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EXPRESSION OF DRUG RESISTANCE-ASSOCIATED *mdr-1*, GST π, AND TOPOISOMERASE II GENES DURING CELL CYCLE TRAVERSE

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Abstract—The expression of drug resistance-associated *mdr-1*, GST π, and topoisomerase II genes was analyzed in cell cycle phase enriched populations of doxorubicin-resistant murine leukemic P388/R-84 cells. Flow cytometric analysis of bromodeoxyuridine (BrdU) incorporation and staining with anti-BrdU antibodies was used to confirm the purity of cell cycle phase enriched populations obtained by centrifugal elutriation. Doxorubicin (DOX) and daunorubicin (DNR) accumulation was significantly lower in S-phase cells, and coincubation with verapamil (VPL) or chlorpromazine (CPZ) enhanced DOX and DNR accumulation more in S-phase than in G_1- and G_2/M-phase cells. While the cellular content of *mdr-1* and topoisomerase II mRNAs changed, GST π mRNA content remained constant during the cell cycle. S-phase cells had about 3-fold higher *mdr-1* mRNA content than G_1- and G_2/M-phase cells. In G_1 cells, P-glycoprotein expression, as determined by C219 monoclonal antibody, was 12% less than that of S and G_2/M cells. Topoisomerase II mRNA content increased with the progression of cell cycle and peaked in G_2/M cells. These observations suggest that cell cycle stage related changes in expression of drug resistance markers may have a major bearing on chemosensitivity of drug-resistant cells.

Key words: multidrug resistance; P-glycoprotein; glutathione *S*-transferase; topoisomerase II; cell cycle; doxorubicin

Although most eukaryotic genes are transcribed at constant rates during the cell cycle, a number of genes, such as those involved in DNA synthesis and cell cycle regulation, display significant variability in their transcription rates. MDR† is caused primarily by decreased drug accumulation mediated by the *mdr-1* gene encoded P-gp, which is believed to be responsible for drug efflux. Amplification and increased expression of the *mdr-1* gene in multidrug-resistant cells have been reported [1–6]. We have reported previously that Adriamycin® cytotoxicity, retention and response to efflux blockers are cell cycle phase and proliferation related [7, 8]. Since drug efflux is mediated by *mdr-1* encoded P-gp, it is quite plausible that *mdr-1* expression may also be cell cycle related. Furthermore, the promoter of *mdr-1* gene resembles the promoters of cell cycle regulated housekeeping genes, such as dihydrofolate reductase [9] and hypoxanthine guanine phosphoribosyltransferase [10], lacking the basic "TATA" box element [11, 12]. The promoter of human *mdr-1* gene is also a target for c-Ha-*ras* 1 oncogene and p53 suppressor gene products, which are expressed at varying rates during the cell cycle [13]. In the present report, we have analyzed *mdr-1* mRNA and P-gp expression in cell cycle phase enriched populations of the DOX-resistant P388/R-84 cell line and correlated the data with cellular drug accumulation and sensitivity to efflux blockers. DOX resistance in P388/R-84 cells is multifactorial, and changes in expression and activity of GST π and topoisomerase II enzymes are believed to contribute partially to cellular drug resistance [14–16]. Therefore, we have also studied the expression of these two drug resistance-associated genes (GST π and topoisomerase II) in cell cycle phase enriched populations.

MATERIALS AND METHODS

Cell lines. DOX-resistant murine leukemic P388/R-84 cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, penicillin and streptomycin. In soft agar clonogenic assays, this cell line is >84-fold more resistant to doxorubicin than the parental P388 cell line [15].

Drugs. Doxorubicin hydrochloride (DOX, Adriamycin, NSC-123127; Adria Laboratories, Columbus, OH), daunorubicin hydrochloride (DNR, Daunomycin, NSC-821151; Wyeth Laboratories, Philadelphia, PA), chlorpromazine (CPZ, Thora-

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† Abbreviations: MDR/mdr, multidrug resistance; GST, glutathione *S*-transferase; BrdU, bromodeoxyuridine; DOX, doxorubicin; DNR, daunorubicin; VPL, verapamil; CPZ, chlorpromazine; P-gp, P-glycoprotein; FITC, fluorescein isothiocyanate; MAb, monoclonal antibody; RT-PCR, reverse transcriptase-polymerase chain reaction; and PKC, protein kinase C.

zine; Smith Kline & Beecham Laboratories, Philadelphia, PA), and verapamil (VPL, Calan; Abbott Laboratories, Chicago, IL) were purchased. [^{14}C]DOX was obtained from the Amersham Corp., Arlington Heights, IL.

Centrifugal elutriation. Log-phase P388/R-84 cells were fractionated into cell cycle phase enriched populations by centrifugal elutriation in a J21-C centrifuge (Beckman Instruments, Palo Alto, CA). Cells (8×10^6) were loaded into a Sanderson separation chamber, and the flow rate was maintained at 7 mL/min. Loading rotor-speed was 2000 rpm, and 200-mL fractions were collected at decreasing rotor speeds. Details of our elutriation procedure have been reported earlier [8]. Aliquots of different fractions were processed by the propidium iodide hypotonic citrate method and analyzed for cell cycle distribution by laser flow cytometry [17].

