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Phorbol Ester-induced P-Glycoprotein Phosphorylation and Functionality in the HTB-123 Human Breast Cancer Cell Line

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ABSTRACT. The discordance between P-glycoprotein (P-gp) expression and functionality [as measured by the efflux of doxorubicin (DOX)] was analyzed in a DOX-sensitive human breast cancer cell line (HTB-123) with high reactivity against four P-gp specific monoclonal antibodies (C219, MRK-16, UIC2, and 4E3). Reverse transcription–polymerase chain reaction (RT–PCR) and Western blotting analyses confirmed the overexpression of MDR1 mRNA and P-gp in this cell line. However, incubation of cells with efflux blockers, verapamil (VPL) or dipyridamole (DPD), did not enhance cellular (DOX) accumulation or cytotoxicity. Upon incubation with 12-*O*-tetradecanoylphorbol-13-acetate (TPA), HTB-123 cells retained less DOX than control cells and were sensitive to the efflux blockers verapamil or dipyridamole. These observations suggest that 12-O-tetradecanoylphorbol-13-acetate-induced P-gp phosphorylation may be associated with induction of P-gp-mediated drug efflux in the HTB-123 cell line. BIOCHEM PHARMACOL **56**;6:709–718, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. multidrug resistance; P-glycoprotein; drug efflux; phorbol ester; phosphorylation

MDR‡ may be one of the reasons for the failure of chemotherapy in refractory breast cancer patients. Several mechanisms responsible for MDR have been described, and energy-dependent drug efflux is one of the major factors contributing to reduced cellular drug accumulation and chemosensitivity [1–4]. Cell membrane-resident 170-kDa P-gp, encoded by the *MDR1* gene, is believed to act as an efflux pump and reduce cellular drug accumulation [2, 5, 6]. Overexpression of P-gp or *MDR1* gene has been reported in drug-resistant tissue culture cells and tumor cells from patients [7–10]. Because P-gp-mediated drug efflux is blocked by several chemicals such as VPL and DPD [11–14], reduced cellular drug accumulation and sensitivity to efflux blockers, which increase drug retention, are important indicators of P-gp function. Anthracyclines such as doxorubicin and daunorubicin are fluorescent, and their cellular accumulation, monitored by laser flow cytometry, has been used as an indicator for P-gp functionality [15].

Earlier reports on enhanced drug sensitivity and phosphorylation in cells incubated with chemosensitizers [16–19] suggest that the drug efflux function of P-gp may be regulated by the phosphorylation/dephosphorylation mechanism. As the linker region separating the two P-gp halves is phosphorylated at several sites, the role of phosphorylation in regulating P-gp-mediated drug transport has been explored by several investigators. Some of these studies have sought to correlate changes in activities and/or levels of different protein kinases with MDR expression and/or drug accumulation [20–24]. Incubation of MDR cells with PKC activators such as phorbol esters (TPA or PMA) have been reported to stimulate P-gp phosphorylation, reduce drug accumulation, and enhance drug resistance [19, 25–28]. In contrast to these reports, some other recent studies have questioned the role of P-gp phosphorylation in drug transport and MDR. Germann *et al.* [29], on the basis of their studies on phosphorylation and dephosphorylation-defective mutant cell lines, have suggested that phosphorylation is not essential for conferring MDR. Goodfellow *et al.* [30] have reported that phosphorylation of P-gp linker in the mutant cell lines does not affect the rate of drug transport. In both of these studies, site-directed mutagenesis was used to develop phosphorylation/dephosphorylation defective mutants of resistant cell lines. In the present study, we report that a DOX-sensitive cell line (HTB-123) has P-gp expression as compared with some other breast

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‡ *Abbreviations:* b_2-MG, b_2-microglobulin; CEM, CCRF-CEM; CEM/VLB, CCRF-CEM/VLB100; DOX/doxorubicin, doxorubicin hydrochloride; DPD, dipyridamole; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; MAb, monoclonal antibody; MCV, mean channel value(s); MDR, multidrug resistance; PBS, calcium and magnesium-free Dulbecco's phosphate-buffered saline; P-gp, P-glycoprotein; PKC, protein kinase C; PMA, 4β-phorbol 2β-myristate-13-acetate; Rh, rhodamine; RT–PCR, reverse transcription–polymerase chain reaction; TPA, 12-O-tetradecanoylphorbol-13-acetate; VLB, vinblastine sulfate; and VPL, verapamil.

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cancer (e.g. MCF-7, HTB-19, and HTB-20) cell lines [31]. However, the P-gp expressed in this cell line is non-functional, and its role as a drug efflux pump can be activated by TPA treatment.

MATERIALS AND METHODS

Cell Lines

HTB-123, a human breast cancer cell line established from a patient with metastatic breast cancer [32], was obtained from the American Type Culture Collection (ATCC). The human leukemic cell line CEM, and its VLB-resistant subline, CEM/VLB, were obtained from Dr. William Beck (University of Illinois). CEM/VLB cells have MDR1 mRNA/P-gp overexpression and MDR phenotype [31]. The cell lines were grown in RPMI 1640 medium supplemented with FBS (20% for HTB-123 and 10% for CEM and CEM/VLB), 2 mM L-glutamine, 100 U/mL of penicillin, and 100 mg/mL of streptomycin in a humidified atmosphere of 5% CO_2 at 37°. CEM/VLB cultures were exposed to 0.1 μM VLB weekly to maintain resistance, and cells were cultured in drug-free medium for at least 4 days before experimentation.

