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Isolation and Preliminary Characterization of Doxorubicin-Resistant Human Myelogenous Leukemia K562

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Abstract

We have produced doxorubicin (DXR)-resistant variant cells (K562/DXR) of the human myelogenous leukemia K562. K562/DXR cells were stable for 2 months in medium without DXR, and were 5-fold more resistant to DXR than the parent K562 cells. K562/DXR cells showed reduction of cellular content of DXR after a 1-hr exposure to DXR when compared with control. During prolonged incubation of control and resistant cells in 1 μM/mL DXR, the DXR content was 40% decreased in resistant cells. The DXR-resistant cells were also resistant to actinomycin D, etoposide and cholchicine, some what resistant to aclarubicin but not resistant to cisplatin and idarubicin. Verapamil, an inhibitor of calcium transport, partially reversed the multiple drug-resistance phenotype by increasing the initial rate of uptake and accumulation of drugs in K562/DXR without an apparent effect on drug efflux. Furthermore, efflux of rhodamine 123 (indicated by increased fluorescent dye that is a substrate for P-glycoprotein and rapidly transported out of multidrug-resistant cells) occurs rapidly from K562 and even more rapidly from K562/DXR. These results support the hypothesis that the development of resistance to DXR in K562 cells is due to a qualitative difference in cell membrane, resulting in an increased permeability to DXR and other compounds from the cell by P-glycoprotein.

Key word —— doxorubicin, human chronic myelogenous leukemia cell (K562), doxorubicin-resistant cells (K562/DXR), cytotoxicity, rhodamine 123 uptake

Introduction

The anthracycline antibiotic doxorubicin (DXR) is one of the most widely used of all anticancer agents because of its broad spec-

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trum of antitumor activity, but the effectiveness of this agent is limited by the equivalent development of drug-resistant cell variants. DXR-resistant tumor cells may show resistance to multiple chemotherapeutic agents. Investigations into the mechanism of multiple drug resistance have utilized the development of cell lines resistant to DXR.

A number of mechanisms of resistance have been identified including defective drug accumulation due to increased active efflux[1], increased intracellular glutathione[2] and decreased amounts of cellular enzymes[3]. DXR resistance is closely associated with the phenomenon of pleiotropic or multidrug resistance[4] where collateral resistance to vincristine and colchicine is observed. This form of resistance is thought to be associated with the presence in the cell membrane of a glycoprotein of 170K molecular weight (P-glycoprotein) and gene amplification[5,6].

As part of our laboratory studies of drug resistance in human leukemia, we wished to produce DXR-resistant sublines of human leukemia cell line. In this paper we describe the derivation of such lines and their partial characterization with the aim of studying the mechanism of multidrug-resistance in a human cell line.

The K562 cell line is a candidate for test of synergy or multidrug resistance; it was derived from a patient with chronic myelogenous leukemia (CML). Like the majority of human CML, K562 cells possess a genotypic abnormality involving dysregulation of the p210 tyrosine kinase activity of the bcr-able fusion oncoprotein[7]. The expression of $p210^{bcr-abl}$ in K562 cells is mainly responsible for their resistance to differentiation and drug-induced apoptosis[7-9]. In addition, the gene products of the antiapoptosis Bcl-x_L and Bcl-2 regulate drug-induced apoptosis[10]. For example, enforced expression of Bcl-x_S in K562 cells significantly enhances the $p210^{bcr-abl}$-mediated inhibition of apoptosis induced by antitumor agents[11].

Materials and Methods

Drugs and chemicals

All chemicals were purchased from Sigma Chemical Co., St. Louis, MO, except where otherwise indicated. All cytotoxic drugs except etoposide and aclarubicin were dissolved in distilled water at 500 μg/mL, filtered via a Millipore membrane (pore size 0.2 μm) and stored in aliquots at −20°C. Etoposide and aclarubicin were dissolved in dimethyl-sulfoxide (Nacalai Tesqu, Inc., Kyoto, Japan) at a concentration of 1 mg/mL. The final concentration of dimethylsulfoxide (0.5% v/v) was shown not to affect drug sensitivity or cell growth. Drugs were thawed and diluted in PBS immediately before use. Cisplatin (500 μg/mL) and cepharanthine (Kaken Natural Products Co. Ltd., 5.0 mg/mL) were obtained in aqueous solution and diluted in PBS. Solutions containing verapamil were protected from light at all times by tin foil wrapping. Idarubicin was kindly supplied by Pharmacia & Upjohn (Milano, Italy).