BrdU labeling and analysis. The procedure for BrdU incorporation, staining and cell cycle analysis described by Dean *et al.* [18] was used with some modifications. Approximately 3×10^6 cells from elutriated fractions were incubated with 10 μM BrdU for 1 hr at 37°, washed with PBS, fixed in 70% cold ethanol, and stored at 4° until analysis. Fixed cells were washed with PBS, resuspended in 0.1% Triton X-100 solution for 1–2 min, followed by a wash in PBS and incubated in 2–3 mL of 4 N HCl for 10 min at room temperature. Cells were washed, resuspended in 100 μL of PBS containing 0.5% Tween-20, 0.5% BSA and 0.5 μg of FITC-labeled anti-BrdU MAb (Boehringer Mannheim Corp., Indianapolis, IN) and incubated for 30 min at room temperature in the dark. FITC-labeled cells were double stained with propidium iodide (5 μg/mL) solution containing RNase (1 mg/mL) for 1 hr and analyzed in a FACscan flow cytometer (Becton Dickinson Corp., San Jose, CA).

Drug efflux and retention. Cells (2×10^6) from each cell cycle phase enriched population were incubated with 3.6 μM DOX or DNR at 37° for 2 hr with shaking and were washed two times with PBS; drug retention in the cells was analyzed by flow cytometry. To determine drug efflux blocking, cells were incubated with DOX or DNR (3.6 μM) in combination with CPZ (100 μM) or VPL (10 μM) at 37° for 1–2 hr. Details of our methods have been reported earlier [8].

To quantitate cellular retention of DOX, 2×10^6 cells were incubated with ^{14}C-labeled DOX (2.5×10^5 cpm) for 2 hr with or without VPL (10 μM) or CPZ (100 μM) at 37°, washed two times with PBS and then three times with 0.85% NaCl. Cellular [^{14}C]DOX was extracted with 50% ethanol–0.3 N HCl solution, and the radioactivity was determined by liquid scintillation spectrometry [19].

For fluorometric determination of cellular DNR content, cells incubated with DNR, with or without efflux blocker (VPL or CPZ), were centrifuged, washed two times with PBS and then three times with 0.85% NaCl solution. DNR was extracted with an alcohol–acid mixture containing 50% ethanol and 0.3 N HCl [19, 20]. DNR fluorescence in the supernatant was determined at excitation and emission wavelengths of 480 and 590 nm, respectively, in a Perkin Elmer LS-3B fluorescence spectrometer (Perkin Elmer Corp., Norwalk, CT). DNR concentration in the sample was derived from a standard curve based on known drug concentration in the extracting solvent.

RNA extraction and RT-PCR assay for mdr-1 *gene expression.* Poly(A)$^+$ RNA was extracted using a Micro-Fast track kit (Invitrogen Corp., San Diego, CA) as per the manufacturer's instructions. The concentration of RNA was determined spectrometrically prior to RT-PCR assay. Reverse transcription was carried out using 0.4 μg poly(A)$^+$ RNA in a total volume of 20 μL containing 5 mM $MgCl_2$, 1 PCR buffer, 1 mM each of four deoxyribonucleotide triphosphates, 1 U/μL of RNase inhibitor, 2.5 U/μL of reverse transcriptase (Perkin Elmer Corp.), and 100 pmol antisense primer (5′-GAAGCACTGGGATGTCCGGT-3′; corresponds to the antisense strand of the *mdr-1* cDNA sequence 3141–3160) [2, 21]. The tubes were incubated in a Perkin Elmer Gene Amp 9600 PCR System at 42° for 15 min, followed by 99° for 5 min, and then chilled at 5° for 5 min. PCR assay was carried out in 100 μL reaction volume containing 2 mM $MgCl_2$, 1 PCR buffer, 2.5 U/μL of AmpliTaq DNA polymerase, 100 pmol each of *mdr-1* sense (5′-ATATCAGCAGCCCACATCAT-3′; corresponds to *mdr-1* cDNA 3007–3026) and antisense (5′-GAAGCACTGGGATGTCCGGT-3′; corresponds to *mdr-1* cDNA sequence 3141–3160) primers, and 1 μL (10 μCi) of [^{32}P]dCTP. This set of primers amplifies a 153 bp fragment in the murine *mdr-1* gene. The reaction mixture was heated at 94° for 75 sec. Amplification was performed in sequential cycles of denaturation at 94° for 15 sec followed by annealing at 60° for 15 sec. After 35 cycles, the samples were extended at 60° for 7 min, followed by soaking at 4° for 5 min. The final product was purified by passage through a Biospin-30 column (Bio-Rad Laboratories, Richmond, CA). PCR products (15 μL each) were electrophoresed on 5% polyacrylamide gels in a Bio-Rad mini-protean apparatus. The gels were washed in fresh electrophoresis buffer, stained with ethidium bromide (5 μg/mL), photographed and exposed overnight at −70° for autoradiography.

Slot blot hybridization. The recombinant plasmid p683 carrying multidrug-resistant gene (*mdr-1*) was obtained from Dr. A. T. Fojo, National Cancer Institute, Bethesda, MD. *mdr-1* cDNA is a 683 bp fragment covering the residues from −27 to + 656 bp of the coding region, cloned into the *Sma* I site of pGEM 3Z (Promega) plasmid vector. GST π (GST π-1) and β-actin (HHC189) clones were purchased from the American Type Culture Collection, Rockville, MD. GST π cDNA is a 725 bp insert cloned into the *Eco* RI site of the pGEM 4 plasmid vector. In slot blot hybridization experiments, human β-actin gene was used as the control. Topoisomerase II clone (pBShTOP 2) was a gift from Dr. J. C. Wang (Harvard University, Cambridge, MA). Topoisomerase II cDNA is a 5.6 kb insert cloned into the *Sma* I site of Bluescript plasmid vector, and the 5.4 kb *Eco* RI digested fragment of pBShTOP 2 was used for making a random primer labeled probe [22]. In northern blots, this probe hybridizes with 6.2 kb mRNA species of P388/R-84 cells and detects only the α-isoform of topoisomerase II. The detailed