Chemicals and Antibodies

DOX, daunorubicin hydrochloride (DNR), Rh, VPL, DPD, and TPA were purchased from the Sigma Chemical Co. Three P-gp specific MAbs, MRK-16, 4E3, and C219, were purchased from commercial sources. UIC2 was a gift from Dr. E. B. Mechetner, Ingenex Inc. Non-specific murine immunoglobulin (for isotype control) and FITC-conjugated F(ab′)2 fragment of sheep-anti-mouse immunoglobulin were purchased from Sigma.

Flow Cytometric Analysis of P-gp Expression

Cells without fixation were used for MRK-16, UIC2, and 4E3 antibody incubation, and methanol-fixed (100%, 30 min, on ice) cells were used for C219 studies. Cells were incubated initially with each antibody or isoform-matched non-specific immunoglobulin (isotype control) for 30 min on ice. After washing with PBS, cells were incubated with FITC-conjugated F(ab′)2 fragment of sheep-anti-mouse immunoglobulin (50 mL of 1:200 dilution). Cellular FITC fluorescence of 10,000 cells per sample was analyzed in a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems). Details of our methods for flow cytometry have been reported earlier [33].

Western Blot Hybridization

Plasma membranes were prepared by a modification of the procedure described by Lever [34]. Cells (5–10 × 10^7) from log-phase cultures were washed twice with PBS, resuspended in 20 mL of medium I (5 mM Tris–HCl, pH 7.5, 0.25 M sucrose, and 0.2 mM $MgCl_2$), centrifuged at 800 *g* for 10 min, washed, and resuspended in medium at 4° for 25 min. The cell pellets were homogenized in a T-7 homogenizer with 3–4 strokes and centrifuged at 800 *g* for 10 min; the supernatant containing plasma membranes and organelles was saved and the nuclear pellet was discarded. The supernatant was centrifuged at 22,000 *g* for 25 min, the supernatant was saved once again, and the pellet containing organelles and plasma membranes was resuspended in 10 mL of medium I containing 1 mM $MgCl_2$. The pellet suspension was layered over 26 mL of a dextran cushion (13% dextran, 5 mM Tris–HCl, 0.25 sucrose, 1 mM $MgCl_2$) and centrifuged at 100,000 *g* for 45 min in a Beckman ultracentrifuge. Plasma membranes banded in the upper layer were transferred to the tube containing the 20 mL of supernatant collected earlier. This pooled fraction was centrifuged again at 100,000 *g* for 45 min, and the pellet containing the plasma membranes was resuspended in 200 μL of PBS. The protein content of the membrane preparation was determined by the Bradford assay [35]. Protein (50 mg) was separated on 5% SDS–PAGE, blotted, and reacted with C219 antibody. A Lumi-phos 530 kit (Boehringer Mannheim) was used to detect the antigen–antibody conjugate according to the manufacturer's protocols [36]. Autoradiographs were scanned in a densitometer, and relative P-gp expression was quantitated.

RT–PCR

MDR1 mRNA expression was analyzed by RT–PCR assay. Poly(A)$^+$ RNA was extracted from tumor cells using the Micro-Fast track kit (Invitrogen Corp.). Reverse transcription was carried out using 0.5 μg of poly(A)$^+$ RNA, 5 mM $MgCl_2$, 1x PCR buffer, 1 mM of each of four deoxyribonucleotide triphosphates, 1 U/μL of RNasin, 5 mM random hexadeoxynucleotide primer, and 2.5 U/μL of superscript reverse transcriptase in a total reaction volume of 20 μL at 37° for 1 hr. PCR was carried out for 30 cycles with 5 μL of cDNA, 300 nM *MDR1* gene specific sense (5′-CCCAT CATTCCAATAGCAGG-3′) and antisense (5′-GTT CAAACTTCTGCTCCTGA-3′) or *MRP* gene specific sense (5′-CCTGGACCCATTCAGCCAGTAC-3′) and antisense (5′-GGCCCTGCAGTTCTGACCAGAT-3′) primers, and 2.5 U/100 μL of AmpliTaq DNA polymerase in a total reaction volume of 100 μL [37]. β_2-*MG* gene amplification was used as an internal control, using a set of sense and antisense primers [37]. PCR products were separated on a 3% NuSieve (FMC BioProducts) agarose gel, and relative *MDR1* gene expression was analyzed. For quantitation, 1 μCi of [α-^{32}P]dCTP was added into each PCR reaction mixture, and radioactivity in each MDR1 mRNA PCR band was counted by liquid scintillation spectrometry.

Cytotoxicity Assay

DOX cytotoxicity was determined by soft agar clonogenic assays. Following incubation with various concentrations of

DOX alone and in the presence of 10 μM VPL at 37° for 2 hr, cells were washed and resuspended in the RPMI medium containing FBS (20% for HTB-123 and 15% for CEM and CEM/VLB) and 0.3% agar, and layered onto a feeder layer containing 0.5% agar and FBS in RPMI medium. After 2 weeks of culture, colonies larger than 50 μm were counted [10, 38]. Each experiment was repeated three times with three replications in every drug concentrations.

Cellular Drug Fluorescence and Effect of Efflux Blockers

Cell suspensions (10^6 mL) were incubated with 5 μM DOX or DNR or 0.26 μM Rh in the presence or absence of efflux blockers, VPL (10 μM), or DPD (25 μM) at 37° for 1 hr. Cellular DOX, DNR, or Rh fluorescence was measured by flow cytometric procedures described earlier [11].

