Cancer Chemother Pharmacol (2002) 49: 391–397
DOI 10.1007/s00280-001-0411-5

ORIGINAL ARTICLE

Zhen-Li Liu · Kenji Onda · Sachiko Tanaka
Tsugutoshi Toma · Toshihiko Hirano · Kitaro Oka

Induction of multidrug resistance in MOLT-4 cells by anticancer agents is closely related to increased expression of functional P-glycoprotein and MDR1 mRNA

Received: 11 July 2001 / Accepted: 16 November 2001 / Published online: 14 February 2002

Abstract *Purpose:* The aim of this study was to investigate the multidrug resistance (MDR) pattern, MDR gene and P-glycoprotein (P-gp) expression, and P-gp function in drug-induced human T-lymphoblastoid leukemia MOLT-4 sublines. *Methods:* The MDR sublines were developed by exposing the parental MOLT-4 cells to stepwise increasing concentrations of anticancer drugs daunorubicin (DNR), vinblastine (VBL) and doxorubicin (DOX). Degrees of resistance were assessed in terms of IC_{50} values in an MTT assay and the P-gp function was evaluated in terms of rhodamine 123 (Rh123) accumulation and efflux. The percentage of cells undergoing apoptosis was determined by flow cytometry after staining with annexin V-FITC and propidium iodide. The levels of P-gp and MDR mRNA expression were estimated using the PE-conjugated anti-P-gp monoclonal antibody 17F9 and quantitative real-time reverse transcription-polymerase chain reaction. *Results:* Three MOLT-4 sublines were established and revealed a 2- to 115-fold resistance to the anticancer reagents DNR, VBL and DOX as compared to the parental cell line. The highest MDR was expressed in MOLT-4/DNR cells, which was overcome by the P-gp modulator, cyclosporin A (CsA). The resistant sublines showed a decreased accumulation and an increased efflux of Rh123 in proportion to the degree of resistance, and these were completely reversed in the presence of 8 μ*M* CsA. The decreased apoptotic response in these cell lines was clearly associated with the degree of drug resistance. P-gp antigen and MDR1 mRNA were highly expressed in both the MOLT-4/DNR and MOLT-4/DOX sublines. Less-resistant MOLT-4/VBL cells expressed lower levels of MDR1 mRNA and P-gp, even though the cell line was established by exposing the parental MOLT-4 cells to VBL for longer (5 months) than to the other two reagents (3 months). *Conclusions:* MOLT-4 cells were able to acquire a high level of drug resistance by culturing the cells in the presence of certain anticancer drugs, and acquisition of the resistance was relatively reagent-specific. The degrees of resistance to the anticancer drugs were well correlated with the expressions of MDR1 mRNA and functional P-gp, and were also associated with a decreased response to apoptosis.

Keywords MOLT-4 · P-glycoprotein · MDR1 mRNA · Apoptosis · Reagent-specific

Z.-L. Liu · K. Onda · S. Tanaka · T. Toma
T. Hirano (✉) · K. Oka
Department of Clinical Pharmacology,
School of Pharmacy,
Tokyo University of Pharmacy and Life Science,
1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan
E-mail: hiranot@ps.toyaku.ac.jp
Tel.: +81-426-765796
Fax: +81-426-765798

Present address: Z.-L. Liu
The Institute of Basic Theory,
China Academy of Traditional Chinese Medicine,
P. R. China, 100700

Introduction

Multidrug resistance (MDR) is recognized as one of the most common causes for failure of cancer chemotherapy. For the study of MDR mechanisms, tumor cell lines have been selected for resistance to vinca alkaloids, anthracyclines, taxanes and epipodophyllotoxins [12, 26]. Intracellular drug accumulation has been found to be decreased as a result of drug efflux in these cell lines [8]. These MDR cell lines usually contain an amplified gene, in humans termed MDR1 [23, 25]. The protein product of this gene is a 170-kDa glycoprotein termed P-glycoprotein (P-gp) which is an ATP-dependent efflux pump that transports a variety of structurally unrelated natural product oncolytics out of cells [1]. It has been demonstrated that intrinsic or acquired expression of P-gp plays a major role in clinical MDR [17]. Several compounds, such as cyclosporin A (CsA), have been shown to be able to overcome MDR by disturbing P-gp function [10].

Some cultured resistant sublines from human leukemia cell lines have been established and each tumor cell

line displays a different selectivity for resistance to anticancer drugs [2, 15, 21]. The human T-lymphoblastoid leukemia MOLT-4 cell line has been used extensively for studies of leukemia cell biology and antileukemia therapy [12, 22], whereas induction of drug resistance in this well-known cell line has been little examined. The only reported resistant subline developed from MOLT-4 cells by anticancer drugs so far was selected by exposure of the cells to doxorubicin (DOX), and the subline cells obtained expressed 0.96- to 4.8-fold resistance to anticancer drugs compared to the parental cell line [9]. However, the selectivity of this cell line to certain anticancer agents to develop MDR is unknown. Therefore, we undertook the current study in order to establish MDR cell lines from MOLT-4 cells. We developed three resistant cell lines by culturing the parental MOLT-4 cells in the presence of one of three anticancer drugs, the anthracycline antibiotics daunorubicin (DNR) or DOX, and the vinca alkaloid vinblastine (VBL), and the characteristics of the resistant sublines compared to those of the parental cell line were studied.

