatured salmon sperm DNA. Hybridization was performed for 16 h in the same solution with the addition of 10% dextran sulfate and 10^7 cpm of cDNA probe, which was the 4.1-kb pHaMDR1/A insert containing the sequence for the *MDR1* cDNA labeled by the random primer method (21). After hybridization, filters were washed for 30 min sequentially with 2 \times SSC and 0.5% SDS at room temperature, with 2 \times SSC and 0.5% SDS at 60°C, with 0.5 \times SSC plus 0.1% SDS at 60°C, then with 0.25 \times SSC plus 0.05% SDS at 60°C. The final wash contained 0.25 \times SSC at room temperature. Blots were visualized by exposing Kodak X-Omat AR film to the filters at -70°C for 12–24 h using an intensifying screen.

Northern blot analysis. Total cellular RNA was isolated using guanidine thiocyanate-cesium chloride gradients (22). RNA samples containing 7% formaldehyde and 50% formamide were denatured by heating at 65°C for 10 min (19). Cytoplasmic RNA from each cell line was size fractionated through 1% agarose/7% formaldehyde gels and blotted onto nitrocellulose sheets. The prehybridization and hybridization conditions were the same as described above. RNA samples for dot blots were treated in a similar manner. The probe for the fetal A γ globin gene was a 0.6-kb cDNA labeled by the random primer method. A ³²P-labeled γ actin probe was used to ensure equal loading of RNA samples in each lane.

Detection of P-glycoprotein. P-glycoprotein was detected by Western blotting and by immunohistochemistry. Plasma membranes were prepared as previously described (23). Membrane proteins were dissolved in sample buffer for 10 min at room temperature and separated in 7.5% SDS-polyacrylamide gels. Transfer of proteins to nitrocellulose was carried out by the method of Towbin et al. (24). The blots were incubated in blocking solution consisting of 5% nonfat dry milk in

TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) at room temperature for 1 h followed by incubation at room temperature overnight with the C219 monoclonal antibody (0.2 µg/ml in TBST), which recognizes an epitope located on the internal plasma membrane. The immunoblots were visualized with goat anti-mouse IgG conjugated to alkaline-phosphatase.

1×10^7 viable cells for flow cytometry, were washed with PBS by centrifugation at 900 g for 10 min, resuspended in 12 ml of 70% methanol for 30 min at -70°C, then washed in PBS. The cells were incubated in 0.5 ml PBS and 5% normal rabbit serum with or without 3 µg/ml C219 monoclonal antibody. Cells were mixed at 4°C for 1 h, then washed three times in ice-cold PBS containing 1% BSA, then reincubated in 0.5 ml PBS and 5% normal rabbit serum containing 5 µl of fluorescein-conjugated goat anti-mouse antibody. Cells were incubated in the dark at 4°C for 45 min and then washed twice in PBS. Finally, cells were resuspended in 0.5 ml of PBS and analyzed using a flowcytometer (FACS IV[®] Becton-Dickinson, San Jose, CA) using an excitation wavelength of 488 nm and an emission wavelength of 520 nm.

Cytoprifuge preparations for immunohistochemistry were air dried and stored at -20°C in a desiccator until use. MRK-16 (Subclass IgG_{2a}) was used as the primary antibody and the biotin-avidin conjugated immunoperoxidase was used as the method of detection (25). Cells with a viability of > 95%, determined by trypan blue exclusion, were suspended in calcium and magnesium-free phosphate-buffered saline containing 0.05% BSA. They were incubated in a blocking solution of dilute normal horse serum (1:200 vol/vol PBS) for 20 min at 4°C, then for 30 min with MRK-16 or dilute normal mouse ascites in a concentration of 5–10 µg/ml. Slides were washed with PBS, incubated with biotinylated rabbit anti-mouse IgG for 30 min, then with biotin-avidin reagent followed by incubation in peroxidase solution, and finally counterstained with hematoxylin.

Inhibition of cell growth. The effect of drugs on the growth of K562 cells was determined by the 3-(4,5-dimethylthiazole-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay (26). Briefly, 3×10^4 cells/well were plated in 100 µl of growth medium in 96-well microtiter plates and allowed to equilibrate for 2 h. Drugs were diluted in medium and added in 100 µl volumes. After 72 h, 50 µl MTT solution (2 mg/ml PBS) was added, and cells were incubated an additional 4 h at 37°C. The medium was aspirated and 150 µl of 100% dimethylsulfoxide was added and plates were thoroughly mixed for 10 min. The optical density of each well was determined by absorbance spectrophotometry at 550 nm using an automatic plate reader (Dynatech Laboratories, Inc., Chantilly, VA). Assays were performed in quadruplicate. IC₅₀ values represent the concentration of drug or combinations of drugs that produced 50% inhibition of Abs₅₅₀ as compared to vehicle treated controls. The effect of drugs alone on the MTT reaction was taken into account in each assay.

The effect of chemosensitizers on drug resistance was studied by exposing cells to a concentration of chemosensitizer that alone produced $\leq 10\%$ inhibition of growth. Dose response curves were corrected for the inhibition of cell growth caused by chemosensitizers alone. The "fold reversal" of MDR for each drug plus chemosensitizer was calculated by dividing the IC₅₀ obtained for chemotherapeutic drug alone by the IC₅₀ obtained for chemotherapeutic drug plus chemosensitizer.