Cells pretreated with 0.2 μM TPA (37°, 2 hr) were subjected to the same procedure. DOX accumulation and sensitivity to the efflux blockers were compared with and without TPA pretreatment.

Fluorometric Analysis of Intracellular DOX Accumulation

The effect of the efflux blockers and TPA pretreatment on DOX accumulation was quantitated by a fluorescence spectrometer. DOX was extracted with an alcohol–acid mixture containing 50% ethanol and 0.3 N HCl [39, 40], and fluorescence was determined at excitation and emission wavelengths of 480 and 590 nm, respectively, in a Perkin Elmer LS-3B fluorescence spectrometer (Perkin Elmer Corp.). DOX concentration in the sample was derived from a standard curve based on the known drug concentration in the extraction solvent.

Phosphorylation of P-gp

To analyze the effect of TPA on phosphorylation, cells were pretreated with 0.2 μM TPA at 37° for 2 hr, followed by labeling of cellular proteins with [^{32}P]orthophosphate (0.2 mCi at 37° for 1 hr). Cells were washed three times with cold PBS, and total lysate was prepared by the procedure of Blobe *et al.* [41]. P-gp in the cell extract was immunoprecipitated with C219 antibody [41] and analyzed by 5% SDS–PAGE. The relative P-gp phosphorylation was determined by scanning of autoradiographs in a Zeineh densitometer.

RESULTS

P-gp/MDR1 Expression

Figure 1 shows flow cytometric analysis of P-gp expression in CEM, CEM/VLB, and HTB-123 cells, reacted with four P-gp specific antibodies. CEM cells had no P-gp expression, as evidenced by lack of reactivity to MRK-16 (A) or C219 (B) antibodies. In contrast, CEM/VLB cells reacted with both MRK-16 (C) and C219 (D) antibodies. The shifts in the MCV of FITC fluorescence between MRK-16-treated cells and the isotype controls, and between C219-treated cells and the isotype controls, were 118- (497 vs 4.2) and 10.6-fold (30.7 vs 2.9), respectively. Patterns of UIC2 or 4E3 staining in CEM and CEM/VLB cells were similar to those of MRK-16 staining (data not shown). HTB-123 cells had significant reactivity with all of the four antibodies; MRK-16 (E), C219 (F), UIC2 (G), and 4E3 (H). The shifts in FITC MCV between antibody-treated cells and isotype controls were 5.0- (72.7 vs 14.6), 3.8- (18.2 vs 5.4), 8.1- (104.8 vs 13.0), and 4.4- (88.9 vs 20.4) fold, for MRK-16, C219, UIC2, and 4E3, respectively.

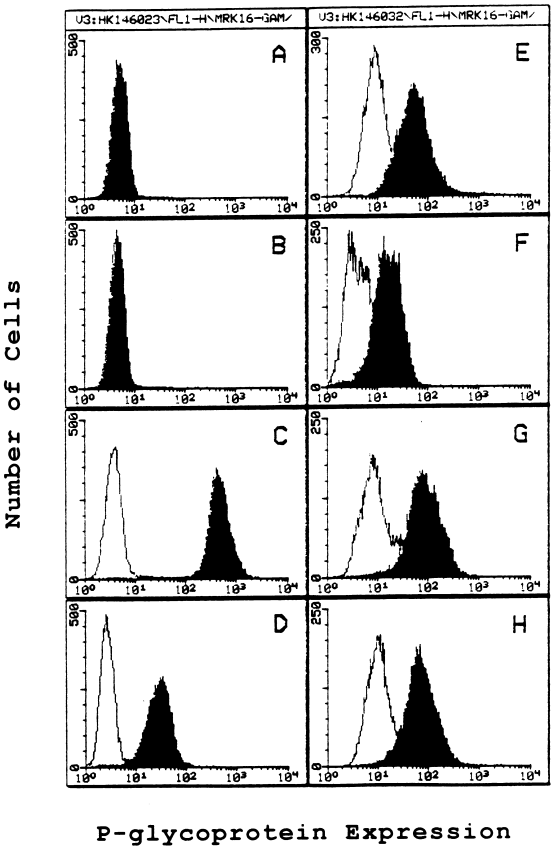

FIG. 1. Flow cytometric analysis of P-gp expression. The horizontal axis records FITC fluorescence of MAb-treated or isotype control cells, while the vertical axis represents number of cells. Histograms in black and those in white are of MAb-treated cells and isotype controls, respectively. (A and B) CEM; (C and D) CEM/VLB; and (E, F, G, and H) HTB-123. (A, C, and E) MRK-16 staining; (B, D, and F) C219 staining; (G) UIC2 staining; and (H) 4E3 staining.

In western blots (Fig. 2), CEM cells showed no antibody binding (lane 1), whereas in CEM/VLB cells, the 170 kDa protein reacted with C219 antibody (lane 2). HTB-123 cells (lane 3) also had the 170 kDa P-gp band reacting with the antibody. P-gp expression in HTB-123 cells was about 57% of that in CEM/VLB cells (HTB-123 = 24.05 ± 1.05 arbitrary densitometric units; CEM/VLB = 42.11 ± 1.90 arbitrary densitometric units).