Materials and methods

Reagents

RPMI-1640 medium and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, N.Y.). A cell proliferation kit I (MTT) was purchased from Roche Diagnostics (Indianapolis, Ind.). Trypan blue, rhodamine 123 (Rh123) and DNR, vinblastine sulfate and doxorubicin hydrochloride were obtained from Sigma Chemical Co. (St. Louis, Mo.). CsA was a gift from Novartis Pharma (Basel, Switzerland). Mouse anti-human P-gp monoclonal antibody 17F9 conjugated with *R*-phycoerythrin (R-PE) and R-PE-conjugated mouse IgG2b isotype control monoclonal antibody were obtained from PharMingen (San Diego, Calif.). The rest of the reagents were of the best available grade.

Cell lines and culture conditions

The MOLT-4 cell line was purchased from ICN Biomedicals (Tokyo, Japan) and the cells were maintained in RPMI-1640 medium containing 10% FBS, 100,000 IU/l penicillin and 100 mg/l streptomycin. For the development of drug-resistant sublines, MOLT-4 cells were initially exposed to the drugs at concentrations that kept the cells at 70% viability. The viable cells were counted in a hemocytometer using trypan blue exclusion. Cells were maintained at this drug concentration until their growth rate approached that of the untreated parental cells. The drug concentration was then increased two or three times. Usually 3 to 5 weeks were required to obtain an adequate growth rate at this drug concentration. Accordingly, three resistant sublines designated as MOLT-4/DNR, MOLT-4/VBL, and MOLT-4/DOX were developed by exposing the parental cells to DNR at concentrations of 10^{-8} *M* to 5×10^{-8} *M* for 3 months, VBL at 10^{-9} *M* to 8×10^{-9} *M* for 5 months, and DOX at 5×10^{-9} *M* to 2×10^{-8} *M* for 3 months, respectively. Investigations of the characteristics of these cell lines were performed on cells grown in drug-free medium for at least 10 days.

Cytotoxicity assays

Cells were washed and suspended in the medium at a density of 5×10^5 cells/ml. Cell suspension (196 µl) were placed in each well of a 96-well flat-bottom plate (Iwaki Company, Chiba, Japan), and 4 µl of each anticancer drug solution or 2 µl of each anticancer drug solution with 2 µl of each modulator solution in ethanol were added to give serial final concentrations. Ethanol (4 µl) was added to the control wells. The cells were incubated for 72 h at 37°C in an atmosphere comprising 5% CO_2/95% air. After the incubation period, cell growth was measured using an MTT assay procedure using the cell proliferation kit I (MTT). Dose-response curves were plotted and the concentrations that gave 50% inhibition of cell growth (IC_{50}) were calculated. The degrees of resistance of the resistant sublines to each drug were determined as the ratio of the IC_{50} of the resistant cells to the IC_{50} of the sensitive parental MOLT-4 cells.

Apoptosis assays

To evaluate the percentage of cells undergoing apoptosis, a flow cytometric analysis was performed to detect phosphatidylserine-exposing cells as we have previously described [13]. Cell suspension (980 µl) was seeded in 24-well flat-bottomed plates (Iwaki Company) at a density of 5×10^5 cells/ml, and 20 µl of each anticancer drug solution in ethanol was added to a final concentration of 2×10^{-6} *M* for DNR, 1×10^{-6} *M* for VLB, and 5×10^{-6} *M* for DOX. After incubation for 1 h at 37°C in an atmosphere comprising 5% CO_2/95% air, the cells were washed twice in medium and resuspended in 1 ml of drug-free medium. The cells were then incubated for another 24 h. After two washes in phosphate-buffered saline (PBS, pH 7.4), the cells were suspended in 100 µl of a binding buffer (10 m*M* Hepes/NaOH, pH 7.4, containing 140 m*M* NaCl and 2.5 m*M* $CaCl_2$) and stained with 5 µl annexin V-FITC (PharMingen) and 10 µl 50 µg/ml propidium iodide (PI) (Sigma) at room temperature for 15 min. A total of 20,000 non-gated cells were analyzed using a FACSCalibur analyzer (Becton Dickinson, Calif.) to obtain dot plot data. These data were further analyzed using CellQuest software (Becton Dickinson) to provide the percentage of cells undergoing apoptosis that were annexin V-FITC-positive and PI-negative.

Evaluation of P-gp function

P-gp function was characterized by determination of the Rh123 accumulation/efflux capacity.

Accumulation experiments. Aliquots of 5×10^5 cells were incubated with 2 µ*M* Rh123 in the presence or absence of 8 µ*M* CsA for 1 h at 37°C in a humidified atmosphere comprising 5% CO_2/95% air. The cells were then washed twice in ice-cold PBS, and Rh123 accumulation was determined using a FACSCalibur analyzer (Becton Dickinson). The green fluorescence of gated populations was collected through a bandpass filter (FL1) to obtain histogram plot data. These data were further analyzed using CellQuest software (Becton Dickinson) to obtain the intracellular Rh123 mean fluorescence intensity for each sample.

Efflux experiments. Aliquots of 5×10^5 cells were incubated in the presence of 2 µ*M* Rh123 and 8 µ*M* CsA for 1 h at 37°C in a humidified atmosphere comprising 5% CO_2/95% air. The cells were then washed twice in ice-cold medium and resuspended in 1 ml medium with or without 8 µ*M* CsA. Following 75 min of dye efflux at 37°C in the medium, the cells were washed twice in ice-cold PBS. The mean retained intracellular Rh123 fluorescence intensity was then estimated using the same procedure as for the accumulation experiments.