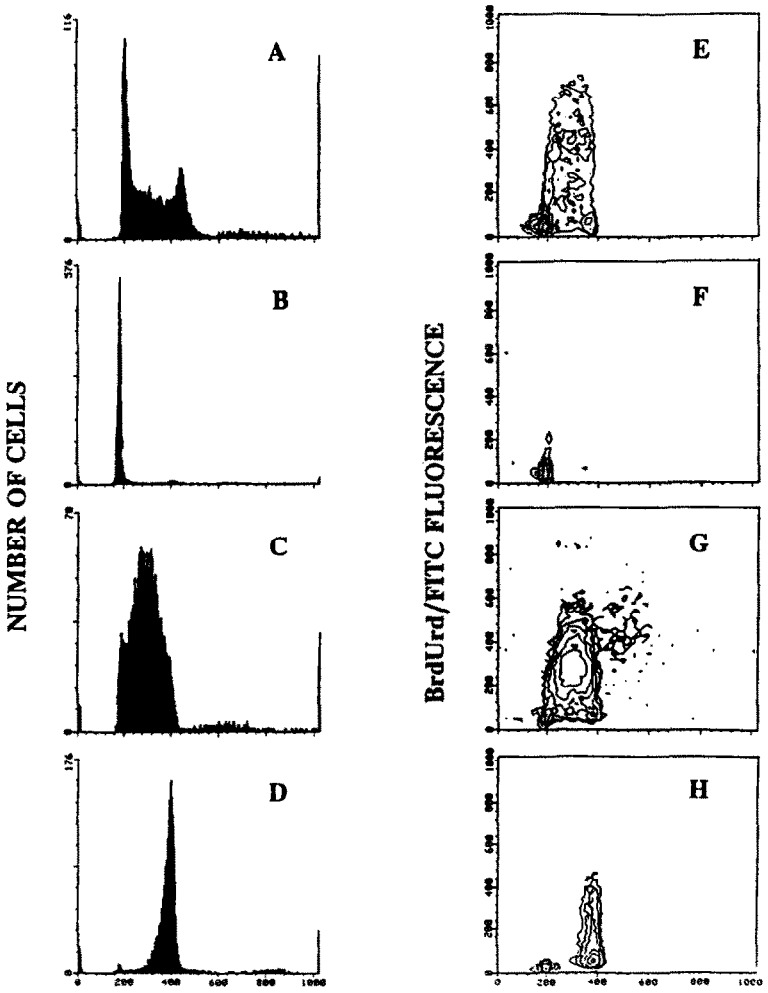

Fig. 1. DNA histograms (A–D) and contour plots of bivariate DNA/BrdU distribution (E–H) of cell cycle phase enriched populations separated by centrifugal elutriation from log-phase cultures of P388/R-84 cells. Histograms are of log-phase cells (A and E), and of elutriated populations of G_1 (B and F), S (C and G) and G_2/M (D and H) phases.

procedure for RNA extraction, preparation of probe, and slot blot hybridization has been described earlier [23]. Slot blot experiments were repeated several times for confirmatory results. Autoradiographs were scanned in a Zeineh soft laser scanning densitometer under tungsten light, and values were expressed as arbitrary units. At least three blots were read in the densitometer, and mean values were calculated.

Flow cytometric determination of P-gp expression. Cells incubated with C219 MAb (Signet Laboratories, Inc., Dedham, MA) were detected by indirect staining with FITC-labeled IgG. Details of our flow cytometric method for P-gp determination have been described earlier [24, 25].

RESULTS

Cell cycle phase enrichment of P388/R-84 cells by centrifugal elutriation. Log-phase P388/R-84 cells were separated into 11–12 fractions by centrifugal elutriation, and fractions containing predominantly G_1, S and G_2/M phases were identified by flow cytometric determination of cellular DNA content after propidium iodide staining. DNA histograms of log-phase and cell cycle phase enriched populations are presented in Fig. 1, A-D. The incorporation of BrdU into DNA of different fractions is shown in Fig. 1, E-H. Histogram 1A is that of log-phase population with about 50% BrdU labeling index (Fig. 1E). The G_1-phase enriched population (Fig. 1B) had about 9% BrdU-labeled cells (Fig. 1F), while S-phase enriched population (Fig. 1C) had 94% labeled cells (Fig. 1G). G_2/M-phase enriched population (Fig. 1D) showed a slight contamination of late S-phase cells, and about 20% of the cells were labeled with BrdU (Fig. 1H).

DOX and DNR accumulation in cell cycle phase enriched populations. The data in Table 1 show DOX and DNR accumulation in cell cycle phase

Table 1. Effect of efflux blockers on [^{14}C]DOX and DNR accumulation in cell cycle phase enriched populations of P388/R-84 cells

	[^{14}C]DOX ($\times 10^2$ cpm)*			DNR (ng/2 $\times$ 10^6 cells)†		
	DOX	DOX + VPL	DOX + CPZ	DNR	DNR + VPL	DNR + CPZ
Log-phase	45.48	86.18 (1.89)‡	98.70 (2.17)‡	9.25	18.95 (2.05)‡	65.0 (7.02)‡
G_1-phase	26.87	76.14 (2.83)	65.53 (2.44)	17.86	44.00 (2.46)	31.43 (1.76)
S-phase	17.85	72.33 (4.05)	66.90 (3.74)	11.77	50.76 (4.31)	106.67 (9.06)
G_2/M-phase	38.94	100.95 (2.59)	102.29 (2.62)	20.71	51.42 (2.48)	105.00 (5.07)

* Cells (2×10^6/mL) were incubated with 2.5×10^5 cpm [^{14}C]DOX at 37° for 1 hr with or without verapamil (10 μM) or chlorpromazine (100 μM). The standard deviation of all estimates from three independent experiments with three replications in each was less than 10% of the mean values.