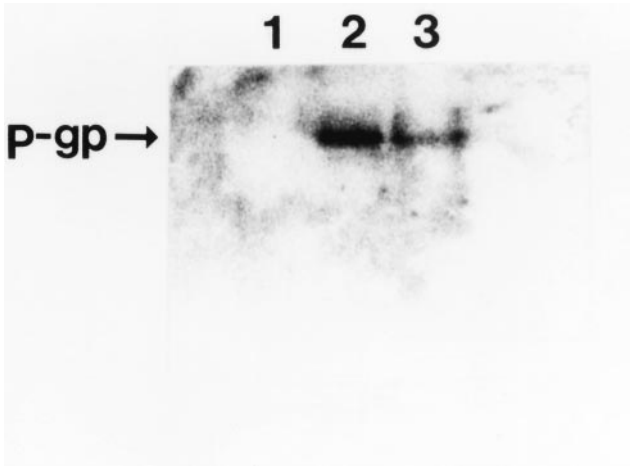

FIG. 2. Western blot analysis of P-gp expression. After the isolation of plasma membrane protein, 50 μg of protein from each sample was separated on 5% SDS–PAGE, blotted, and hybridized with P-gp specific C219 MAb. Lane 1, CEM; lane 2, CEM/VLB; and lane 3, HTB-123.

Figure 3 shows the results of RT–PCR analysis of MDR1 mRNA expression in CEM, CEM/VLB, and HTB-123 cells. The expected 167 base fragment of MDR1 mRNA was amplified from CEM/VLB cells (lane 2) and HTB-123 cells (lane 3) but not from CEM cells (lane 1). MDR1 mRNA content in HTB-123 cells was approximately 50% of that in CEM/VLB cells. CEM cells had very little MDR1 mRNA expression.

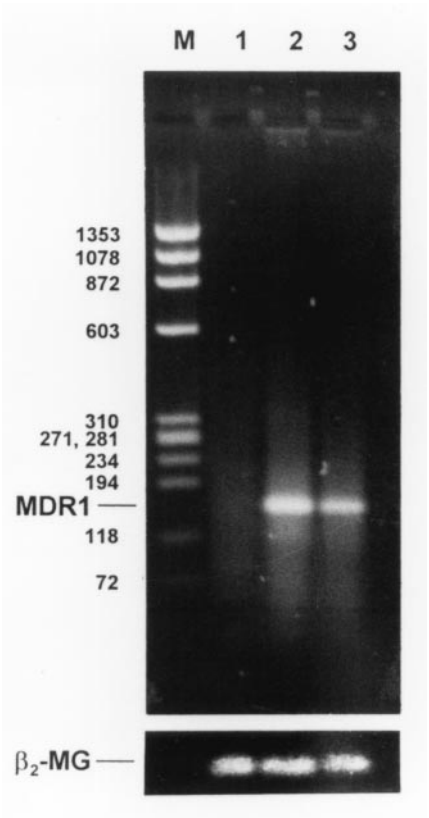

FIG. 3. RT–PCR analysis of MDR1 mRNA expression. After reverse transcription, PCR was carried out for 30 cycles. b_2-MG mRNA amplified was used as the internal control (lower bands). Lane M, marker; lane 1, CEM; lane 2, CEM/VLB; and lane 3, HTB-123.

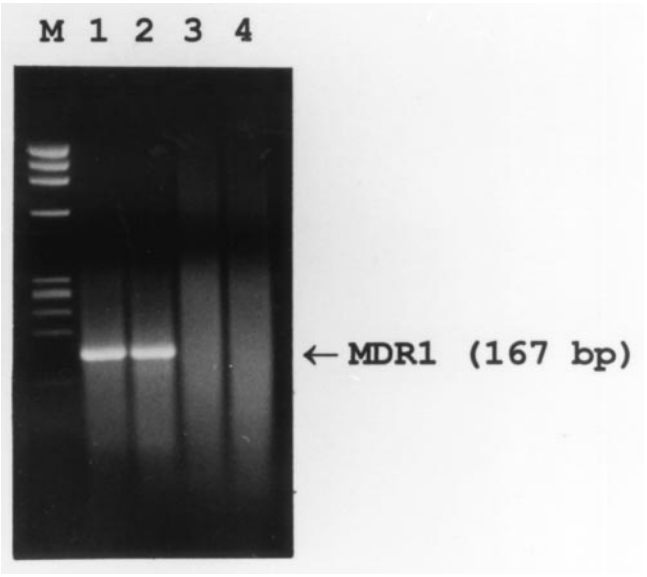

FIG. 4. RT–PCR analysis of MDR1 and MRP mRNA expression in untreated and TPA pretreated HTB-123 cells. Lane M, marker; lane 1, total RNA of untreated HTB-123 cells amplified with *MDR1* primers; lane 2, total RNA from TPA pretreated cells amplified with *MDR1* primers; lane 3, RNA from untreated cells amplified with *MRP* primers; lane 4, total RNA from TPA pretreated cells amplified with *MRP* primers.

Figure 4 shows the results of RT–PCR amplification of MDR1 (lanes 1 and 2) and MRP mRNA (lanes 3 and 4) in untreated and TPA pretreated HTB-123 cells. TPA pretreatment of HTB-123 cells for 2 hr caused no change in the MDR1 mRNA levels. HTB-123 cells neither expressed MRP nor was its expression induced by a 2-hr TPA pretreatment. P-gp expression was also not altered by TPA pretreatment (Fig. 5).