Cellular accumulation of doxorubicin. 2.5×10^6 cells in a total volume of 1.5 ml were incubated at 37°C with 0–20 µM doxorubicin for 3 h in the presence and absence of cyclosporin A (0.8 µM) or trans-flupenthixol (5 µM). Cells were washed three times in PBS by centrifugation at 900 g for 10 min, resuspended in 1.5 ml of 0.3 N HCl in 50% ethanol, sonicated with 10 pulses at 200-W s with a cell sonicator (Tekmar/Hereaus, Cincinnati, OH), and then centrifuged at 1,000 g for 30 min. The supernatant fraction was removed and assayed for doxorubicin content with a spectrofluorometer (model 512; Perkin-Elmer Corp., Norwalk, CT) using an excitation wavelength of 475 nm and an emission wavelength of 575 nm (8).

Effect of drugs on cellular differentiation. The expression of fetal γ globin mRNA and concentration of hemoglobin was used as markers of erythroid differentiation. The ability of hemin and cytosine arabinoside to induce hemoglobin production in K562 cells was assayed as previously described (27, 28). Cells were grown in RPMI 1640 medium at 37°C for varying periods of time with or without hemin or cytosine arabinoside, then collected by centrifugation at 900 g for 10 min, washed once with ice-cold PBS, resuspended at a concentration of 1×10^5 cells per μ l, and lysed in 5 mM sodium phosphate buffer (pH 7.4) containing 1% NP-40. The lysates were centrifuged at 12,000 g for 30 min at 4°C and hemoglobin measured by the tetramethylbenzidine method (28).

Statistical analysis. Statistical analysis of each dose response curve was performed by the method of Finney (29). The IC_{50} values \pm standard errors for the inhibition of cellular proliferation by drugs alone or in combination were determined by linear regression analysis of the logit-transformed data. The significance of each "fold reversal" was then determined by Student's *t* test and was expressed in terms of *P* values.

Drugs and chemicals. Cyclosporin A and *trans*-flupenthixol were supplied by Sandoz Laboratories (Sandoz Pharmaceuticals, East Hanover, NJ) and Dr. John Hyttel of H. Lundbeck (Copenhagen, Denmark), respectively. C219 and MRK-16 monoclonal antibodies recognizing P-glycoprotein, were generous gifts from Centocor Diagnostics, Inc. (Malvern, PA) and Dr. T. Tsuruo, Japanese Foundation of Cancer Research (Tokyo), respectively. The following compounds were purchased from commercial sources including: vinblastine, Eli Lilly Co. (Indianapolis, IN); doxorubicin, Ben Venue Laboratories (Bedford, OH); etoposide, Bristol Laboratories (Evansville, IN); Lambda HindIII, alkaline phosphatase-conjugated goat anti-mouse IgG, Promega (Madison, WI); a random primer DNA labeling kit, Boehringer Mannheim (Mannheim, Germany); [α - 32 P]-dCTP (3,000 Ci/mmol), Amersham Corporation (Arlington Heights, IL); a fetal A γ globin gene 0.6-kb cDNA was kindly supplied by Dr. Ajay Bhargava (Yale University, New Haven, CT); fluorescein-conjugated goat anti-mouse antibody from Becton Dickinson; MTT, dimethylsulfoxide, mechlorethamine, hydroxyurea, hemin, hemoglobin, 3,3',5,5'-tetramethyl benzidine, and cytosine arabinoside, Sigma Chemical Co., (St. Louis, MO); 2-amino-6-purinethiol, Aldrich Chemical Co. (Milwaukee, WI); lipofectin TM reagent, Bethesda Research Laboratories, (Gaithersburg, MD) and; γ actin probe, American Type Culture Collection (Rockville, MD). All other chemicals were of reagent grade and purchased from commercial sources.

Results

Transfection of K562 cells by pHaMDR1/A DNA. The transfected clones were 20–30-fold resistant to vinblastine, 23–35-fold resistant to doxorubicin, and three- to fivefold resistant to etoposide (Table I). The transfected clones retained their sensitivity to mechlorethamine, hydroxyurea, and 6-thioguanine. The nontransfected resistant clone, K562/Vbl₂, showed a similar pattern of resistance to that of the transfected cells.

Transfection of the pHaMDR1/A DNA resulted in the incorporation of the *MDR1* cDNA into genomic DNA in two of the three clones (Fig. 1). A 4.1-kb *MDR1* cDNA fragment prepared from the pHaMDR1/A vector by digestion with SacII and XhoI strongly hybridized to 4.3-kb and 2.2-kb DNA fragments in K562/Vbl₁ and Vbl₃ clones. There was no detectable hybridization to these fragments in the parental line or in the Vbl₂ clone. The hybridization pattern revealed no structural differences in the endogenous *MDR1* gene in drug-sensitive and -resistant cells. Resistant K562 clones also overexpressed *MDR1* mRNA whereas no significant hybridization

Table I. Effect of Chemotherapeutic Drugs on Sensitive and Resistant K562 Clones

Drug	Cell line			
	K562/S	K56S/Vbl ₁	K562/Vbl ₂	K562/Vbl ₃
	<i>IC₅₀</i> *			
Vinblastine (nM)	2.7 \pm 1.8	55 \pm 9	80 \pm 15	70 \pm 5.3
Doxorubicin (μ M)	0.4 \pm 0.1	9.2 \pm 4.5	9.9 \pm 3.6	14 \pm 6.5
Mechlorethamine (μ M)	2.2 \pm 0.5	2.2 \pm 0.3	2.0 \pm 0.2	6.7 \pm 1.9
Etoposide (μ M)	5.6 \pm 0.6	16 \pm 2.5	15 \pm 2.5	30 \pm 0.5
Hydroxyurea (mM)	3.0 \pm 1.1	4.0 \pm 0.6	6.4 \pm 0.9	4.6 \pm 1.1
6-Thioguanine (μ M)	1.2 \pm 0.2	0.5 \pm 0.2	0.9 \pm 0.3	0.8 \pm 0.5

* Values represent the mean \pm SEM from two to three experiments.

was seen with mRNAs from the parental cells (data not shown).