P-gp expression

Aliquots of 1×10^6 cells were washed twice in ice-cold PBS and resuspended in 50 µl binding buffer and then incubated with either 20 µl R-PE-conjugated mouse anti-human P-gp monoclonal antibody 17F9 or 20 µl R-PE-conjugated mouse IgG2b isotype control monoclonal antibody for 30 min at 4°C. After incubation and a further two washes in ice-cold PBS, the cells were resuspended in ice-cold binding buffer and kept on ice in the dark until analysis.

P-gp expression was determined using a FACSCalibur analyzer (Becton Dickinson). The fluorescence was transmitted through a bandpass filter (FL2) to acquire histogram plot data.

Extraction of poly(A) + RNA and quantitative real-time PCR

Poly(A) + RNA was isolated from cultured cells using a QuickPrep Micro mRNA purification kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions. cDNA was constructed by reverse transcription in 50 µl buffer containing 1×TaqMan buffer, 5.5 m*M* $MgCl_2$, 500 µ*M* of each dNTP, 2.5 µ*M* of random hexamer, 0.4 U/µl of RNase inhibitor, and 1.25 U/µl of MultiScribe reverse transcriptase. Reverse transcription was performed with a stepwise reaction at 25°C for 10 min, 48°C for 30 min and 95°C for 5 min. For the subsequent PCR procedure, 2.5 µl cDNA mixture was used.

The PCR was performed using real-time SYBR green technology and analyzed by an ABI 7700 sequence detector (Applied Biosystems). Primer pairs for MDR1 and *β*-actin were designed to span an intron sequence and to amplify 128-bp and 148-bp products, respectively. Primer sequences were as follows: *MDR1* 5′-GGCCTAATGCCGAACACATT-3′ and 5′-CAGCGTCTGGCCCTTCTTC-3′, *β-actin* 5′-CCCAGGCACCAGGTAGTGAT-3′ and 5′-TGCCAGATTTTCTCCATGTCG-3′. In order to prepare the calibration concentration curve, lambda DNA amplicon (140 bp) was constructed by PCR using lambda DNA-specific primer pairs (5′-AGTTCTGGCTGGAGTCAGTATGG-3′ and 5′-GGCTGTACCGGACAATGAGTG-3′). A calibration curve was obtained using 1:10 dilutions of a lambda DNA template (TaKaRa, Kyodo, Japan). The quantity of the MDR1 and *β*-actin transcript in each sample was determined from the calibration curve. To compare the expression level of MDR1 mRNA among the cell lines, the ratio of MDR1 transcript to *β*-actin transcript was calculated. Each sample was analyzed in triplicate and all PCR products were subjected to 2% agarose gel electrophoresis to ensure that no unspecific amplicon was obtained. To further confirm their sequences, the resultant PCR products were applied to the sequencing reaction.

Statistical analysis

The data were analyzed by Bonferroni/Dun multiple comparison. Calculated *P*-values of less than 0.05 were considered to be significant. The degree of correlation between two categories of data was determined with Pearson's correlation coefficient test. Statistical comparisons were supported by 95% confidence intervals for differences.

Results

Establishment of drug-resistant MOLT-4 sublines

Resistant sublines designated as MOLT-4/DNR, MOLT-4/VBL, and MOLT-4/DOX were developed by exposure of parental MOLT-4 cells to the medium containing DNR, VBL, and DOX, respectively. The parental MOLT-4 cells were uniformly round single cells (Fig. 1A), growing in stationary suspension culture with occasional aggregates seen only at high density ($> 1.2\times10^6$ cells/ml). In contrast, the resistant MOLT-4/DNR cells exhibited an altered culture morphology (Fig. 1B): aggregates of several cells occurred even at relatively low density (about 3×10^5 cells/ml). However, these MOLT-4/DNR cells individually were similar to the parental MOLT-4 cells.

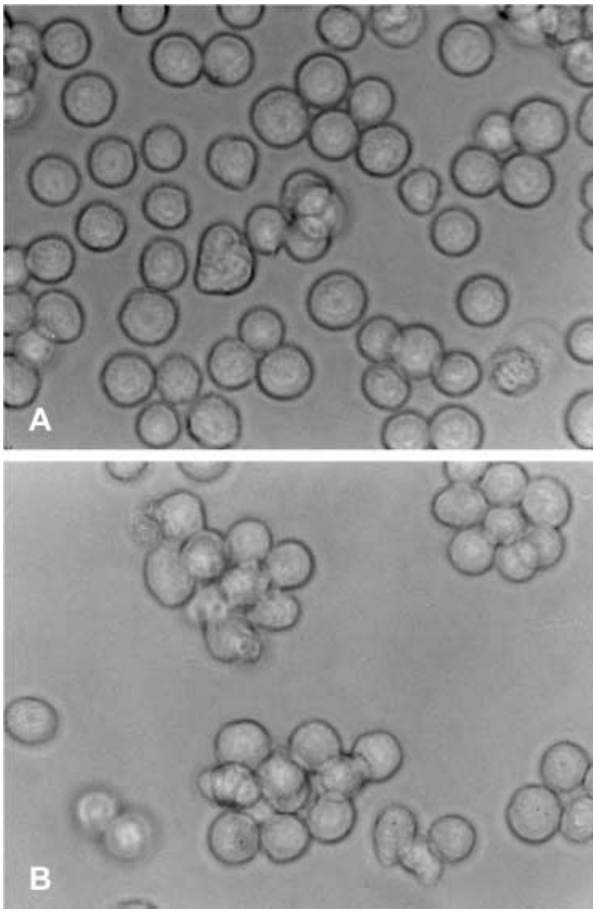

Fig. 1A, B. Morphological features of the parental MOLT-4 cells (**A**) and the resistant MOLT-4/DNR cells (**B**) as shown by light microscopy (×40). Cell density: 5×10^5 cells/ml

Degrees of resistance to anticancer drugs and reversal of drug resistances by MDR modulator

The values of IC_{50} obtained from the growth inhibition dose-response curves demonstrated that when compared with the parental MOLT-4 cells, MOLT-4/DNR cells were 24- to 61-fold, MOLT-4/VBL cells were 2- to 3-fold, MOLT-4/DOX cells were 11- to 115-fold more resistant to DNR, VBL, and DOX, respectively. CsA at a concentration of 5 µ*M* enhanced the cytotoxicity of DNR, VLB and DOX from 22- to 66-fold in the resistant MOLT-4/DNR cells, but had little or no effect on the cytotoxicity of these anticancer drugs in the parental MOLT-4 cells (Table 1).