DOX Cytotoxicity

Data on DOX cytotoxicity in the presence or absence of VPL (10 μM) in CEM (A), CEM/VLB (B), and HTB-123 (C) cells are presented in Fig. 6. The IC_{50} values are given in Table 1. DOX IC_{50} in CEM cells was 0.033 μM, and co-incubation with VPL did not change DOX cytotoxicity significantly. In the multidrug-resistant CEM/VLB cells (approximately 70-fold resistant), co-incubation with VPL decreased DOX resistance to 10-fold ($P < 0.05$ by Student's *t*-test). HTB-123 cells were more sensitive to DOX than CEM cells with an IC_{50} value of 0.010 μM, which was approximately three-fold less than that of the CEM cells. Co-incubation with VPL did not change the DOX sensitivity of HTB-123 cells significantly (Table 1).

To determine the effect of TPA pretreatment on DOX cytotoxicity to HTB-123 cells, a colony-forming assay was performed and compared with untreated cells. TPA pretreatment of HTB-123 cells caused a significant (100%)

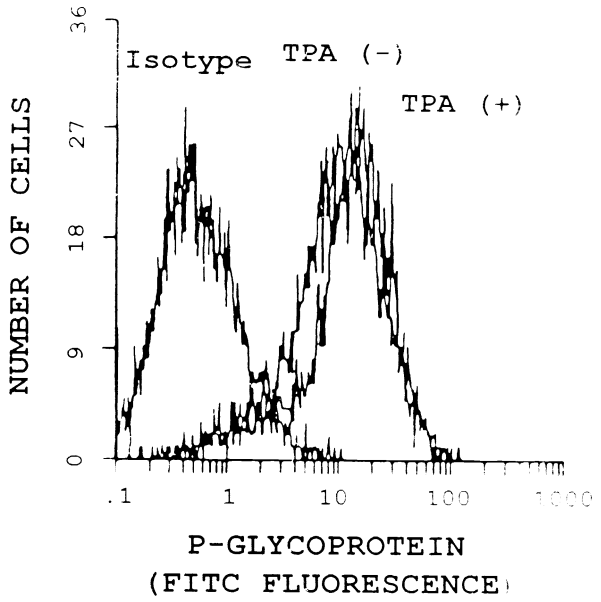

FIG. 5. Flow-cytometric analysis of P-gp expression in untreated (TPA−) and TPA pretreated (TPA+) HTB-123 cells.

increase in the DOX IC_{50} value (0.020 ± 0.002 μM) as compared with the untreated cells (0.010 ± 0.001 μM), in which there appeared to be low, but significant induction of DOX resistance ($P < 0.05$ by Student's *t*-test).

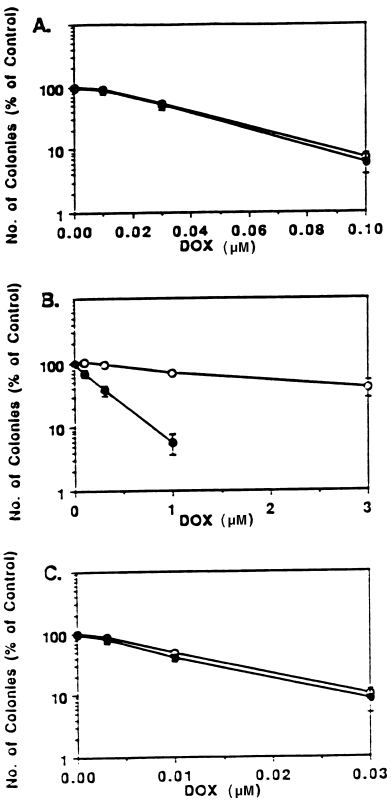

FIG. 6. DOX concentration–response curve in soft agar clonogenic assay. Data are mean values ± SE of three different experiments. (A) CEM; (B) CEM/VLB; and (C) HTB-123. Key: (○) DOX alone, and (●) DOX + VPL (10 μM).

TABLE 1. DOX cytotoxicity of TPA-pretreated and untreated tumor cells in the presence and absence of VPL

Cell lines	VPL (10 μM)	DOX IC_{50} (μM) TPA (−)	DOX IC_{50} (μM) TPA (+)*
CEM	−	0.033 ± 0.005	0.035 ± 0.004
	+	0.031 ± 0.004	
CEM/VLB	−	2.40 ± 0.400	2.450 ± 0.270
	+	0.22 ± 0.050†	
HTB-123	−	0.010 ± 0.001	0.020 ± 0.002‡
	+	0.009 ± 0.002	

Analyzed by soft agar clonogenic assay. All estimates are means ± SE (N = 3). Statistical significance was measured using Student's *t*-test.

*Tumor cells were pretreated with TPA at 37° for 2 hr in a CO_2 incubator followed by DOX at various concentrations in the presence or absence of VPL. See Materials and Methods for the detailed procedure. TPA (−), without TPA pretreatment; TPA (+), with TPA pretreatment.

†$P < 0.05$ vs VPL(−) value.

‡$P < 0.05$ vs TPA(−) value.