Resistant clones overexpressed P-glycoprotein as measured by several techniques. Fig. 2A demonstrates the results of Western blots using the C219 antibody, which recognizes an internal P-glycoprotein epitope. All three clones expressed the protein, although the nontransfected clone, Vbl₂, showed less reactivity. Results of flow cytometry using the C219 antibody and cell permeabilization are shown in Fig. 2B. Sensitive cells showed no additional fluorescence over background in the presence of antibody, whereas the resistant clone, Vbl₃, was clearly identifiable. Cells incubated with C219 alone, or with fluorescein-conjugated goat anti-mouse antibody alone produced no change compared to background fluorescence. Results of im-

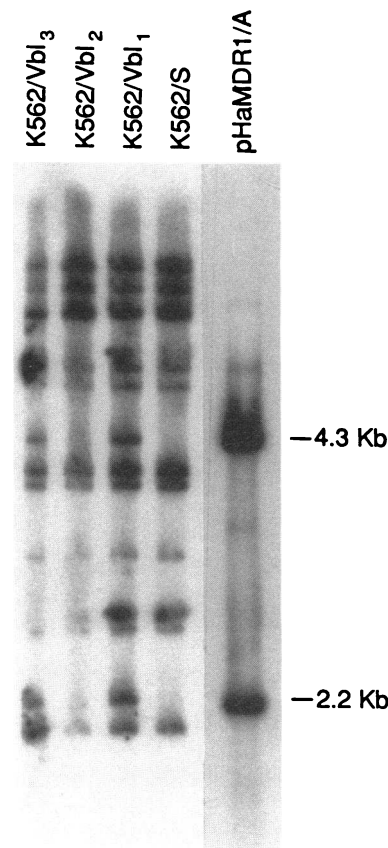


Figure 1. Identification of the *MDR1* cDNA in sensitive and resistant K562 cells by Southern blot analysis. 15 μ g of genomic DNA derived from each cell line were digested to completion with HindIII, electrophoresed in 0.8% agarose gels, transferred to nitrocellulose, and hybridized with the 4.1-kb 32 P-labeled *MDR1* cDNA probe as described in Methods. The DNA size markers are HindIII-digested λ -DNA.

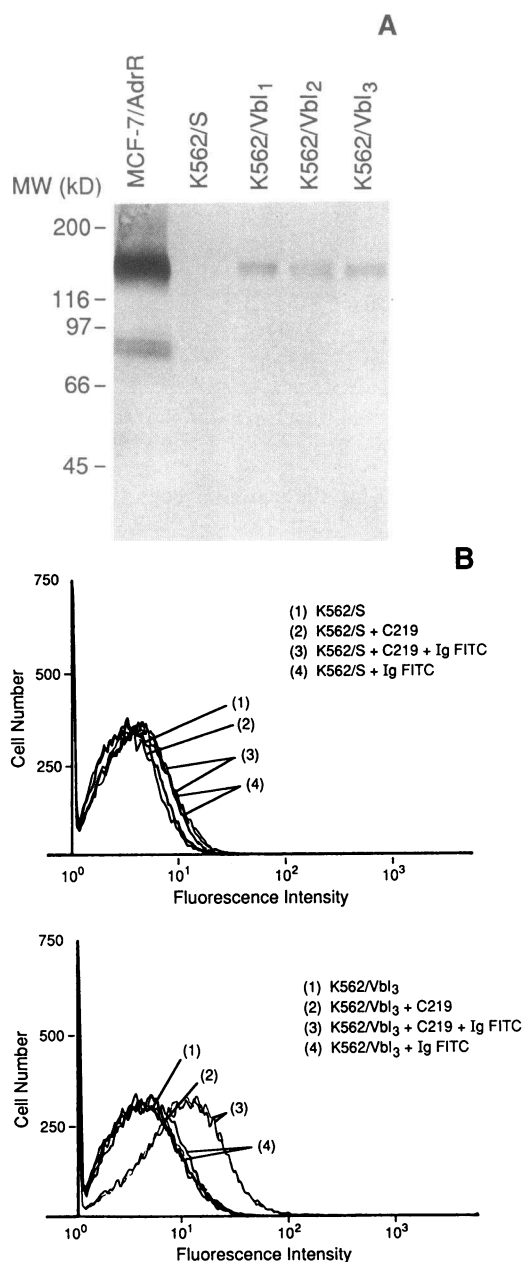


Figure 2. Expression of P-glycoprotein in sensitive and resistant K562 cells. (A) Immunoblots: Membrane proteins (50–100 μ g per lane) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with the C219 monoclonal antibody as described in Methods. Size markers were myosin, 200,000; *Escherichia coli* β -galactosidase, 116,250; Rabbit muscle phosphorylase b, 97,400; BSA, 66,200; hen egg white ovalbumin, 45,000; bovine carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; and hen egg white lysozyme, 14,400. MCF-7/AdrR cells were used as positive controls. (B) FACS[®] analysis. 1×10^7 cells were fixed in methanol, incubated in PBS containing 3 μ g/ml of C219 antibody then counterstained with 5 μ l of a fluorescein-conjugated goat anti-mouse antibody as described in Methods. FACS[®] analysis was carried out using excitation and emission wavelengths of 488 nm and 520 nm, respectively. *Top panel*, K562/S; *bottom panel*, K562/Vbl₃. (C) (Continued on next page) Immunohistochemistry. Cytoentrifuge preparations of sensitive and resistant cells were air dried then incubated in PBS containing 5–10 μ g/ml of MRK-16 monoclonal antibody and visualized by the avidin-biotin reaction as described in Methods. *Top panel*, K562/Vbl₃; *bottom panel*, K562/S.

munohistochemistry, using the MRK-16 antibody that recognizes an external P-glycoprotein epitope are shown in Fig. 2 C. All clones demonstrated immunoreactivity. In contrast, the sensitive parental line, K562/S, showed no evidence of expression of P-glycoprotein by Western blots or immunohistochemistry.