To confirm that the enhanced cytotoxicity with CsA (Table 1) was due to modulation and not due to cytotoxicity of the agent itself, the IC_{50} values of CsA on cell proliferation were determined for both the parental MOLT-4 cells and the resistant MOLT-4/DNR cells, and were found to be 13.5 and 14.5 µ*M*, respectively. This result demonstrated that 5 µ*M* CsA had little cytotoxicity to the cells.

Table 1. IC_{50} values (M) for anticancer drugs in the parental and resistant MOLT-4 cell lines. The numbers in parentheses indicate the relative resistance compared with the parental MOLT-4 cells treated with each anticancer drug alone. Each value represents the mean of three independent experiments (*DNR* daunorubicin, *VBL* vinblastine, *DOX* doxorubicin, *CsA* cyclosporin A)

Drug	MOLT-4	MOLT-4/DNR	MOLT-4/VBL	MOLT-4/DOX
DNR	3.63×10^{-8}	2.21×10^{-6} (61)	5.81×10^{-8} (2)	4.95×10^{-7} (14)
DNR + CsA[a]	3.47×10^{-8} (< 1)	5.62×10^{-8} (2)		
VLB	6.48×10^{-9}	5.22×10^{-7} (81)	2.12×10^{-8} (3)	7.43×10^{-7} (115)
VLB + CsA[a]	5.65×10^{-9} (< 1)	7.9×10^{-9} (1)		
DOX	3.15×10^{-8}	7.46×10^{-7} (24)	5.57×10^{-8} (2)	3.38×10^{-7} (11)
DOX + CsA[a]	3.16×10^{-8} (1)	3.36×10^{-8} (1)		

[a]CsA at 5 μ*M*

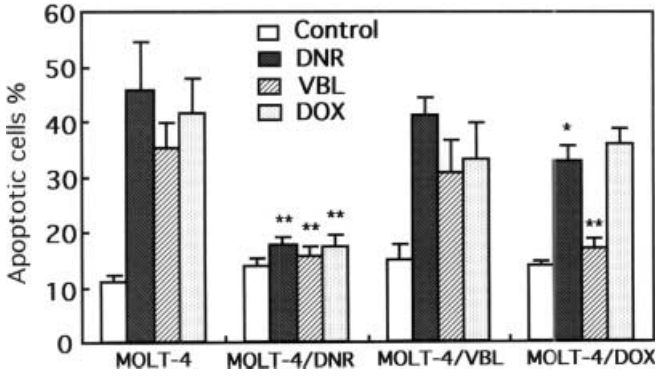

Fig. 2. Percentages of apoptotic cells in the parent MOLT-4 and the resistant sublines after treatment with anticancer drugs. Cells were incubated with 2×10^{-6} *M* DNR, 1×10^{-6} *M* VBL, or 5×10^{-6} *M* DOX for 1 h, then washed and incubated in drug-free medium for 24 h. Ethanol was added to the control group. After staining the cells with annexin V-FITC and PI, a FACSCalibur analyzer was used to determine the percentage of cells undergoing apoptosis. The results are expressed as the means obtained from three wells. $^{*}P<0.05$, $^{**}P<0.01$, vs parental MOLT-4 cells