Cell line and culture conditions

A human chronic myelogenous leukemia cell line, K562 was obtained from Cancer Cell Repository (Tohoku University). Cells were grown in Rosewell Park Memorial Institute Medium 1640 (RPMI-1640, Gibco/BRL Ltd.), supplemented with 10% (v/v) fetal calf serum (FCS, Gibco/BRL Ltd.,) and 1% (v/v) penicilline/streptomycin solution (Sigma). Cultures

were maintained in a humidified incubator at 37°C in a controlled 5% CO_2/95% air atmosphere and were used for experiments during the exponential phase of growth. The K562 cell line resistant to DXR used in our study was selected in the presence of increasing doses of DXR without prior mutagenization. These resistant cells were termed K562/DXR. The K562/DXR cell line was maintained in medium containing 1 μM/mL DXR. The DXR was washed out at least 2 day before the experiments.

Evalution of cell survival by MTT Assay

The MTT [3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium] colorimetric assay performed in a 96-well plate was used for an *in vitro* chemosensitivity test[12]. The assay is dependent on the reduction of MTT by the mitochondrial dehydrogenase of viable cells to a blue formazan product which can be measured spectrophotometrically. Briefly, cells were inoculated into each well of 96-well plates with 50 μL of the culture medium at 1×10^5 cells. After an overnight incubation, 50 μL of antitumor drug solution, at final concentrations of indicated dose were added in triplicate and the plates incubated for 24 hr. Then 10 μL of MTT (5.0 mg/mL phosphate-buffered saline) was added to each well and incubated for 2 hr. The resulting formazan was dissolved with 100 μL of dimethylsulfoxide (DMSO) after aspiration of the culture medium. Plates were placed on a plate shaker for 5 min and read immediately on an ELISA reader (Immuno Mini NJ-2300, Inter Med) at 570 nm. Controls for the MTT assays were as follows: medium alone served as controls for experiments using DXR and medium with 0.5% dimethylsulfoxide served as controls for experiments using etoposide. The median concentration inhibiting tumor cell growth by 50% (IC_{50}) was determined by plotting the logarithm of the drug concentration versus the growth rate (percentage of control) of the treated cells.

Measurement of cellular uptake of doxorubicin by flow cytometry

For determination of flow cytometric intracellular DXR concentration, a FACScan (Becton Dickinson, Mountain View, CA) equipped with an argon laser using a 488-nm line operating at 15 mM was used. Light was collected through a 585/20-nm filter for the DXR uptake and retention studies. For uptake studies, a concentration of 1×10^6 cells/mL were incubated with DXR at a concentration of 1 or 10 μM/mL. After 60 min of incubation, cells were washed twice in ice-cold phosphate-buffer saline (PBS) without Ca^{2+} and Mg^{2+} (Gibco/BRL) and resuspended in 0.5 mL ice-cold PBS with 5% FCS. DXR fluorescence was immediatelly measured using the FACScan with an appropriate filter.

Effect of resistance modifiers

To determine whether the addition of verapamil and cepharanthine significantly increased intracellular DNA retention, cells were incubated for 1 hr with DXR at a concentration of 1 or 10 μM/mL plus verapamil 30 μM/mL or cepharanthine 2.0 μg/mL; cells were then washed twice and resuspended in fresh medium containing verapamil or cepharanthine. These concentrations were previously selected by us to represent two to three times the clinically achievable peak level, and to cause a 20-fold increase in DXR-sensitization in the H-69/LX4 line and Ch^R-24[13,14]. Control tumors were set up simultaneously containing 100 μL of the appropriate solvent. Flow cytometric analysis was then performed as above using the appropriate filter. Experiments were performed 3

times. Controls were medium alone and medium with verapamil 30 μM/mL or cepharanthine 2.0 μg/mL.