Cross-resistance patterns and effects of chemosensitizers. *Trans*-flupenthixol and cyclosporin A sensitized the resistant cells to doxorubicin and vinblastine (Table II). Cyclosporin A was more potent and more effective than *trans*-flupenthixol. For example, 0.8 μ M cyclosporin A sensitized the resistant cells to doxorubicin by 27–47-fold and to vinblastine by 12–26-fold. In contrast, 5 μ M *trans*-flupenthixol sensitized the resistant cells to doxorubicin by 6–12-fold and to vinblastine by 8–10-fold. The chemosensitizers had no significant effect on the parental line.

Drug accumulation in the transfected clones. Sensitive K562 cells accumulated twice as much doxorubicin as resistant cells (Fig. 3). *Trans*-flupenthixol and cyclosporin A increased the cellular accumulation of doxorubicin to concentrations approximately equal to that of the parental line (Fig. 3). Chemosensitizers had no effect on the accumulation of doxorubicin in the sensitive lines.

Influence of the MDR phenotype on erythroid differentiation. Sensitive and resistant K562 cells were equally sensitive to the effects of hemin on erythroid differentiation (Fig. 4). Fig. 4 A demonstrates that fetal γ globin mRNA increased in both K562/S and K562/Vbl₃ cells after exposure to hemin. Hemoglobin content increased with increasing concentrations of hemin in both cell lines until the concentration of hemin exceeded 25 μ M (Fig. 4 B). At 50 μ M, hemin was more effective in inhibiting the growth of parental cells (data not shown) while being more effective in inducing hemoglobin production in resistant cells (Fig. 4 B).

We also studied the effects of cytosine arabinoside on erythroid differentiation in K562 cells. Fig. 5 demonstrates that the sensitive K562/S cells and the resistant K562/Vbl₃ cells were equally sensitive to growth inhibition by cytosine arabinoside (Fig. 5 A), and that the resistant cells were more sensitive to the effects of this drug on erythroid differentiation (Fig. 5 B).

To study the effect of differentiation on the expression of the *MDR1* gene, we measured the expression of *MDR1* mRNA and P-glycoprotein after exposure to hemin or cytosine arabinoside. These compounds had no effect on the expression of *MDR1* mRNA or P-glycoprotein in the sensitive cell line. In contrast, hemin increased the expression of *MDR1* mRNA by 130–290% (Fig. 6 A) and cytosine arabinoside increased the expression of *MDR1* mRNA by 140% (Fig. 7 A). Similarly, treatment with hemin (Fig. 6 B) or cytosine arabinoside (Fig. 7 B) selectively increased the expression of P-glycoprotein in the resistant cells.

Discussion

The transfection of K562 cells with a human *MDR1* cDNA produced clones of cells that display the MDR phenotype. During the selection process, we also isolated a clone that was multidrug resistant, but unlike K562/Vbl₁ and K562/Vbl₃, clone K562/Vbl₂ had not incorporated the transfected *MDR1* cDNA (Fig. 1). All clones express P-glycoprotein when mea-

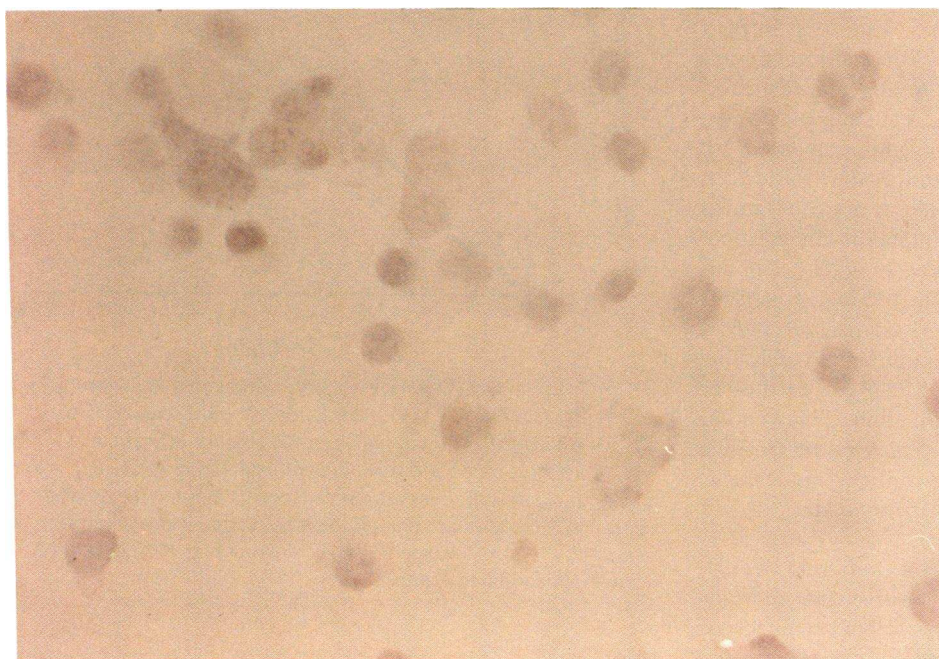
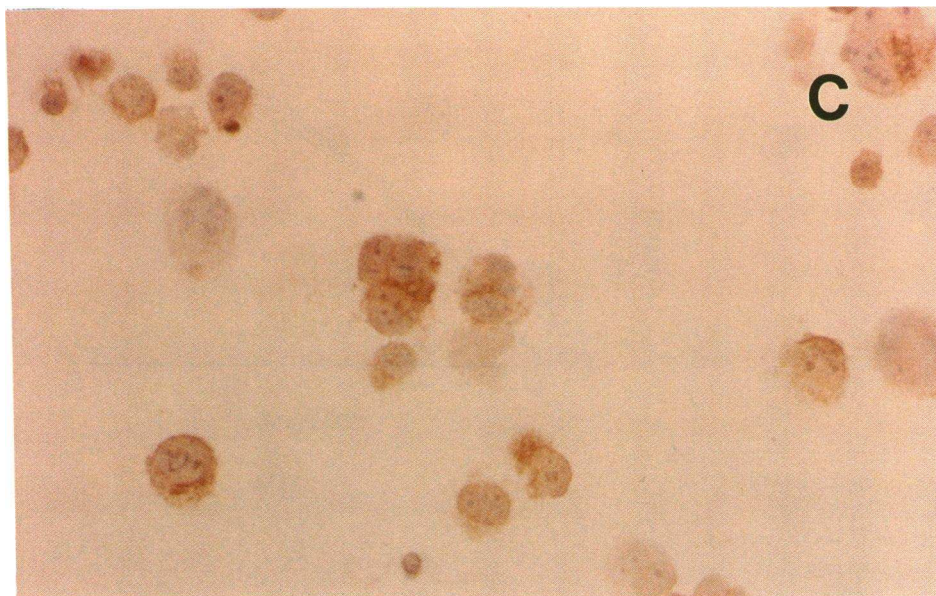