† Cells (2×10^6) were incubated with 2 μg/mL of DNR at 37° with or without efflux blockers, verapamil (10 μM) or chlorpromazine (100 μM). The standard deviation of all estimates from three separate experiments with three replications in each was less than 10% of the mean values.

‡ Values in parentheses represent fold-increase in DOX or DNR accumulation in the presence of efflux blockers (VPL or CPZ).

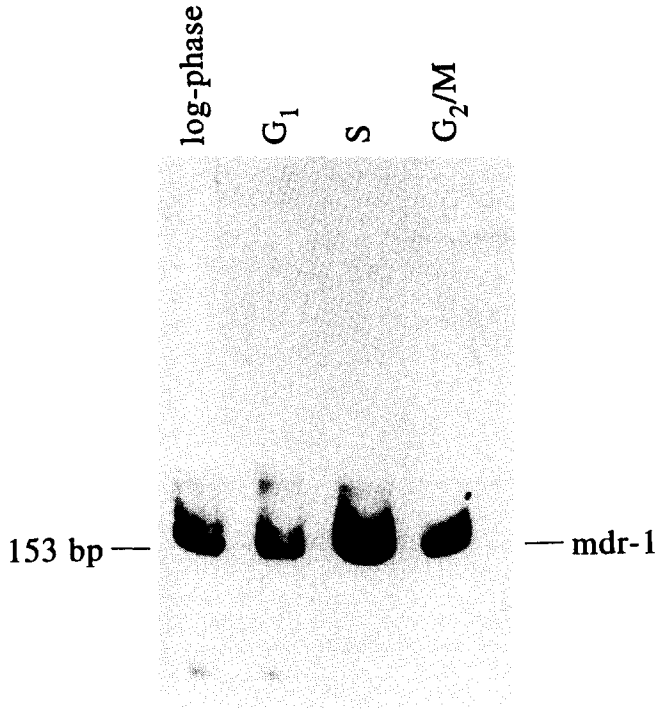

Fig. 2. RT-PCR assay of *mdr-1* mRNA expression in log-phase cells and enriched populations of G_1-S-, and G_2/M-phase cells. PCR was performed with [α-^{32}P]dCTP for 35 cycles. After polyacrylamide gel electrophoresis of PCR products, gels were dried and exposed to X-ray film overnight at −80°. Note that S-phase cells had the highest content of *mdr-1* mRNA.

enriched populations of P388/R-84 cells in the presence or absence of efflux blockers (VPL or CPZ). DOX accumulation in S-phase cells was significantly lower than that of G_1- or G_2/M-phase cells. Coincubation with VPL or CPZ enhanced DOX accumulation in all cell cycle phases. The increase in cellular DOX accumulation after efflux blocking ranged from 1.89- to 4.05-fold with VPL and from 2.17- to 3.74-fold with CPZ. Both VPL and CPZ had significantly higher efflux blocking effects in S-phase cells compared with G_1- and G_2/M-phase cells (Table 1). VPL and CPZ increased DOX accumulation 4.05- and 3.74-fold, respectively, in S-phase cells.

Fluoremetric data on DNR content of cells incubated with or without efflux blockers were similar to the data on DOX accumulation. S-phase cells accumulated significantly lower amounts of DNR (11.77 ng/2 $\times$ 10^6 cells) than G_1 (17.86 ng/2 $\times$ 10^6 cells) or G_2/M (20.71 ng/2 $\times$ 10^6 cells) cells. VPL and CPZ increased DNR accumulation in S-phase cells by 4.31- and 9.06-fold, respectively. On the other hand, VPL enhanced DNR accumulation in G_1- and G_2/M-phase cells by only about 2.5-fold. CPZ enhanced DNR accumulation in G_1- and G_2/M-phase cells by 1.76- and 5.07-fold, respectively. Flow cytometric analysis of DOX and DNR accumulation in the presence and absence of VPL or CPZ also showed similar results (data not shown).

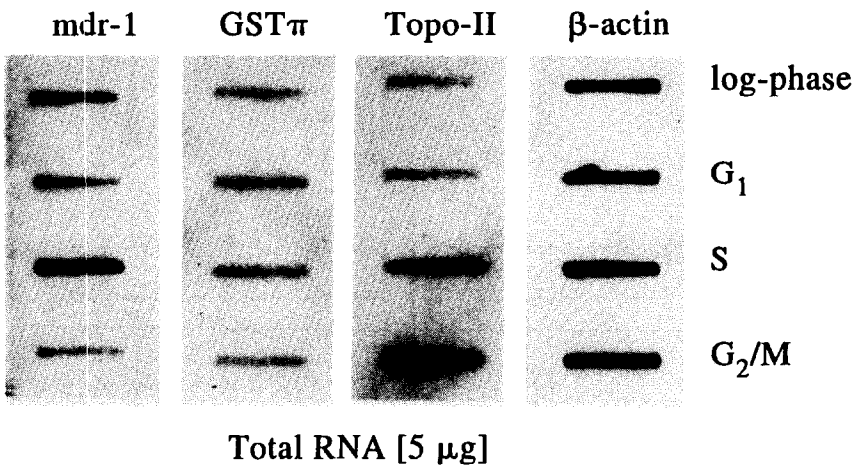

Fig. 3. Slot blot hybridization for *mdr-1*, GST π, topoisomerase II and β-actin mRNA levels in log-phase cells and enriched populations of G_1-, S-, and G_2/M-phase cells. Total RNA at serial dilutions (3, 4 and 5 μg; only 5 μg lane is shown) was blotted and hybridized with ^{32}P-labeled cDNA probes of *mdr-1*, GST π, topoisomerase II, and β-actin. Note that the highest level of *mdr-1* mRNA expression was in S-phase cells.