Measurement of rhodamine 123 uptake by flow cytometry

We essentially followed the procedure described by Twentyman et al.[15]. Briefly, 1×10^5 cells were seeded onto plates and incubated for 1 day. Rhodamine 123 (Sigma) was dissolved in water and added at a final concentration of 1.0 μg/mL. After 1 hr, 500 μL of cells were taken, washed with medium once, and resuspended in 500 μL of medium. Viable cells were analyzed for the accumulation of rhodamine 123 on a Beckton Dickinson flow cytometer.

Results

Isolation of a K562/DXR cell line

By increasing DXR concentrations stepwise (2-3 nM at a time) in the culture medium as described under Materials and Methods, a K562 cell line tolerant to chronic 1 μM DXR concentration was selected which had viability and growth characteristics similar to those of the parental K562 cells. Survival curves for K562/DXR revealed a 5.0-fold enhanced resistance to DXR compared to the parent line. The IC_{50} values are 0.7 and 3.5 μM against K562 and K562/DXR cells, respectively. A K562 cell chronically tolerant to 1 μM DXR also showed stable growth characteristics, although K562/DXR had a lightly longer doubling time. The doubling times of K562 and K562/DXR were in the range of 18-20 hr (Fig. 1 and Table 1).

Stability of resistance

We tested the resistance to DXR of subline K562/DXR after 3 and 9 weeks growth in the absence of drug. K562/DXR cells were stable for at least 2 months during culture without DXR. At 3 weeks the IC_{50} of DXR for K562/

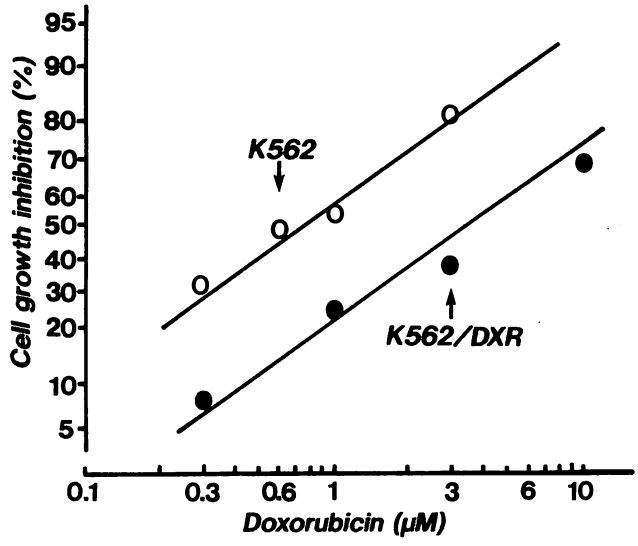

Fig. 1 Effect of Doxorubicin on the Survival of K562 and K562/DXR cells
Cells were inoculated in plates and incubated for 24 hr at 37°C. Doxorubicin at the indicated concentration was then added and incubated for 1 day before MTT assay as described in "Materials and Methods".

Table 1. Drug Concentration at Which Resistant Lines were Maintained, Response of Cell Lines to Drug in Terms of IC_{50}, and Degree of Resistance and Cross-Resistance

Cell line	IC_{50} (μM/mL)	Degree of resistance to						Population doubline time (hr)
		DXR	ACD	COL	ETP	IDR	CDDP	
K562	0.7	1	1	1	1	1	1	18
K562/DXR	3.5	5.0	5.6	6.7	5.9	1.0	1.0	20

Cells were cultured for 24 hr with increasing drug concentrations. Cells viability was assessed as described in "Materials and Methods". IC_{50} is expressed as the drug concentration required to inhibit cell growth by 50% as compared to the control (cells cultured without antitumor drugs). Resistance is expressed as the ratio of IC_{50} values for resistant line (K562/DXR) to parental line (K562); ratios were calculated as the antilog of the difference between log IC_{50} values obtained by computer. The relative resistance is the mean of duplicate determinations. Abbreviations: DXR; doxorubicin, ACD: actinomycin D, COL: colchicine, ETP: etoposide, IDR: idarubicin, CDDP: cisplatin