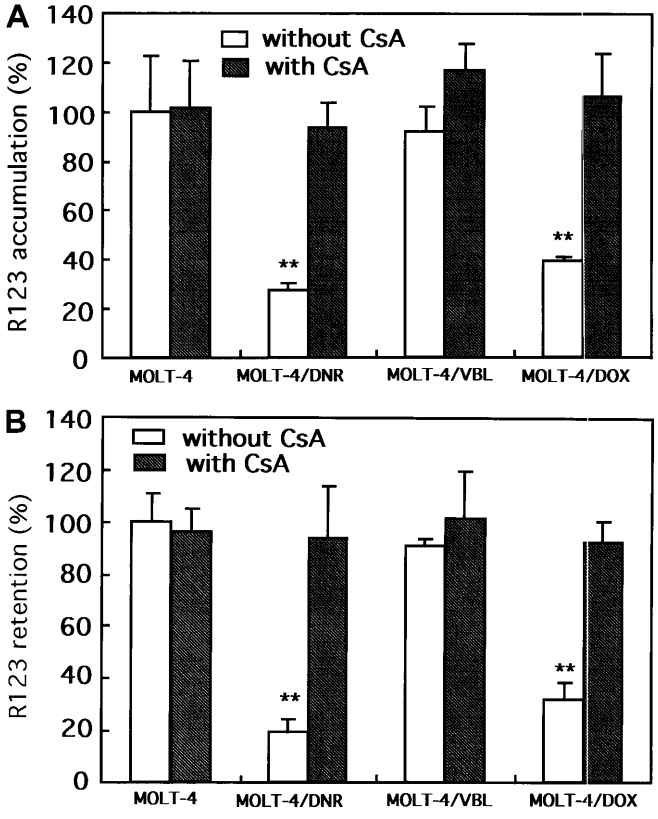

Fig. 3A, B. Rh123 accumulation (**A**) and retention (**B**) in cells of the parental MOLT-4 line and the resistant sublines with or without CsA. **A** Cells were stained with Rh123 for 1 h in the presence or absence of CsA, washed, and the Rh123 mean fluorescence intensities were determined by flow cytometry. **B** Cells were incubated with Rh123 in the presence of CsA for 1 h, washed, and then resuspended in Rh123-free medium in the presence or absence of CsA and left to stand for 75 min. Mean retained intracellular Rh123 fluorescence intensities were determined by flow cytometry. The mean fluorescence intensities were normalized to the parental MOLT-4 cell level (100%), and the values presented are the means from three independent experiments. $^{**}P<0.01$ vs parental MOLT-4 cells

Apoptosis induction by anticancer drugs

The percentages of cells undergoing apoptosis were determined after treatment of the cells with DNR, VBL or DOX at 2×10^{-6} *M*, 1×10^{-6} *M*, or 5×10^{-6} *M*, respectively (Fig. 2). Compared to the parental MOLT-4 cells, the MOLT-4/DNR cells exhibited significant resistance to the apoptosis-inducing effect of the three drugs ($P<0.01$), whereas the MOLT-4/DOX cells showed significant resistance to DNR and VBL ($P<0.05$, $P<0.01$). In contrast, the degree of resistance in the MOLT-4/VBL cells was lower than in the other resistant sublines (Fig. 2).

Figure 3A shows intercellular Rh123 accumulation in the parental MOLT-4 cells and the resistant sublines after 1 h incubation with Rh123 in the presence or absence of CsA. In the absence of CsA, the MOLT-4/DNR and MOLT-4/DOX cells demonstrated a significant decrease in accumulation of Rh123. The average Rh123 accumulation was 27% and 40%, respectively, compared that in the parental MOLT-4 cells (100%), while the accumulation in the MOLT-4/VLB cells was 92% of that in parental MOLT-4 cells. However, CsA at 8 μ*M* completely restored Rh123 accumulation in the MOLT-4/DNR and MOLT-4/DOX cells. The accumulated amount of Rh123 in MOLT-4/VBL cells changed little in the presence of CsA. This concentration of CsA showed little cytotoxicity to the cells.

In order to prove whether the rapidly decreasing dye accumulation in MOLT-4/DNR cells and MOLT-4/

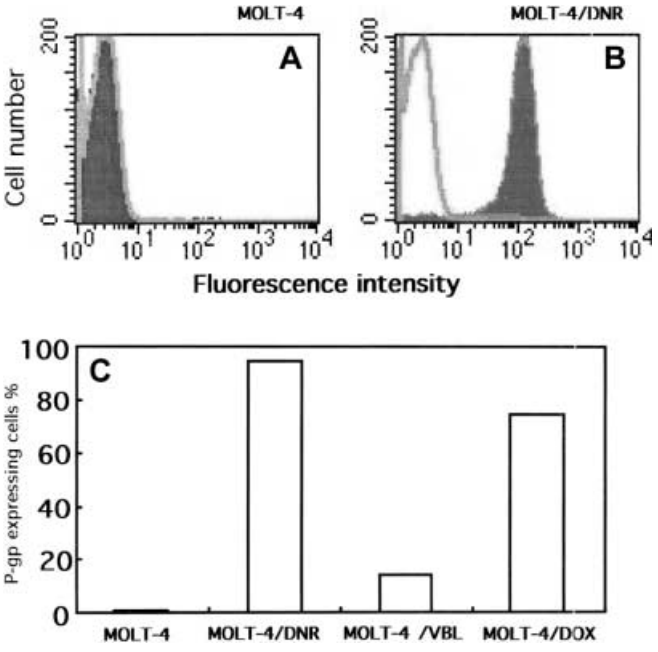

Fig. 4A–C. P-gp expressions in cells of the parental MOLT-4 line and the resistant sublines. Cells were stained with monoclonal antibodies as described in Materials and methods and then analyzed with a flow cytometer. **A, B** Monodimensional histograms of the fluorescence derived from PE-conjugated anti-P-gp monoclonal antibody 17F9 (*dark curve*), demonstrating P-gp-expressing cells and background fluorescence from control cells incubated with PE-conjugated mouse IgG2b isotype control (*light curve*) (**A** MOLT-4 cells, **B** MOLT-4/DNR cells). **C** Percentages of P-gp expressing cells in the parental and the resistant MOLT-4 cell lines. The values presented are the means of two independent experiments

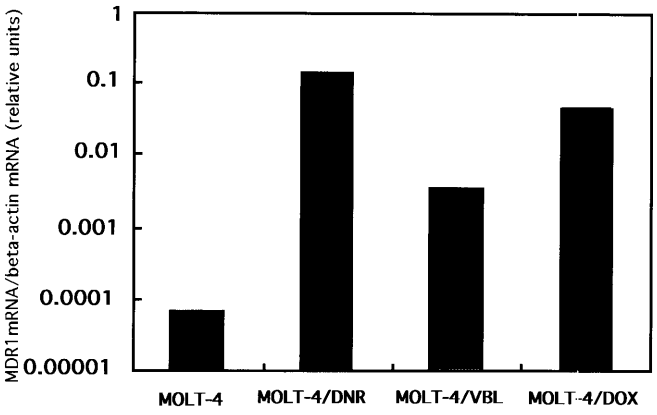

Fig. 5. MDR1 mRNA expressions in cells of the parental MOLT-4 and the resistant sublines. The quantities of MDR1 and β-actin transcripts were determined using real-time PCR as described in Materials and methods. To compare the expression levels among the cell lines, the results are expressed as the ratio of MDR1 to β-actin transcript. The values presented are the means of three independent experiments. The SD of each column was too low to express in the figure