Table 2. Expression of *mdr-1*, GST π, and topoisomerase II mRNAs during cell cycle

	mdr-1 mRNA*	GST π mRNA*	Topoisomerase II mRNA*
Log-phase	23.38 ± 2.26	21.08 ± 1.90	15.98 ± 4.10
G_1-phase	10.70 ± 3.17	20.50 ± 1.05	15.60 ± 5.20
S-phase	39.78 ± 4.38	21.48 ± 1.95	25.63 ± 2.91
G_2/M-phase	13.32 ± 1.05	21.58 ± 2.30	34.03 ± 5.50

All estimates are arbitrary units (means ± SD, N = 3).
* Analyzed by slot blot hybridization.

mdr-1, *GST π and topoisomerase II mRNA expression.* Data in Fig. 2 (RT-PCR), Fig. 3 (slot blot), and Table 2 (densitometric) clearly indicate that in comparison to G_1- and G_2/M-phase cells, S-phase cells had the highest expression of *mdr-1* mRNA. In the RT-PCR assay, the expected 153 bp *mdr-1* band was very prominent, especially in S-phase cells, and absent in drug-sensitive P388 cells (data not shown). In the densitometric data from the slot blots (Table 2), S-phase cells had approximately 3-fold (39.78 AU) higher *mdr-1* mRNA expression than G_1 (10.70 AU) and G_2/M (13.32 AU) cells.

Slot blots of RNA hybridized with GST π and topoisomerase II cDNA probes are presented in Fig. 3, and the mean densitometric readings of autoradiographs are given in Table 2. GST π mRNA content remained constant throughout the cell cycle without any significant differences between the cell cycle phase enriched populations. Topoisomerase II mRNA content, on the other hand, increased with the progression of the cell cycle, reaching a maximum in G_2/M-phase. G_2/M-phase cells (34.03 AU) had more than 2-fold higher topoisomerase II mRNA content than G_1-phase cells (15.60 AU). S-phase cells also showed 64% higher topoisomerase II mRNA content (25.63 AU) than G_1-phase cells.

P-gp expression. Figure 4 shows the flow cytometric analysis (dot plots and histograms) of P-gp and cellular DNA content in cell cycle phase enriched fractions. In dot plots, the X-axis shows DNA content, while the Y-axis records the amount of P-gp expression as determined by C219 antibody reactivity. The horizontal line indicates the electronic gate used to exclude 99% of FITC-labeled cells in the isotype control. Dots above this line were recorded as the percentage of positive cells. The insets in the right-hand top corner of each figure are histograms of C219-FITC fluorescence. The open histograms are of isotype controls, while the filled ones are of C219-FITC-stained cells.

In Chinese hamster AUX B_1 drug-sensitive cells (Fig. 4A), only a small number of cells (3%) were P-gp positive, in contrast to CH^RC5 cells (Fig. 4B) where 70% of cells had C219 positive expression. Log-phase population of P388/R-84 (Fig. 4C) had 83% P-gp positive cells with a mean fluorescence channel value (MFCV) of log 6.92. Among the cell