DOX Accumulation and Efflux

In Fig. 7, histograms of DOX accumulation in CEM (A), CEM/VLB (B), HTB-123 (C), and TPA pretreated HTB-123 (D) cells are shown. Histograms labeled "a" in the four panels are of the cellular autofluorescence. The MCV of DOX fluorescence are given in Table 2. In CEM cells, co-incubation with DOX + VPL or DOX + DPD did not increase DOX fluorescence over that of cells incubated with DOX alone (Fig. 7A; Table 2). In CEM/VLB cells, co-incubation with VPL increased DOX accumulation with an increase of MCV from 43.3 to 93.9, as shown in Fig. 7B and Table 2. DOX fluorescence histograms of HTB-123 cells incubated with DOX alone or DOX + VPL (b and c in Fig. 7C) were similar. Thus, the efflux blockers (VPL or DPD) had no effect on DOX accumulation in HTB-123 cells. Panel D of Fig. 7 shows DOX accumulation in HTB-123 cells pretreated with TPA and incubated with DOX alone (b) or DOX + VPL (c). TPA pretreatment caused a reduction in DOX accumulation in HTB-123 cells that was reversed by co-incubation with efflux blocker (VPL). DPD also reversed the reduction of DOX accumulation in TPA pretreated HTB-123 cells (Table 2). These observations suggested that HTB-123 cells under normal conditions have very little DOX efflux, but when pretreated with TPA for 2 hr, they have reduced DOX accumulation that can be reversed by co-incubation with efflux blockers. TPA treatment did not change cellular DOX fluorescence or sensitivity to efflux blockers in CEM or CEM/VLB cells (data not shown).

Data on intracellular DOX accumulation quantitated by the fluorescence spectrometer are presented in Fig. 8. These results confirmed the flow cytometric data in Fig. 7 and Table 2. VPL did not increase DOX accumulation of HTB-123 cells (cellular DOX in B = 858 ± 45 vs A = 852 ± 35 ng/mg protein). However, in TPA-pretreated HTB-123 cells, DOX accumulation was reduced by approximately 40% (C = 537 ± 43 vs A = 852 ± 35 ng/mg protein) compared with untreated cells. Co-incubation of

TABLE 2. MCV of cellular DOX fluorescence by co-incubation with or without efflux blockers

Cells	MCV			
	DOX (−) (autofluorescence)	DOX alone	DOX + VPL	DOX + DPD
CEM	3.5	130	134	129
CEM/VLB	2.7	43.3	93.9	88.0
HTB-123	3.4	171	166	150
TPA pretreated HTB-123	3.6	91.5	143	161

Mean values were calculated from three separate experiments.

TPA-pretreated HTB-123 cells with DOX + VPL enhanced DOX accumulation by approximately 35% as compared with TPA-pretreated cells incubated with DOX alone (D = 729 ± 55 vs C = 537 ± 43 ng/mg of protein).

Cellular DNR or Rh accumulation data, presented in Fig. 9, showed similar results. TPA pretreatment of HTB-123 cells caused a reduction in cellular DNR and Rh accumulation that could be reversed by co-incubation with VPL.

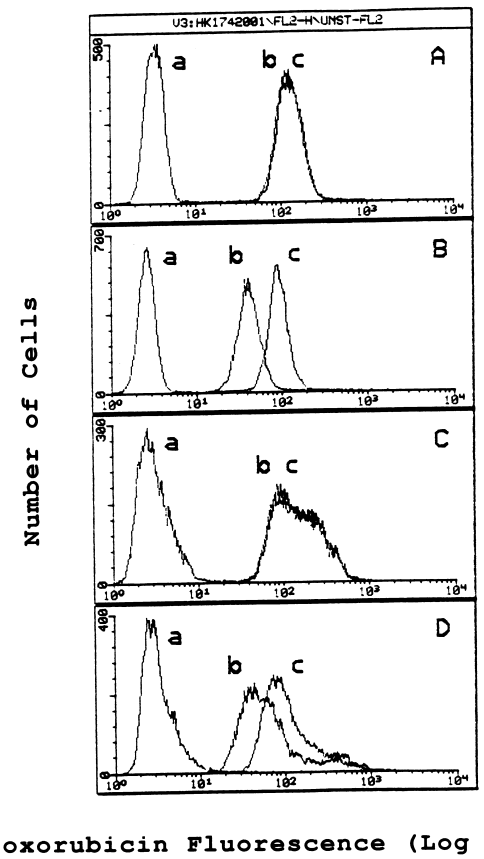

FIG. 7. Flow-cytometric analysis of cellular DOX fluorescence after 2 hr of incubation with and without VPL. In each overlaid histogram, the horizontal axis records DOX fluorescence while the vertical axis represents number of cells. Histograms of cellular autofluorescence without any drugs (a), with DOX alone (b), and DOX + VPL (c) are indicated in the figure. (A) CEM without TPA pretreatment; (B) CEM/VLB without TPA pretreatment; (C) HTB-123 without TPA pretreatment; and (D) HTB-123 with TPA treatment.

P-gp Phosphorylation

Figure 10 shows the results of P-gp phosphorylation experiments. [^{32}P]-labeled proteins immunoprecipitated with C219 showed a distinct 170 kDa P-gp band. Lanes 1, 2 and 3 in TPA-untreated (TPA−) and TPA pretreated (TPA+) are of HTB-123, CEM/VLB, and CEM cells, respectively. There was no P-gp expression in CEM cells (Fig. 2), and TPA treatment (2 hr) induced neither P-gp expression (data not shown) nor P-gp phosphorylation. In CEM/VLB cells, which have functional P-gp, TPA treatment increased the P-gp phosphorylation by 30% (compare lane 2 in TPA− vs TPA+). In HTB-123 cells, P-gp was not phosphorylated as indicated by the absence of any 170 kDa band (lane 1 in TPA−). However, in HTB-123 cells pretreated with TPA, P-gp was phosphorylated by about 26% (lane 1 in TPA+) as compared with that in TPA