Figure 2 (Continued)

sured by immunoblotting (Fig. 2 *A*), by flow cytometry (Fig. 2 *B*), or by immunohistochemistry (Fig. 2 *C*). These results point out that even a brief exposure to vinblastine can give rise to resistant clones of cells expressing the endogenous *MDR1* gene. The evaluation of P-glycoprotein by immunohistochemistry in these lines is simple and sensitive, since the two antibodies against P-glycoprotein are of mouse origin, and therefore, the secondary antibodies directed against the mouse immunoglobulin do not produce high background staining of the human cells.

All three clones display the MDR phenotype (Table I) and maintain the phenotype for ≤ 2 mo when grown in drug-free medium. The pattern of cross-resistance in the transfected and the nontransfected clones are similar. They are most resistant to vinblastine and doxorubicin and are less resistant to etoposide.

These studies demonstrate that K562 human CML blasts expressing P-glycoprotein are as sensitive to cytosine arabinoside (Fig. 5 *A*), mechlorethamine, 6-thioguanine, and hydroxyurea as the parental line (Table I). Since these drugs fail to

Table II. Effect of *Trans*-flupenthixol and Cyclosporin A on the Sensitivity of Parental and Resistant K562 Clones to Doxorubicin and Vinblastine

Cell line	Drug	Fold reversal	
		<i>Trans</i> -flupenthixol	Cyclosporin A
K562/S	Doxorubicin	0.6*	1.3*
K562/Vbl ₁	Doxorubicin	10 [§]	47
K562/Vbl ₂	Doxorubicin	6 [§]	33 [§]
K562/Vbl ₃	Doxorubicin	12 [‡]	27 [§]
K562/S	Vinblastine	0.4*	1.0*
K562/Vbl ₁	Vinblastine	8	26 [§]
K562/Vbl ₂	Vinblastine	10 [‡]	15 [‡]
K562/Vbl ₃	Vinblastine	9 [‡]	12 [§]

Cells were exposed to doxorubicin (0.1–33 μ M) or vinblastine (0.1–200 nM) in the absence or presence of *trans*-flupenthixol (5 μ M) or cyclosporin A (0.8 μ M). Fold reversal represents the IC₅₀ of the cytotoxic drug alone divided by the IC₅₀ of the cytotoxic drug in the presence of the chemosensitizer. Each value represents the mean of at least three experiments. * Not significant compared to cytotoxic drug alone. [‡] $P < 0.05$. [§] $P < 0.01$. ^{||} $P < 0.001$.

produce sustained remissions in patients with CML in blast crisis, these data strongly suggest that additional mechanisms of resistance are present in the clinical setting, and that overcoming resistance mediated by P-glycoprotein may only be the first step toward improving treatment. An important feature of the resistant K562 clones, whether transfected or selected, is that resistance mediated by P-glycoprotein is not complicated by increased cellular content of glutathione or altered topoisomerase II (Hait, W. N., and Y.-c. Cheng, unpublished observations), two other mechanisms known to produce cross-resistance to chemotherapeutic drugs (30, 31). Since selection of the clones required growth in low concentrations of vinblastine for short periods of time, we have not totally excluded other changes such as altered microtubular function (32). In addition, because of the low IC₅₀ for doxorubicin in the sensitive clone (0.4 μ M), it was not possible to compare intracellular accumulation of doxorubicin between sensitive and resistant clones at equitoxic drug concentrations. Finally, since PCR analysis was not done, one cannot definitively rule out the possibility that differences between sensitive and resistant clones were caused purely by differences in the expression of P-glycoprotein. However, the pattern of cross-resistance, the defect in cellular accumulation of drug (Fig. 3), and the restoration of sensitivity by the chemosensitizers (Table II) strongly suggest that expression of P-glycoprotein is the major cause of the MDR phenotype in these cells.