DXR (drug free culture) was 2.6 μM/mL compared with 0.7 μM/mL for parental line K562 and 3.5 μM/mL for K562/DXR maintained in DXR. After 9 weeks the IC_{50} of DXR for K562/DXR (drug free culture) was 3.0 μM/mL compared with 0.8 μM/mL for K562 and 3.4 μM/mL for K562/DXR maintained in DXR. The resistance factors [i.e. IC_{50} (resistant line)/IC_{50} (parental line)] for K562/DXR out of drug were therefore 3.5 and 3.7 after 3 and 9 weeks respectively compared with 5 and 5.5 for K562 in drug. Partial loss of resistance therefore occurs within 3 weeks but not further loss is seen between 3 and 9 weeks of drug-free growth.

Multidrug resistance of K562/DXR

To determine whether K562/DXR exhibited the multiple drug resistant phenotype, the relative resistance of K562/DXR compared to K562 was assessed with a panel of drugs (Table 1). In Figure 1 the IC_{50} values are 0.7 and 3.5 μM/mL for K562 and K562/DXR, respectively. K562/DXR selected for resistance to 1 μM/mL DXR is five times as resistant to DXR as the K562 cell line and cross-resistant to actinomycin D, colchicine and etoposide. But K562/DXR is similar to other human multidrug-resistant lines in that it displays little or no cross-resistant to idarubicin and cisplatin. Furthermore, cross-resistance was found for mitoxantrone, epirubicin, amsacrine, vincristine and vinblastine, but not for 5-fluorouracil, bleomycin, cytarabine or mechlorethamine (data not shown).

Effect of verapamil

To investigate whether the calcium channel blocker, verapamil, and bisbezylisoquinoline-type alkaloid, cepharanthine could overcome the resistance to DXR, a series of experiments was carried out as previously described but with the addition of 30 μM verapamil and 2.0 μg cepharanthine to matched wells. This dose of verapamil and cepharanthine has been used extensively[13,16,17] in this type of investigation and we found it to have no growth inhibitory effects on its own. We were in fact able to use doses of verapamil alone as high as 30 μM without causing significant inhibition of growth in K562 cells. The results are shown in Fig. 2. In all three experiments, the addition of verapamil and cepharanthine led to a large drop in but not the total loss of resistance from line K562/DXR whilst having little or no effect on the sensitivity of K562 cells.

Cellular accumulation of DXR

Flow cytometry was used to quantitate the cellular accumulation of DXR in K562 and K562/DXR cells. Recorded data are displayed as histograms of cell number versus fluorescence intensity (Fig. 3). Initial experiments (not shown) indicated that when cells were exposed at 10^6 cells/mL to 1 or 10 μg/mL of DXR, the DXR content per cell rose over the first 15-30 min but did not increase further up to 1 hr. Subsequent experiments have, therefore, compared the DXR content per cell of K562 and K562/DXR cells exposed to DXR for 1 hr in order to determine the equilibrium content for short-term exposure. A typical fluorescence histogram of cells incubated without any drug is also displayed. The peak of fluorescence distribution of DXR clearly shifted to the left when the agent was incubated in the presence of K562 cells as compared to K562/DXR cells in a dose dependent manner. Clearly, cellular accumulationis weaker in the K562/DXR resistant subline than in sensitive K562 parental cells. Similar results were obtained in four replicate experiments although the level of uptake differd between experiments. Furthermore, the effects of verapamil and cephranthine on the accumulation of the DXR was determined in both cells. Figure 4 demonstrates the marked increase in cellular DXR content in the K562/DXR cells in the present of either verapamil or cepharanthine. But, cellular DXR content in K562/DXR cells treated with verapamil was higher than for the cepharanthine-treated cells. This contrasts markedly with the minimal effects seen for the corresponding