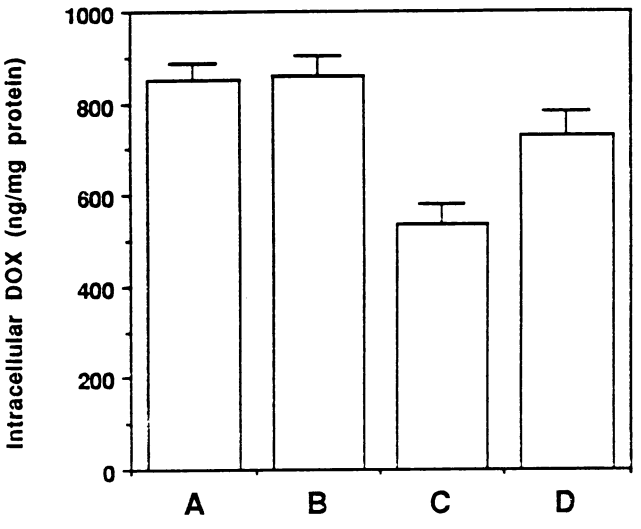

FIG. 8. Intracellular DOX accumulation in HTB-123 cells measured by a fluorescence spectrophotometer. Data are mean values ± SE of three different experiments. (A and B) without TPA pretreatment; (C and D) with TPA pretreatment. (A and C) incubation with DOX alone; (B and D) co-incubation with DOX + VPL. TPA pretreatment of HTB-123 cells (C) significantly reduced ($P < 0.05$ by Student's *t*-test) cellular DOX accumulation compared with untreated cells (A). Co-incubation of TPA pretreated cells with DOX + VPL (D) significantly enhanced and restored cellular DOX accumulation ($P < 0.05$ by Student's *t*-test) compared with TPA pretreated cells incubated with DOX alone (C).

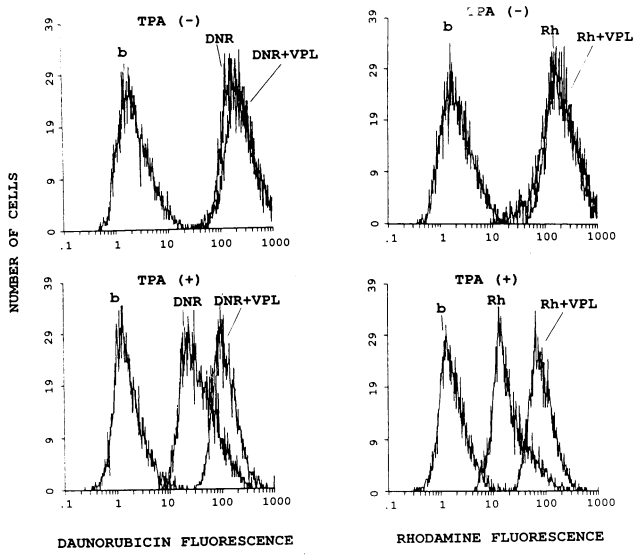

FIG. 9. Flow-cytometric analysis of cellular DNR and Rh fluorescence in untreated and TPA pretreated HTB-123 cells after a 1-hr incubation with or without VPL. In each overlaid histogram, the horizontal axis records DNR or Rh fluorescence while vertical axis represents number of cells. Histograms of cellular autofluorescence without any drug (b), with DNR or Rh alone and DNR + VPL or Rh + VPL are indicated in the figure. TPA (−), untreated; TPA (+), TPA pretreated.

pretreated CEM/VLB cells (HTB-123 = 23.75 ± 2.20 densitometric units; CEM/VLB = 91.01 ± 4.38 densitometric units).

DISCUSSION

Cellular resistance may be one of the major reasons for the failure of chemotherapy in human breast cancer. Energy-dependent drug efflux involving P-gp is believed to result in reduced drug accumulation and sensitivity to a variety of

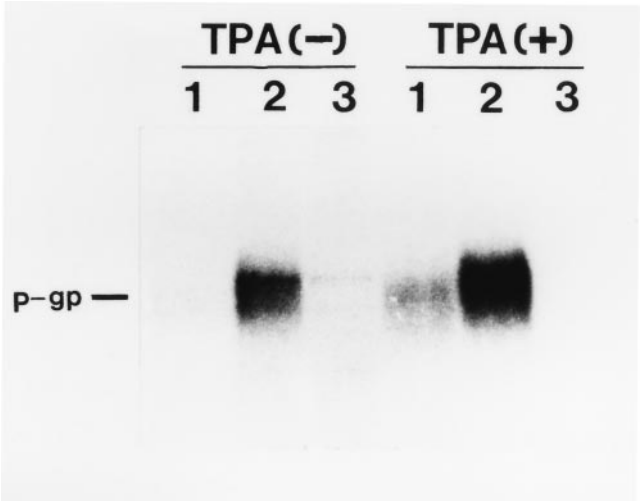

FIG. 10. Effect of TPA on P-gp phosphorylation in CEM, CEM/VLB, and HTB-123 cells. Untreated (TPA−) and pretreated (TPA+) cells are indicated in the figure. Lane 1, HTB-123; lane 2, CEM/VLB; and lane 3, CEM.

natural products used in the therapy of breast cancer. During the last decade, numerous studies have reported on the expression of P-gp, a 170-kDa transmembrane protein encoded by the *MDR1* gene, in human breast cancer cells [42–53]. Most of these studies have reported MDR1 mRNA expression or the presence of P-gp in tumor biopsies or paraffin-embedded tumor samples. Verelle *et al.* [50] reported that 85% of tumors from patients with primary locally advanced breast carcinoma have high P-gp expression, which correlates with lack of response to therapy and disease progression or recurrence. Ro *et al.* [43] suggested that intrinsic rather than acquired drug resistance may play a role in the failure of chemotherapy in locally advanced breast cancer.