Trans-flupenthixol and cyclosporin A increase sensitivity of resistant K562 cells to vinblastine, doxorubicin, and etoposide (Table II). Cyclosporin A fully sensitized both the transfected and nontransfected K562 cell lines, whereas *trans*-flupenthixol was somewhat less effective (Table II). At higher concentrations, *trans*-flupenthixol displayed intrinsic cytotoxicity, making it impossible to distinguish this effect from its effect on P-glycoprotein mediated drug resistance.

The pattern of reversal seen in transfected clones did not consistently differ from that observed in the selected clone. For

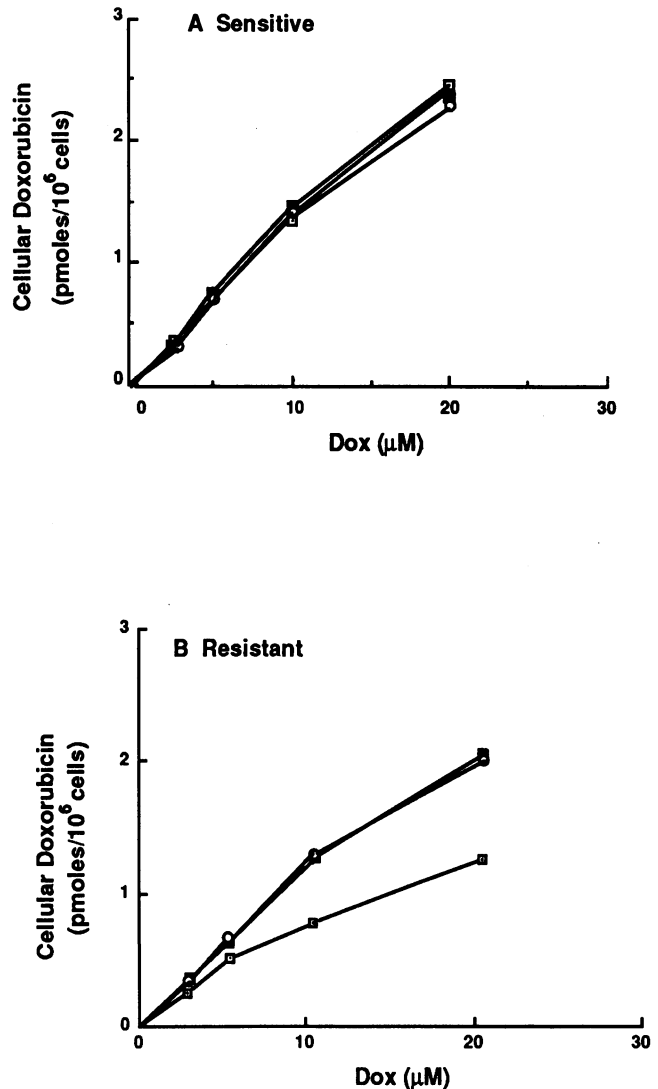


Figure 3. Effect of chemosensitizers on accumulation of doxorubicin in sensitive and resistant K562 cells. 2.5×10^6 cells were incubated for 3 h with 0–20 μ M doxorubicin in the absence or presence of 0.8 μ M cyclosporin A or 5 μ M *trans*-flupenthixol. Cell-associated doxorubicin (pmol/ 10^6 cells) was determined spectrofluorometrically as described in Methods. Each point is the mean of duplicate determinations. —□—, Doxorubicin; —■—, doxorubicin + cyclosporin A; —○—, doxorubicin + flupenthixol.

example, Vbl₂ (selected) was less affected by *trans*-flupenthixol plus doxorubicin than Vbl₃ (transfected), yet Vbl₂ was more sensitive to cyclosporin plus vinblastine than Vbl₃. Furthermore, Vbl₂ was at least as sensitive to *trans*-flupenthixol plus vinblastine as both transfectants and as sensitive to cyclosporin plus vinblastine as Vbl₃.

Trans-flupenthixol and cyclosporin A produce identical increments in drug accumulation in the Vbl₃ cells (Fig. 3), yet cyclosporin A supersensitizes these cells to chemotherapeutic drugs (Table II). Similar results were obtained with all clones studied. *Trans*-flupenthixol and cyclosporin A are believed to work through interference with the function of P-glycoprotein, since both chemosensitizers can displace [³H]azidopine from P-glycoprotein (10, 33). The current results support previous

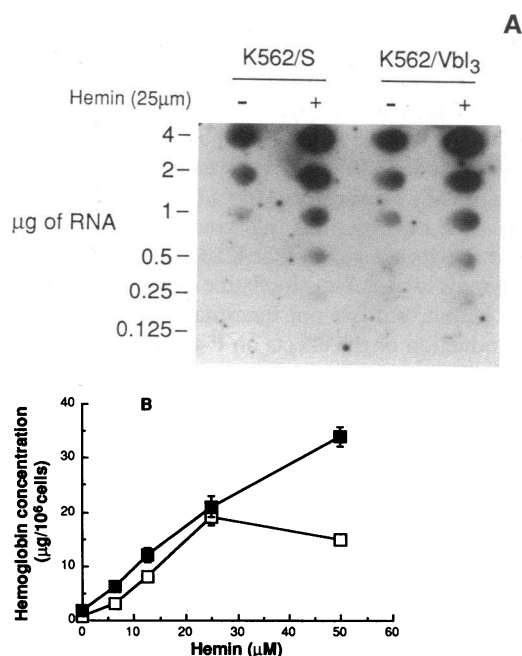


Figure 4. Effect of hemin on the differentiation of K562 cells. (A) Effect on fetal γ globin RNA in sensitive and resistant K562 cells: After exposure of sensitive and resistant K562 cells to 25 μ M hemin for 4 d, RNA was prepared as described in Methods. 0.125–4 μ g of RNA was blotted onto nitrocellulose and probed using a fetal A γ globin 0.6-kb cDNA labeled by the random primer method. (B) Effect on hemoglobin content: 1.5×10^5 cells/ml were incubated for 4 d in the presence of 6.25–50 μ M hemin or vehicle. Hemoglobin content was determined using tetramethylbenzidine as described in Methods. Each point represents the mean \pm SEM of three experiments each run in triplicate. — \square — K562/S; — \blacksquare —, K562/Vbl₃.