DOX cells was in fact caused by an active outward transport, efflux experiments were carried out. Figure 3B illustrates that without CsA, a rather rapid decrease in intracellular Rh123 level was observed in the MOLT-4/DNR cells and MOLT-4/DOX cells after incubation in Rh123-free medium for 75 min. The average percentage of Rh123 retention was 20% and 32%, respectively, compared to the parental MOLT-4 cells (100%). However, in the presence of CsA, Rh123 efflux was completely inhibited in both cell lines, whereas the parental MOLT-4 cells retained the dye. Similar to the case of the parental cells, there was less efflux activity in the MOLT-4/VBL cells.

To evaluate the expression of P-gp antigen recognized by 17F9 on the parental sensitive cell line and the resistant sublines, immunofluorescence analysis was performed. Figure 4 indicates that 0.48% of MOLT-4 cells and 94.2% of MOLT-4/DNR cells expressed the antigen, whereas 13.9% of MOLT-4/VBL cells and 74.7% of MOLT-4/DOX cells expressed P-gp.

MDR1 mRNA was expressed in the three resistant sublines at different levels (Fig. 5), while only a very weak expression was observed in the parental MOLT-4 cells. It has been reported that some drug-sensitive tumor cells express a relatively low level of resistance-associated MDR1 transcript [14]. The MOLT-4/DNR cells showed the highest level of MDR1 mRNA and P-gp, and the MOLT-4/VBL cells the lowest. There was a positive correlation between the expression of the MDR1 mRNA and P-gp level determined with the monoclonal antibody.

Finally, we examined the relationships between P-gp expression and P-gp function and between P-gp expression and the IC_{50} values of the anticancer agents in the parental MOLT-4 line and the resistant sublines (Fig. 6). P-gp expression (expressed as a percentage) in these four cell lines was significantly correlated with Rh123 accumulation (Fig. 6A), Rh123 retention (Fig. 6A), and the IC_{50} values of the anticancer agents (Fig. 6B).

Discussion

We describe here the characteristics of three drug-resistant sublines derived from the human T-lymphoblastoid leukemia cell line MOLT-4 selected for resistance to the anticancer drugs DNR, VBL, and DOX. The leukemia cell line was able to acquire a high level of drug resistance, and the cells showed drug selectivity for developing resistance. The anthracyclines DNR and DOX induced the parental MOLT-4 cells to express high levels of P-gp, while the vinca alkaloid VBL did not. Among the three drugs used, DNR induced the greatest degree of resistance producing the MOLT-4/DNR subline. Although we exposed the parental MOLT-4 cells to medium containing VBL for the longest time (5 months), the MOLT-4/VBL cell line developed showed the lowest degree of resistance to drugs of the sublines studied. Moreover, the resistant sublines MOLT-4/DNR and MOLT-4/DOX showed higher