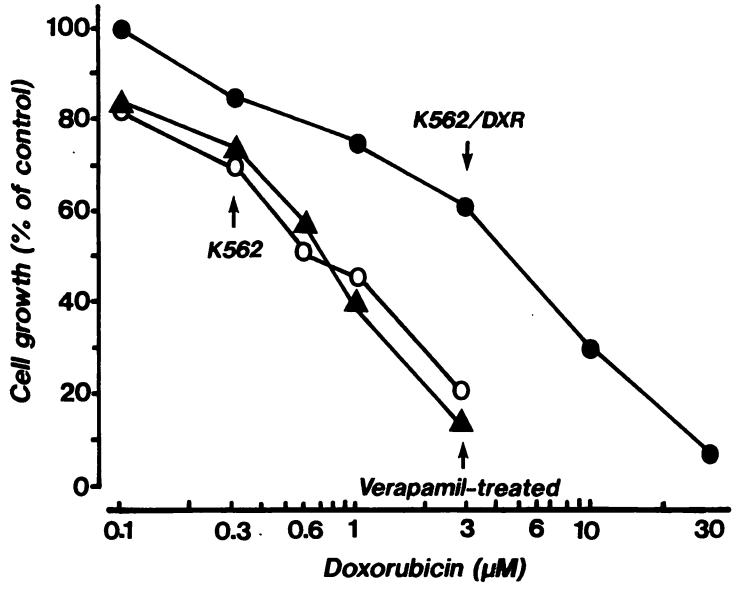

Fig. 2 Effect of Verapamil on the Growth Inhibitory Action of Doxorubicin in K562 and K562/DXR cells

Cells were inoculated in plates and incubated for 24 hr at 37°C. Then, they were treated with doxorubicin at the indicated concentrations in the absence or presence of either verapamil (30 μM), and incubated for 1 day before MTT assay as described in "Materials and Methods". Open circls, doxorubicin only, in K562 cells. Closed circles, doxorubicin only; closed triangles, doxorubicin plus verapamil, in K562/DXR cells.

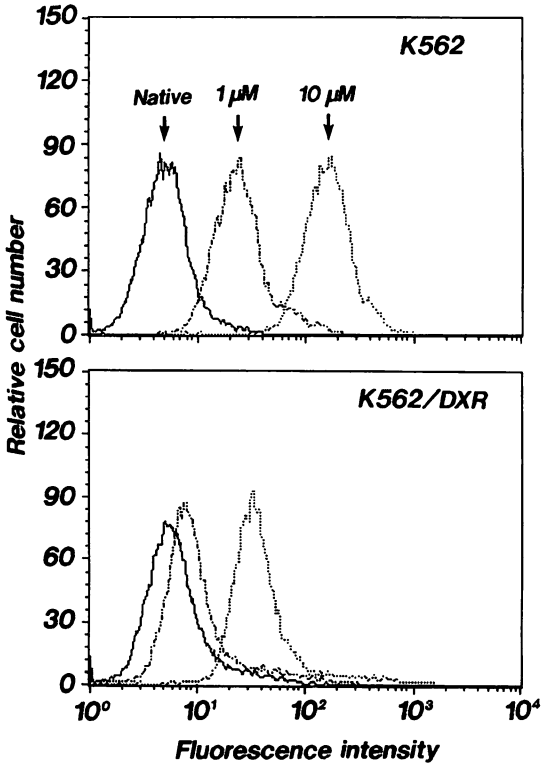

Fig. 3 Cellular Accumulation of Doxorubicin in K562 and K562/DXR Cells
Fluorescence intensity histograms were obtained by flow cytometry after incubation of K562 and K562/DXR cells (10^6cells/mL) with doxorubicin (1 or 10 μM/mL) at 37°C. Fluorescence intensity is displayed on a log scale. A total of 10,000 cells were counted for each histogram. Experiments were repeated 4 times and gave essentially the same profiles as the ones shown here.