observations by us and others that cyclosporin A appears to have additional mechanisms of action. For example, cyclosporin A increased the sensitivity of “nonresistant” cells to

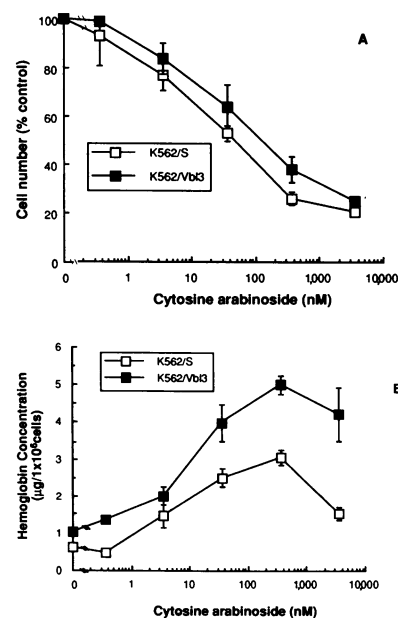


Figure 5. Effect of cytosine arabinoside on the growth and differentiation of K562 cells. 1.5×10^5 cells/ml were incubated for 4 d in the presence of 0.36–3,600 nM or vehicle (media). After 4 d, cell number was determined by electronic counting (A) and hemoglobin content with tetramethylbenzidine (B) as described in Methods. Each point represents the mean \pm SEM of three experiments each run in triplicate.

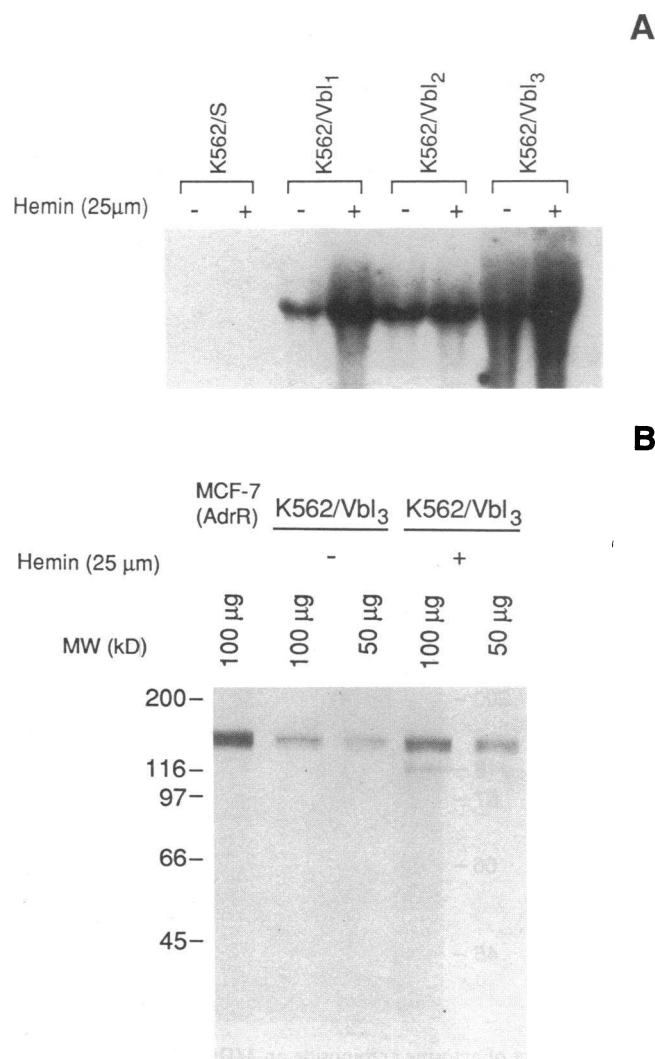


Figure 6. Effect of hemin on *MDR1* gene expression in sensitive and resistant K562 cells. (A) *MDR1* mRNA: After exposure of sensitive and resistant K562 cells to 25 μ M hemin for 4 d, RNA was prepared as described in Methods. 20 μ g of RNA was electrophoresed in 1% agarose/7% formaldehyde gels then transferred to nitrocellulose. Blots were probed using a 4.1-kb *MDR1* cDNA insert prepared from the pHaMDR1/A retroviral vector. (B) P-glycoprotein. K562/Vbl₃ cells were exposed to 25 μ M hemin for 4 d. Membranes were prepared as described in Methods. Proteins were solubilized and electrophoresed on 7.5% SDS gels, transferred to nitrocellulose and probed with the C219 antibody. MCF-7/AdrR cells were used as positive controls.

anthracyclines (34, 35) and altered membrane potentials (36) and synthesis of nucleolar proteins (37).