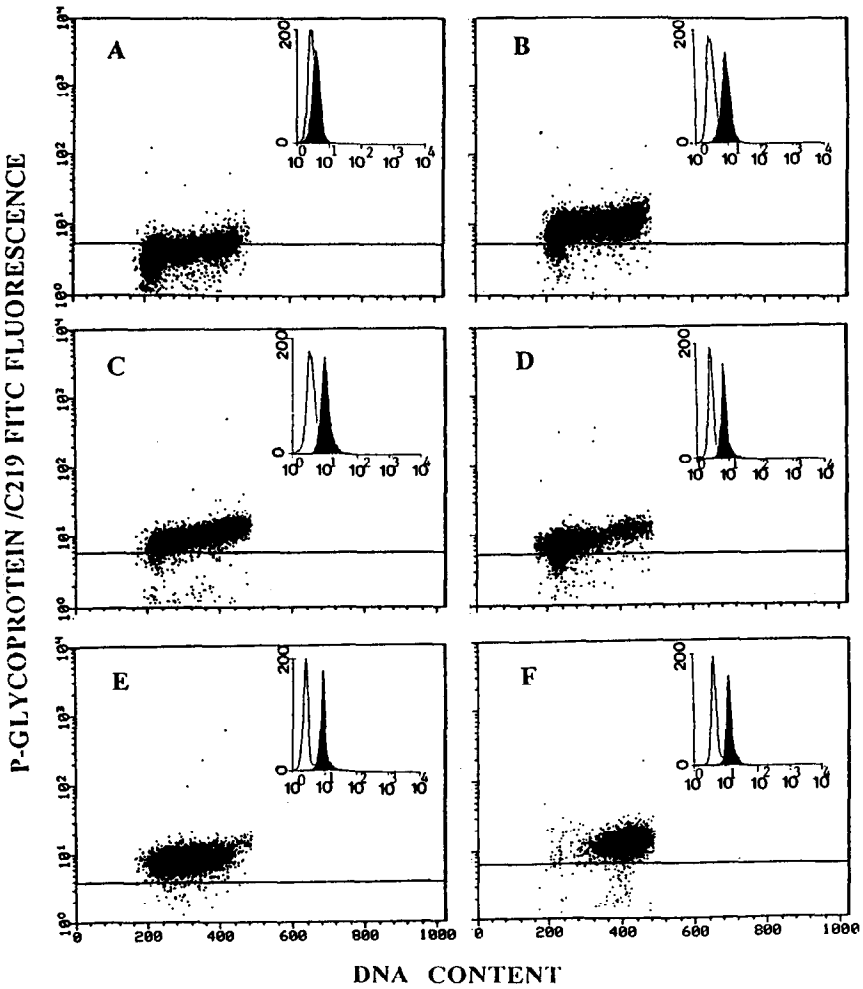

Fig. 4. Two parameter dot plots and single parameter histograms (insets) of P-gp negative AUX B_1 (A), P-gp positive CH^RC5 (B), log-phase P388/R-84 (C), and G_1- (D), S- (E), and G_2/M-phase (F) cells stained for P-gp expression (log FITC fluorescence) with C219 MAb. The horizontal lines represent electronic gates used to exclude 99% of fluorescent cells in the isotype control. Single parameter insets show FITC fluorescence of the isotype controls (open histogram) and C219 positive cells (filled histogram). The percentages of P-gp positive cells were 83, 74, 86, and 86% in log-phase, G_1-, S-, and G_2/M-populations, respectively.

cycle phase enriched P388/R-84 populations, G_1 cells showed the lowest percentage of cells with P-gp (74%) expression (Fig. 4D). P-gp expression in S- and G_2/M-phase enriched populations was highest with about 86% P-gp positive cells (Fig. 4, E and F). The difference between mean fluorescence channel values of IgG control and C219-reacted cells also increased with progression of the cell cycle (G_1 = log 4.88; S = log 6.62; and G_2/M = log 7.05).

DISCUSSION

In earlier studies, we had shown that cellular retention of doxorubicin and its efflux are cell cycle and proliferation related [7, 8]. The present study was undertaken to investigate the possible molecular mechanism involved in this phenomenon. The present study demonstrates the cell cycle related expression of the three markers of MDR in doxorubicin-resistant murine leukemic P388/R-84 cells. It is possible that cell lines selected for high level of resistance *in vitro* may not be physiologically relevant for studies on normal regulation of *mdr-1*, GST π, and topoisomerase II genes. However, the P388/R-84 MDR model was developed *in vivo* and, as we reported earlier [14–16, 22, 23], it is by far one of the best characterized systems for the study of multiple markers of resistance *in vivo* and *in vitro*. Further, this model cell line does not need the continuous presence of drug for expression of resistance. We used the cell cycle enriched populations separated by centrifugal elutriation for our study to avoid the biochemical artifacts that may be introduced by other non-mechanical methods of cell synchronization. The cells at S-phase had significantly higher *mdr-1* mRNA content than G_1-

and G_2/M-phase cells. Generally, there are three major factors controlling the mRNA content in cells: (a) rate of transcription, (b) nuclear turnover, processing and transport, and (c) mature RNA stability. Mickley *et al.* [26] reported that *mdr-1* mRNA has a short half-life of less than 3 hr and this short half-life is not increased by modulation of *mdr-1* mRNA expression by differentiating agents such as sodium butyrate. Recently, in colon carcinoma cells (LS-180-AD50) induced to express high *mdr-1* mRNA content with P-gp antagonists, Herzog *et al.* [27] failed to detect any increase in *mdr-1* transcription in nuclear run-off transcription experiments or in the stabilization of mRNA after actinomycin D inhibition of new RNA synthesis. Further studies are needed to determine the mechanism(s) controlling *mdr-1* mRNA content in tumor cells traversing through cell cycle. Since the promoter of human *mdr-1* gene is a target for c-Ha-*ras* 1 oncogene and p53 tumor suppressor gene products, both of which are expressed discontinuously during cell cycle [13], it is possible that *mdr-1* gene expression may also be cell cycle related.

In parallel with mRNA content, P-gp expression also changed during cell cycle progression, and G_1-phase enriched population had approximately 12% less P-gp positive cells than did S and G_2/M populations. In contrast, the S-phase cells had much higher *mdr-1* mRNA content than G_1 cells. These results may be expected due to the longer half-life of 24 hr [26] or 72 hr [28] for P-gp than *mdr-1* mRNA (3 hr) [26].

P-gp acts as an energy-dependent efflux pump for transporting xenobiotics and possibly other secondary products out of the cells and decreases the cellular retention of DNR and DOX in P388/R-84 cells [24, 29]. Furthermore, a number of efflux blockers, such as verapamil and chlorpromazine, can compete for this pump and can be used to enhance DOX and DNR retention in P388/R-84 cells [8, 29]. In the present study, DOX and DNR accumulation in P388/R-84 cells was significantly lower in S-phase cells than in G_1- and G_2/M-phase cells. Further, the efflux-blocking effect of VPL and CPZ was significantly higher in S-phase cells than in G_1- and G_2/M-phase cells. The drug efflux and efflux-blocking effect correlate very well with *mdr-1* mRNA expression, but not entirely with total P-gp content. Based on the long half-life of P-gp [26, 28] and the sensitivity of flow cytometric analysis of P-gp levels, one can argue that P-gp content should be the same throughout the cell cycle. However, the differences in drug accumulation and sensitivity to efflux blockers cannot be explained by similar P-gp levels at different cell cycle phase enriched populations. This paradox may be better explained by the post-translational modifications needed for the functionality of P-gp. The level of P-gp does not predict a given level of drug accumulation. Post-translational modifications such as glycosylation and phosphorylation are critical for the functionality of P-gp. Alteration in glycosylation is quite unlikely because of the surface localization of P-gp, and may not affect significantly the function of P-gp *per se* [6, 26]. On the other hand, the primary sequence of P-gp indicated the presence of 37 potential phosphorylation sites indicating 14 potential PKC sites, some or all of which could act to modulate drug transport [30]. Several investigators have suggested that more than one P-gp site can be phosphorylated by several different kinases [31–34]. Perhaps, PKC genes involved in P-gp phosphorylation may also be under cell cycle regulation, and their role in activation of the efflux pump needs to be investigated.