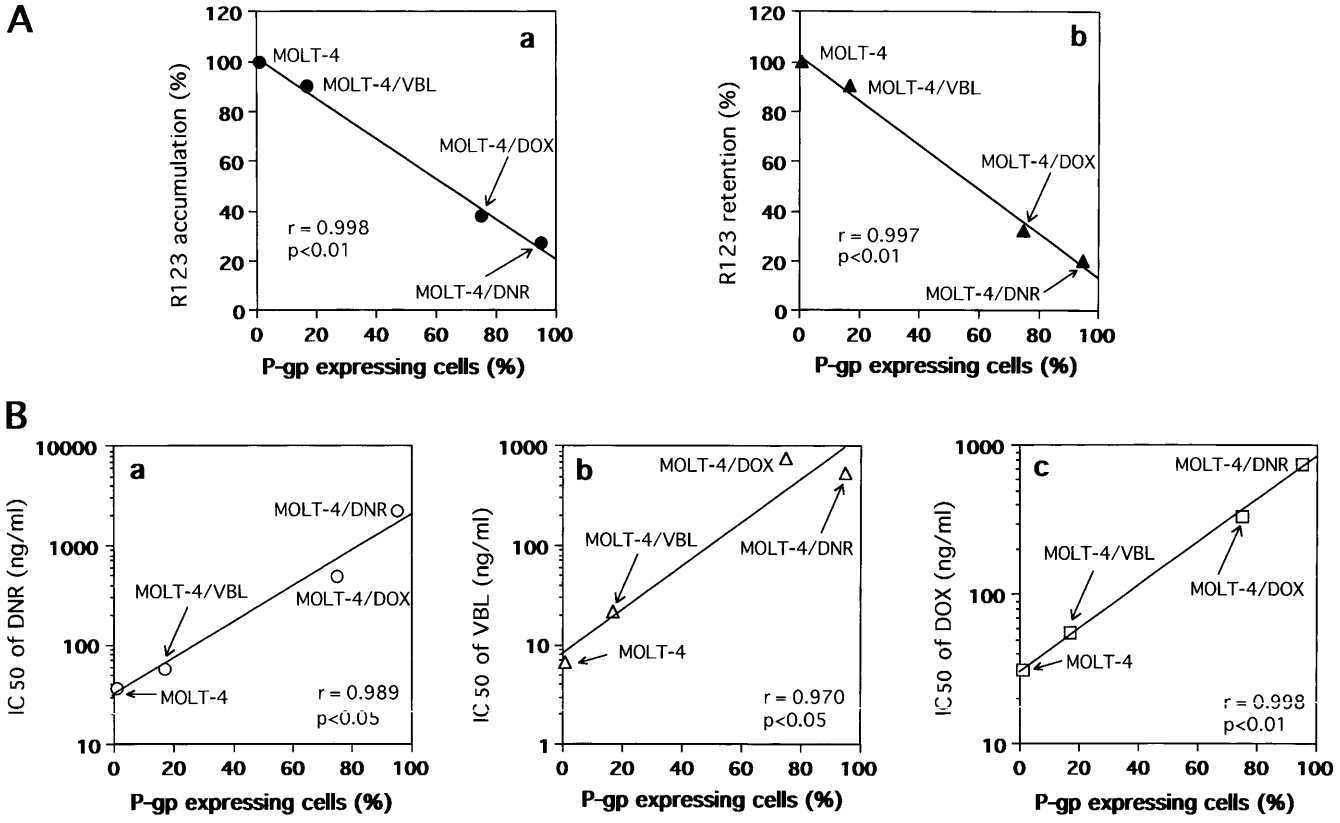

Fig. 6A, B. Correlations between P-gp expression and R123 accumulation (**A***a*), R123 retention (**A***b*), and IC_{50} values of the three anticancer agents (**B***a*, *b*, *c*) in four MOLT-4 sublines. The percentages of P-gp-expressing cells were determined as described for Fig. 4. The percentages of cells accumulating and retaining R123 were calculated as described for Fig. 3. The IC_{50} values used in **B** are those presented in Table 1

degrees of cross-resistance to VBL than to their selecting agents, DNR and DOX. This observation is contrary to that observed by Arkin et al. [2] who found that the greatest resistance occurs against the selecting agent and cross-resistance to other classes of drug is less. However, our results are consistent with a report of a DOX-resistant RPMI 8226 human myeloma cell line [6]. In addition, previous studies have shown that each MDR cell line displays a different pattern of cross-resistance [7, 10, 23]. Drug-induced MDR-resistant subpopulations appear to be most resistant to their selecting agents, but this is not the case for vinca alkaloids [6, 23] and gramicidin D [20]. It is possible that the variable and complex pattern of cross-resistance in MDR phenotypes may be a result of differences in membrane alterations and the diverse selection pressures applied to obtain these phenotypes [3].

Apoptosis is a common mechanism of death in malignant cells treated with anticancer drugs [16]. In addition to its function in the efflux of drugs, P-gp also plays a role in regulating drug-resistant tumor cells from multiple forms of caspase-dependent apoptosis pathways [27]. Clinical observations and in vitro experiments have shown a decreased apoptotic response to be associated with drug resistance [24]. The comparison of apoptosis in sensitive and resistant cells in the present study demonstrated that decreased apoptotic responses of the resistant sublines were associated with the degree of resistance to drugs (Fig. 2). The current results are in agreement with previously reported results that MOLT-4/Adr cells selected for resistance to DOX express a similar degree of resistance to DOX and etoposide as assessed by growth inhibition and apoptosis assays [9].

Rh123 has been observed to accumulate in the mitochondria of cells and is used as a standard functional indicator of MDR [4, 18]. The function of P-gp can also be evaluated by inhibition of Rh123 efflux and increase in Rh123 accumulation with MDR modulators such as verapamil and CsA [19]. The resistant sublines showed a decreased accumulation and an increased efflux of Rh123 in proportion to the degree of resistance, and this was extensively reversed by the MDR modulator CsA (Fig. 3). This result demonstrates that a decreased Rh123 accumulation in resistant cell lines was in fact the result of P-gp-mediated efflux. Our results are consistent with the previous observation that there is an inverse correlation between MDR1 expression and accumulation of Rh123 [15].

We showed that increased expression of MDR1 mRNA was well correlated with the percentage of cells expressing P-gp. Moreover, these genotypic characteristics of the present resistant cell lines appeared to correlate well with their increased drug resistance. Our results also suggest that P-gp function was directly correlated with the amount of P-gp antigen on the cell

surface, and that the sensitivity (resistance) of these sublines depended on the amount of P-gp expressed on these cells (Fig. 6). In addition, the substrate specificity in these sublines appeared to be similar. Thus, our present findings correspond with those of a previous study showing a close correlation between drug resistance and the overexpression of the MDR1 gene or P-gp.

Numerous human leukemia sublines with drug resistance have been established. These cell lines provide information on the characteristics of drug-resistant mechanisms in leukemia. The reported periods of exposing sensitive cells to medium containing anticancer drugs for developing resistant sublines vary from several months to 2 years, depending both on the drug used for induction of resistance and on the particular cell type [5, 21]. In the current study MOLT-4 cells were highly selective to DNR, less selective to DOX and least selective to VBL. The resistance pattern of MOLT-4 cells after a more prolonged exposure to VBL is, however, unknown. Whether this drug selectivity is clinically relevant remains to be determined, and detailed investigation are necessary to elucidate the mechanism behind each drug-induced effect.

In summary, we demonstrated that human leukemia MOLT-4 cells showed specific selectivity to anticancer drugs for developing drug resistance. The degree of resistance to an anticancer drug was closely correlated with the expression of MDR1 mRNA and functional P-gp.