Expression of P-glycoprotein in K562 cells does not interfere with erythroid differentiation. This was true in both the transfected (K562/Vbl₁ and Vbl₃) and nontransfected clones (K562/Vbl₂). In fact, the resistant cells produce more hemoglobin in response to cytosine arabinoside than the parental cells (Fig. 5 B). This suggests that *MDR1* gene expression per se does not interfere with early steps in hematopoietic cellular differentiation. This data may be relevant to ongoing attempts to create multidrug resistant bone marrow by transfection of the *MDR1* gene into early hematopoietic precursors, since suc-

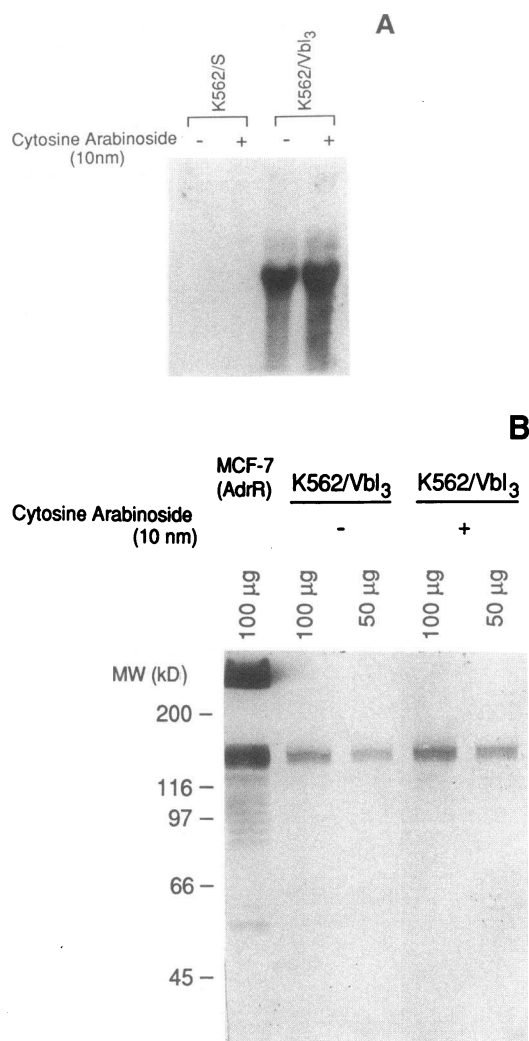


Figure 7. Effect of cytosine arabinoside on *MDR1* gene expression in sensitive and resistant K562 cells. (A) *MDR1* mRNA. After exposure of sensitive and resistant K562 cells to 10 nM cytosine arabinoside for 4 d, 20 μ g RNA was prepared and blots were probed as described in Methods and in Fig. 6. (B) P-glycoprotein. K562/Vbl₃ cells were exposed to 10 nM cytosine arabinoside for 4 d. Membranes were prepared, blotted, and probed as described in Fig. 6.

cessful marrow reconstitution would depend on normal proliferation and differentiation.

Because of the ability to induce erythroid differentiation in the resistant K562 clones, we were able to study the effect of differentiation on the expression of the *MDR1* gene. Differentiation along the erythropoietic pathway increased the production of *MDR1* mRNA (Figs. 6 A and 7 A) and the synthesis of P-glycoprotein in resistant cells, but had no effect on the expression of this gene in the sensitive line (Figs. 6 B and 7 B). The increase in *MDR1* was most pronounced in clone Vbl₁. The induction of *MDR1* is complex and may be related to cellular stress induced by heat shock, heavy metals, or DNA damage as described by Chin et al. (38, 39), or through the induction of protein kinase C (40). It will be interesting to determine whether these responses differ in the Vbl₁ clone compared to the others.

These studies are consistent with those of Biedler and col-

leagues, who found that multidrug resistant neuroblastoma cells induced to differentiate with retinoic acid increased expression of P-glycoprotein (41), and with those of Fojo's group, who found increased P-glycoprotein in colon cancer cells induced to differentiate with sodium butyrate (42). These data also show that induction of differentiation in certain cells that do not intrinsically overexpress *MDR1* (i.e. K562/S cells) does not induce the expression of this gene product. Okabe-Kado et al. had previously shown that K562 cells selected for resistance by chronic exposure to vincristine could differentiate in response to hemin, but did not quantitate the changes in hemoglobin production or the effects on P-glycoprotein (43). Furthermore, unlike previous studies the current results are unlikely to be confounded by cellular alterations that occur when cell lines are selected for long periods of time in cytotoxic drugs.

An alternative explanation of these findings is that the drugs that induce differentiation are substrates for P-glycoprotein, and therefore, further upregulate the drug-efflux pump as a protective mechanism, rather than as a consequence of differentiation. In fact, the resistant cells were less sensitive to the growth inhibitory effects of hemin than the resistant ones. However, this explanation seems unlikely, since sensitivity to cytosine arabinoside is not affected by P-glycoprotein (Fig. 5 A), yet this drug also increased *MDR1* expression (Fig. 7).

Studies with transfected K562 cells may provide direction for selecting drugs for the treatment of the blast crisis of CML. For example, it would be important to determine whether treatment of patients with cyclosporin A plus an anthracycline is superior to that of the anthracycline alone, or if a combination of non-cross-resistant drugs, such as cytosine arabinoside and 6-thioguanine given with an anthracycline, or etoposide plus a chemosensitizer, is superior to currently available treatments. In addition, these results suggest that the MDR phenotype does not produce cross-resistance to certain differentiating agents, and offers a potential alternative to cytoreductive therapy in drug-resistant CML. Therefore, the ultimate test of the current observations will require carefully designed clinical trials that rigorously evaluate the presence of P-glycoprotein in CML blasts and the effect of chemosensitizers used with drugs affected by this form of drug resistance.

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