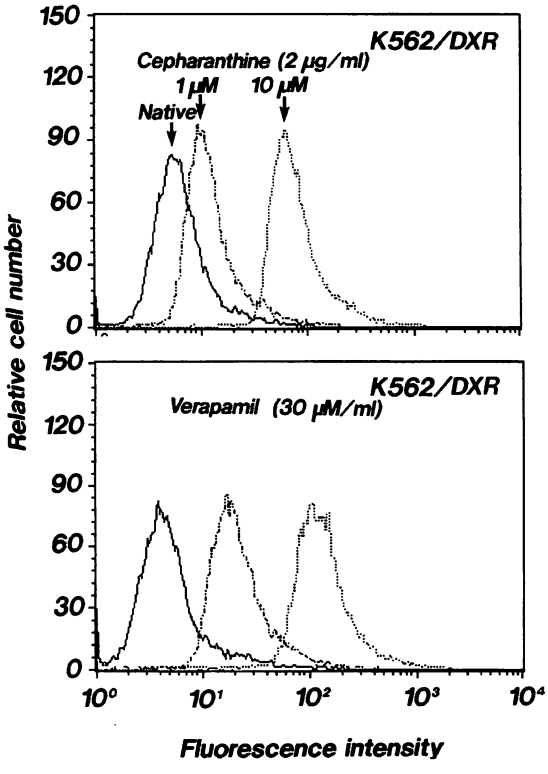

Fig. 4 Effect of Verapamil and Cepharanthine on the Cellular Accumulation of Doxorubicin in K562/DXR Cells
Cells were inoculated in plates and incubated for 24 hr at 37°C. Doxorubicin (1 or 10 μM) and verapamil (30 μM) or cepharanthine (2 μg/mL) was then added, and incubated for 1 hr before flow cytometry assay as described in "Materials and Methods".

parent K562 cells (data not shown).

Rhodamine 123 accumulation

Rhodamine 123 is a fluorescence dye that is a substrate for P-glycoprotein and rapidly transported out of multidrug resistance cells. Thus, rhodamine 123 uptake can be used as a simple and convenient way of assessing the impact of various treatments on the multidrug resistance phenotype. We evaluated the effect of resistance on drug transport in K562 cells by monitoring rhodamine 123 using a flow cytometric assay[14]. Accumulation of rhodamine 123 on K562 and K562/DXR cells as determined by flow cytometry is shown in Figure 5. There was little or no efflux of rhodamine 123 from parental cells over a period of 60 min, whereas the resistance cell line lost 70% of its rhodamine 123 content during this time.

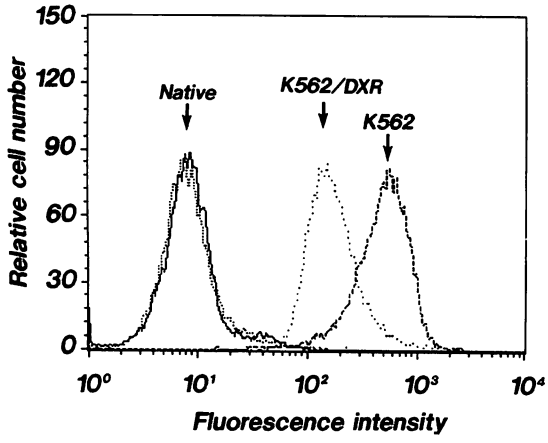

Fig. 5 Cellular Accumulation of Rhodamine 123 in K562 and K562/DXR cells
Fluorescence intensity histograms were obtained by flow cytometry after incubation of K562 and K562/DXR cells (10^6cells/mL) with rhodamine 123 (1.0 μg/mL) at 37°C. Fluorescence intensity is displayed on a log scale. A total of 10,000 cells were counted for each histogram. Experiments were repeated 4 times and gave essentially the same profiles as the ones shown here.

Discussion

In this paper we have described the isolation and partial characterization of a DXR-resistant subline of human leukemia cell line. These cells were selected as a model for drug resistance in a human leukemia cell line because they are usually refractory to multidrug treatment. Several authors have previously isolated similar rodent and human lymphoblastoid lines, and reports concerning drug accumulation in this cell have appeared[8,18,19].