Doxorubicin resistance in P388/R-84 cells may partly involve overexpression of GST π and decreased topoisomerase II activity, even though the actual mechanism of resistance attributable to either of these factors is not conclusively proven [14–16, 35]. The present study indicates that while GST π mRNA expression was consistent during cell cycle traverse, topoisomerase II mRNA content increased with progression of cell cycle, reaching a maximum in G_2/M cells. Our observations on GST π mRNA content support the observations of Tihan *et al.* [36], who reported that total GST activity in cell cycle phase enriched fractions remains almost constant. Heck *et al.* [37] reported that topoisomerase II content increases just prior to or at the onset of DNA replication and continues to increase through S- and G_2-phases, peaking in the late G_2/M. On the other hand, Hsiang *et al.* [38] reported that the DNA topoisomerase II level remained constant throughout the late G_1, S, G_2, and M phases, and the level of DNA topoisomerase II is regulated primarily in the G_1-phase of the cell cycle. Our results indicated that in P388/R-84 cells, topoisomerase II mRNA expression is cell cycle related. The high expression of topoisomerase II mRNA may, in part, modulate (reduce) the increased resistance of P388/R-84 cells contributed by higher *mdr-1* mRNA expression in S-phase cells than in G_1- and G_2/M-phase cells. However, we believe that this modulating effect may be insignificant because of the unproven and minor role of topoisomerase II in drug resistance of P388/R-84 cells. Previous investigators have reported variations in the half-life of topoisomerase II protein between normal and transformed cells [37]. It would be worthwhile to investigate whether such a difference in the stability of topoisomerase II mRNA and protein exists between drug-resistant and -sensitive tumor cells on one hand, and between tumor and normal cells on the other.

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REFERENCES

1. Ueda K, Cardarelli C, Gottesman MM and Pastan I, Expression of a full-length cDNA for human "*MDR1*" gene confers resistance to colchicine, doxorubicin and vinblastine. *Proc Natl Acad Sci USA* **84**: 3004–3008, 1987.
2. Gros P, Neriah YB, Croop JM and Housman DE, Isolation and expression of a complementary DNA that confers multidrug resistance. *Nature* **323**: 728–731, 1986.
3. Scotto KW, Biedler JL and Melera PW, Amplification and expression of genes associated with multidrug