Several investigators seeking to correlate P-gp expression with clinical outcome have reported the absence of any apparent relationship between MDR1 mRNA, P-gp expression, and response to therapy [46, 48]. However, it should be noted that most of these investigations measured either MDR1 mRNA and/or P-gp expression in tumor cells. None of these studies analyzed functionality of the P-gp as an efflux pump. Several recent studies along with data from the present report suggest that it may not be sufficient to measure mRNA or P-gp expression alone without determining the functionality of the pump and its role in reducing cellular drug accumulation and chemosensitivity.

In an earlier publication, we reported that human melanoma cell lines (FCCM-2 and FCCM-9) with 2.7- and 6.1-fold DOX resistance (as compared with a DOX-sensitive HM-1 melanoma cell line) had high MDR1 mRNA and P-gp expression that was not accompanied by low cellular DOX accumulation or sensitivity to efflux blockers [10]. Similarly, Xie *et al.* [54] reported that P-gp positive acute myeloid leukemia (AML) cells were not sensitive to the efflux blocking action of cyclosporin A, and no correlation between P-gp expression and drug efflux was seen.

Data from the present study would suggest that the discordance between P-gp expression in DOX-sensitive HTB-123 human breast cancer cells and the lack of their sensitivity to efflux blockers may be related to the non-functionality of the P-gp and the efflux pump. P-gp in this cell line may be activated and functionality of the pump restored by treatment with TPA, a known phosphorylation inducer. A similar discordance may be present in tumor cells from clinical specimens where high P-gp expression may not correlate with enhanced drug accumulation and cytotoxicity [54, 55].

In CEM/VLB cells also, TPA pretreatment increased P-gp phosphorylation by 30%. However, unlike HTB-123 cells, TPA treatment could not further reduce drug accumulation or DOX cytotoxicity in this MDR cell line. In CEM/VLB, P-gp is already phosphorylated, and functionality may be at a threshold maximum level. Perhaps an enhancement of P-gp phosphorylation by TPA treatment may not be causing any detectable increase in drug efflux.

The role of phosphorylation on P-gp functionality has been debated since the initial observation by Carlsen *et al.*

[56] showing that P-gp is phosphorylated. Several earlier studies have demonstrated that exposure of multidrug-resistant cells to PKC agonists, such as TPA, enhances P-gp phosphorylation, reduces drug accumulation, and enhances resistance [19–28]. However, two recent important studies based on site-directed mutagenesis have argued against the importance of phosphorylation in P-gp functionality [29, 30]. Studies on phosphorylation- and dephosphorylation-defective mutant cell lines suggested that P-gp phosphorylation is not essential for having a resistant phenotype. Goodfellow *et al.* [30] reported that mutations of the phosphorylation sites in the linker region did not alter P-gp expression or its subcellular localization. Since the drug transport properties of mutant and wild-type P-gp were indistinguishable, they suggested that phosphorylation of P-gp linker sites by PKC does not affect the rate of drug transport. Both of these studies have used DOX-resistant cell lines for disabling P-gp phosphorylation by site-directed mutagenesis.

Brief exposure of mammalian cells to TPA results in PKC activation, whereas prolonged exposure has been reported to increase *MDR1* gene expression at both the mRNA and the protein levels [57]. TPA pretreatment of HTB-123 cells for 2 hr did not cause any up-regulation of MDR1 mRNA or P-gp expression. This is quite plausible, if we take into account the long half-lives of MDR-1 mRNA (4 hr) and P-gp (24–72 hr) [58, 59]. TPA pretreatment also did not induce the expression of the other drug transporter, MRP. It would be worthwhile to study the expression of TPA-activated proteins, which, in turn, may regulate P-gp functionality. Besides activating P-gp phosphorylation, TPA induces several biological cellular events such as differentiation and causes reduced drug influx [60]. TPA also induces a reversible translocation of PKC on the plasma membrane and increased P-gp phosphorylation in KB-VI cells [26]. Further studies are needed to determine whether TPA induces any of the above-mentioned cellular events resulting in increased P-gp phosphorylation or functionality.

The HTB-123 cell line, unlike the drug-resistant cell lines employed by other investigators [29, 30], was established from a cutaneous metastatic nodule of a patient with advanced breast cancer; to the best of our knowledge, this lineage was not exposed either *in vivo* or *in vitro* to DOX [32] and, thus, must have high intrinsic P-gp expression. In HTB-123 cells, pretreatment with TPA results in reduced DOX accumulation, which can be enhanced by incubation with efflux blockers. Our data would indicate that TPA treatment induces phosphorylation, resulting in the activation of the non-functional drug efflux pump. The HTB-123 cell line, with its high DOX accumulation and P-gp expression accompanied by a non-functional efflux pump that can be activated by phosphorylation, may be a good model for studying efflux pump activation.

Clinical drug resistance, unlike that of cell lines selected *in vitro*, is very low. This low level of resistance is sufficient for leading into chemotherapy failure. Therapeutic modulations that can block cellular drug efflux and enhance cellular accumulation and toxicity may result in enhanced responses. However, before one attempts to correlate the outcome of these modulations with therapeutic response, it is essential not only to know if the tumors have MDR1 mRNA and P-gp expression but also to see if they have a functional drug efflux pump.

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