A variety of techniques have been used to produce DXR-resistant sublines of murine cells including growth in low concentrations for many months followed by cloning[20], a double cloning over 6-8 weeks[21] or growth of cells in rapidly increasing concentration of DXR over 6-8 weeks[22]. We were able to obtain an DXR-resistant subline of the K562 tumor cell line by growth *in vitro* by increasing the concentration of DXR over 6 weeks. DXR-resistant human leukeima cell K562/DXR subline with 5- to 6-fold the resistance to DXR of the parent line exhibits a spectrum of cross-resistance including resistance to epirubicin, amsacrine, vincristine, vinblastine, actinomycin D, colchicine, mitoxantrone and etoposide, consistent with the multidrug-resistant phenotypes, and cell lines expressing increased levels of P-glycoprotein mRNA and protein product[5,6]. K562/DXR expresses the multidrug-resistant phenotype as it is cross-resistant to anthracycline analogues including epirubicin, mitoxantrone and amsacrine, as well as to etoposide and colchicine, and the vinca alkaloids, vincristine and binblastine. The resistant phenotype of K562/DXR was stable for at least 2 months during culture without DXR. The doubling time of the DXR-resistant subline was approximately 20 hr, slightly longer than that of the parental cell line being 18 hr.

Cross-resistance of DXR-resistant lines to etoposide, actinomycin D, vincristine, vinblastine, colchicine and amsacrine has been reported for *in vivo* systems by Schabel et al.[23]. Lack of cross-resistance to cisplatin by DXR resistant lines has also been described[24]. A lack of cross-resistance to idarubicin in DXR-resistant human colon adenocarcinoma cell line LOVO DX has been reported by Mariagrazia et al.[25] and the results of Jean-Michel et al.[26] suggest that DXR-resistant cells are less than fully cross-resistant to aclarubicin. Similarly, though the data for nitrosourea BCNU is not available, the closely related nirosourea cytarabin was found to be fully active against an

DXR-resistant line[23]. In general therefore, the cross-resistance patterns shows by our DXR-resistant human cells are similar to results previously described for rodent cells. A lack of cross-resistance for idarubicin have reported preliminary observations on lack of cross-resistance between DXR and a family of novel anthracyclines by Berman and McBride[27].

The total cellular fluorescence of DXR at the steady correlates well with cytotoxicity[28,29] and is generally used to gain rapid information on the ability of pharmacological agents to reverse multidrug resistance[30,31]. We measured the accumulation of DXR (as measured by flow cytometry for DXR) in sensitive and resistant K562 cells. Our results confirm this because there is a close correlation between DXR accumulation measured by flow cytometry and the cytotoxicity of DXR in K562 cells, as indicated in Fig. 1 and Table 1.

Verapamil, which were observed to attenuate resistance of tumors in animal studies, have been shown to inhibit the efflux of chemotherapeutic competitively by binding P-glycoprotein, thereby increasing the accumulation of chemotherapeutics in tumor cells[16,17,32,33]. The induction of the mRNA for the multidrug resistance pump, as well as the increase in P-glycoprotein (as measured by flow cytometry for rhodamine 123) itself, therefore indicates that the resistance reduces rhodamine 123 retention by increasing the efficiency of the multidrug resistance pump.

Our observations that DXR-resistant human leukemia cells show a reduced drug content after a given exposure are in accordance with previous observations in rodent cells[1]. The reduced DXR content is believed to be due to an increased efficiency of active drug efflux by P-glycoprotein[31]. Use of a calcium transport blocker, verapamil, to block such active efflux has been described by Tsuruo et al.[16,17]. Studies of the relationship between cellular DXR accumulation and cytotoxicity are currently in progress and will shed light on the significance of this observation. Furthermore, cepharanthine, an agent known to decrease the level of drug resistance in DXR-resistant cell lines, also reversed DXR resistance in our cell lines.

A number of studies have indicated that multidrug resistance is frequently associated with gene amplification and changes in cellular protein composition[5,6]. The present investigations suggest that the resistance to DXR development in human leukemia cells exposed to various concentrations of DXR is related to decreased penetration of drug into the cell. Our continuing studies are therefore directed towards identification and characterization of genetic changes and associated differences in protein composition is normal and drug-resistant human leukemia cells.

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