References

1. Ambudkar SV, Dey S, Hrycyna CA, Ramachandra M, Pastan I, Gottesman MM (1999) Biochemical, cellular, and pharmacological aspects of the multidrug transporter. Annu Rev Pharmacol Toxicol 39:361
2. Arkin H, Ohnuma T, Kamen BA, Holland JF, Vallabhajosula S (1989) Multidrug resistance in a human leukemic cell line selected for resistance to trimetrexate. Cancer Res 49:6556
3. Bhalla K, Hindenburg A, Taub RN, Grant S (1985) Isolation and characterization of an anthracycline-resistant human leukemic cell line. Cancer Res 45:3657
4. Chaudhary PM, Robinson IB (1991) Expression and activity of P-glycoprotein, a multidrug efflux pump, in human hematopoietic stem cells. Cell 66:85
5. Chaudhary PM, Robinson IB (1993) Induction of multidrug resistance in human cells by transient exposure to different chemotherapeutic drugs. J Natl Cancer Inst 85:632
6. Dalton W, Durie BG, Alberts DS, Gerlach H, Cress AE (1986) Characterization of a new drug-resistant human myeloma cell line that expresses P-glycoprotein. Cancer Res 46:5125
7. Fichtner I, Stein U, Hoffmann J, Winterfeld G, Pfeil D, Hentschel M (1994) Characterization of four drug-resistant P388 sublines: resistance/sensitivity in vivo, resistance- and proliferation-markers, immunogenicity. Anticancer Res 14:1995
8. Fojo A, Akiyama S, Gottesman MM, Pastan I (1985) Reduced drug accumulation in multiply drug-resistant human KB carcinoma cell lines. Cancer Res 45:3002
9. Frankfurt OS, Seckinger D, Sugarbaker EV (1994) Pleiotropic drug resistance and survival advantage in leukemic cells with diminished apoptotic response. Int J Cancer 59:217
10. Fukuda T, Kamishima T, Kakihara T, Ohnishi Y, Suzuki T (1996) Characterization of newly established human myeloid leukemia cell line (KF-19) and its drug resistant sublines. Leuk Res 20:931
12. Hirano T, Gotoh M, Oka K (1994) Natural flavonoids and lignans are potent cytostatic agents against human leukemic HL-60 cells. Life Sci 55:1061
13. Horigome H, Homma M, Hirano T, Oka K, Niitsuma T, Hayashi T (2001) Magnolol from *Magnolia officinalis* inhibits 11beta-hydroxysteroid dehydrogenase without increases of corticosterone and thymocyte apoptosis in mice. Planta Med 67:33
14. Kartner N, Evernden-Porelle D, Bradley G, Ling V (1985) Detection of P-glycoprotein in multidrug-resistant cell lines by monoclonal antibodies. Nature 316:820
15. Koizumi S, Konishi M, Ichihara T, Wada H, Matsukawa H, Goi K, Mizutani S (1995) Flow cytometric functional analysis of multidrug resistance by Fluo-3: a comparison with rhodamine-123. Eur J Cancer 31A:1682
16. Lennon SV, Martin SJ, Cotter TG (1991) Dose-dependent induction of apoptosis in human tumour cell lines by widely diverging stimuli. Cell Prolif 24:203
17. Ling V (1997) Multidrug resistance: molecular mechanisms and clinical relevance. Cancer Chemother Pharmacol 40:S3
18. Ludescher C, Gattringer C, Drach J, Hofmann J, Grunicke H (1991) Rapid functional assay for the detection of multidrug-resistant cells using the fluorescent dye rhodamine 123. Blood 78:1385
19. Ludescher C, Thaler J, Drach D, Drach J, Spitaler M, Gattringer C, Huber H, Hofmann J (1992) Detection of activity of P-glycoprotein in human tumour samples using rhodamine 123. Br J Haematol 82:161
20. Mirski SE, Gerlach JH, Cole SP (1987) Multidrug resistance in a human small cell lung cancer cell line selected in adriamycin. Cancer Res 47:2594
21. Ohnuma T, Lo RJ, Scanlon KJ, Kamen BA, Ohnishi T, Wolman SR, Holland JF (1985) Evolution of methotrexate resistance of human acute lymphoblastic leukemia cells in vitro. Cancer Res 45:1815
22. Renner S, Kuci Z, d'Cruze H, Niethammer D, Bruchelt G (2000) Isotachophoretic analysis of the dihydrofolate reductase reaction in the presence of methotrexate and ascorbic acid. Electrophoresis 21:2828
23. Riordan JR, Deuchars K, Kartner N, Alon N, Trent J, Ling V (1985) Amplification of P-glycoprotein genes in multidrug-resistant mammalian cell lines. Nature 316:817
24. Robertson LE, Chubb S, Meyn RE, Story M, Ford R, Hittelma WN, Plunkett W (1993) Induction of apoptotic cell death in chronic lymphocytic leukemia by 2-chloro-2′-deoxyadenosine and 9-beta-D-arabinosyl-2-fluoroadenine. Blood 81:143
25. Scotto KW, Biedler JL, Melera PW (1986) Amplification and expression of genes associated with multidrug resistance in mammalian cells. Science 232:751
26. Shen DW, Cardarelli C, Hwan J, Cornwell M, Richert N, Ishii S, Pastan I, Gottesman MM (1986) Multiple drug-resistant human KB carcinoma cells independently selected for high-level resistance to colchicine, adriamycin, or vinblastine show changes in expression of specific proteins. J Biol Chem 261:7762
27. Smyth MJ, Krasovskis E, Sutton VR, Johnstone RW (1998) The drug efflux protein, P-glycoprotein, additionally protects drug-resistant tumor cells from multiple forms of caspase-dependent apoptosis. Proc Natl Acad Sci U S A 95:7024