resistance in mammalian cells. *Science* **232**: 751–755, 1986.
4. Van der Bliek AM, Baas F, Van der Velde-Koerts T, Biedler JL, Meyers MB, Ozols RF, Hamilton TC, Joenje H and Borst P, Genes amplified and overexpressed in human multidrug-resistant cell lines. *Cancer Res* **48**: 5927–5932, 1988.
5. Shen D-w, Fojo A, Chin JE, Roninson IB, Richert N, Pastan I and Gottesman MM, Human multidrug-resistant cell lines: Increased *mdr*1 expression can precede gene amplification. *Science* **232**: 643–645, 1986.
6. Chen C, Chin JE, Ueda K, Clark DP, Pastan I, Gottesman MM and Roninson IB, Internal duplication and homology with bacterial transport proteins in the *mdr*1 (P-glycoprotein) gene from multidrug-resistant human cells. *Cell* **47**: 381–389, 1986.
7. Krishan A and Frei E III, Effect of adriamycin on the cell cycle traverse and kinetics of cultured human lymphoblasts. *Cancer Res* **36**: 143–150, 1976.
8. Krishan A, Sauerteig A and Wellham L, Flow cytometric studies on modulation of cellular adriamycin retention by phenothiazine. *Cancer Res* **45**: 1046–1051, 1985.
9. Farnham PJ and Schimke RT, Transcriptional regulation of mouse dihydrofolate reductase in the cell cycle. *J Biol Chem* **260**: 7675–7680, 1985.
10. Melton DW, Konecki DS, Brennand J and Caskey CT, Structure, expression and mutation of the hypoxanthine phosphoribosyltransferase gene. *Proc Natl Acad Sci USA* **81**: 2147–2151, 1984.
11. Kohno K, Sato S, Uchiumi T, Takano H, Kato S and Kuwano M, Tissue-specific enhancer of the human multidrug-resistance (MDR1) gene. *J Biol Chem* **265**: 19690–19696, 1990.
12. Madden MJ, Morrow CS, Nakagawa M, Goldsmith ME, Fairchild CR and Cowan KH, Identification of 5′ and 3′ sequences involved in the regulation of transcription of the human *mdr*1 gene *in vivo*. *J Biol Chem* **268**: 8290–8297, 1993.
13. Chin K-V, Ueda K, Pastan I and Gottesman MM, Modulation of activity of the promoter of the human *MDR*1 gene by Ras and p53. *Science* **255**: 459–462, 1992.
14. Singh SV, Nair S, Ahmad H, Awasthi YC and Krishan A, Glutathione *S*-transferases and glutathione peroxidases in doxorubicin-resistant murine leukemic P388 cells. *Biochem Pharmacol* **38**: 3505–3510, 1989.
15. Nair S, Singh SV, Samy TSA and Krishan A, Anthracycline resistance in murine leukemic P388 cells: Role of drug efflux and glutathione related enzymes. *Biochem Pharmacol* **39**: 723–728, 1990.
16. Maniar N, Krishan A, Israel M and Samy TSA, Anthracycline-induced DNA breaks and resealing in doxorubicin-resistant murine leukemic P388 cells. *Biochem Pharmacol* **37**: 1763–1772, 1988.
17. Krishan A, Rapid flow cytometric analysis of mammalian cell cycle by propidium iodide staining. *J Cell Biol* **66**: 188–193, 1975.
18. Dean PN, Dolbeare F, Gratzner H, Rice GC and Gray JW, Cell-cycle analysis using a monoclonal antibody to BrdUrd. *Cell Tissue Kinet* **17**: 427–436, 1984.
19. Bachur NR, Moore AL, Bernstein JG and Liu A, Tissue distribution and disposition of daunomycin (NSC-82151) in mice: Fluorometric and isotopic methods. *Cancer Chemother Rep* **54**: 89–94, 1970.
20. Ganapathi R, Reiter W and Krishan A, Intracellular adriamycin levels and cytotoxicity in adriamycin-sensitive and adriamycin-resistant P388 mouse leukemic cells. *J Natl Cancer Inst* **68**: 1027–1032, 1982.
21. Fuqua SAW, Fitzgerald SD and McGuire WL, A simple polymerase chain reaction method for detection and cloning of low-abundance transcripts. *Biotechniques* **90**: 206–211, 1990.
22. Ramachandran C, Samy TSA, Huang XL, Yuan ZK and Krishan A, Doxorubicin-induced DNA breaks, topoisomerase II activity and gene expression in human melanoma cells. *Biochem Pharmacol* **45**: 1367–1371, 1993.
23. Ramachandran C, Yuan ZK, Huang XL and Krishan A, Doxorubicin resistance in human melanoma cells: *MDR-1* and glutathione *S*-transferase π gene expression. *Biochem Pharmacol* **45**: 743–751, 1993.
24. Ramachandran C, Sauerteig A, Sridhar KS, Thurer RJ and Krishan A, MDR-1 gene expression, anthracycline retention and cytotoxicity in human lung-tumor cells from refractory patients. *Cancer Chemother Pharmacol* **31**: 431–441, 1993.
25. Krishan A, Sauerteig A and Stein JH, A comparison of three commercially available antibodies for flow cytometric monitoring of P-glycoprotein expression in tumor cells. *Cytometry* **12**: 731–742, 1991.
26. Mickley LA, Bates SE, Richert ND, Currier S, Tanaka S, Foss F, Rosen N and Fojo AT, Modulation of the expression of a multidrug resistance gene (*mdr*-1/P-glycoprotein) by differentiating agents. *J Biol Chem* **264**: 18031–18040, 1989.
27. Herzog CE, Tsokos M, Bates SE and Fojo AT, Increased *mdr*-1/P-glycoprotein expression after treatment of human colon carcinoma cells with P-glycoprotein antagonists. *J Biol Chem* **268**: 2946–2952, 1993.
28. Richert N, Aldwin L, Nitecki D, Gottesman MM and Pastan I, Stability and covalent modulation of P-glycoprotein in multidrug-resistant KB cells. *Biochemistry* **27**: 7607–7613, 1988.
29. Krishan A, Sauerteig A, Gordon K and Swinkin C, Flow cytometric monitoring of cellular anthracycline accumulation in murine leukemic cells. *Cancer Res* **46**: 1768–1773, 1986.
30. Barioch A, *PROSITE, a Dictionary of Protein Sites and Patterns*. Fifth Release. University of Geneva Press, Geneva, Switzerland, 1990.
31. Chambers TC, Chalikonda I and Eilon G, Correlation of protein kinase C translocation, P-glycoprotein phosphorylation and reduced drug accumulation in multidrug resistant human KB cells. *Biochem Biophys Res Commun* **169**: 253–259, 1990.
32. Mellado W and Horwitz SB, Phosphorylation of the multidrug resistance associated glycoprotein. *Biochemistry* **26**: 6900–6904, 1987.
33. Fine RL, Pastel J and Chabner BA, Phorbol esters induce multidrug resistance in human breast cancer cells. *Proc Natl Acad Sci USA* **85**: 582–586, 1988.
34. Staats J, Marquardt D and Center MS, Characterization of a membrane-associated protein kinase of multidrug-resistant HL60 cells which phosphorylates P-glycoprotein. *J Biol Chem* **265**: 4084–4090, 1990.
35. Goldenberg GJ, Wang H and Blair GW, Resistance to adriamycin: Relationship of cytotoxicity to drug uptake and DNA single- and double-strand breakage in cloned cell lines of adriamycin-sensitive and -resistant P388 leukemia. *Cancer Res* **46**: 2978–2983, 1986.
36. Tihan T, Chiba P, Eher R, Goebl R, Pernerstorfer T, Kraupp M and Kaiser E, Influence of cell cycle on glutathione-*S*-transferase, selenium-dependent glutathione peroxidase, superoxide dismutase and glutathione levels in human myeloid leukemia cell lines. *Carcinogenesis* **10**: 1709–1712, 1989.
37. Heck MMS, Hittelman WN and Earnshaw WC, Differential expression of DNA topoisomerases I and II during the eukaryotic cell cycle. *Proc Natl Acad Sci USA* **85**: 1086–1090, 1988.
38. Hsiang Y-H, Wu H-Y and Liu LF, Proliferation-dependent regulation of DNA topoisomerase II in cultured human cells. *Cancer Res* **48**: 3230–3235, 1988.