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DOXORUBICIN RESISTANCE IN HUMAN MELANOMA CELLS: *MDR-1* AND GLUTATHIONE *S*-TRANSFERASE π GENE EXPRESSION

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Abstract—Cellular drug resistance is believed to involve P-glycoprotein-related drug efflux as well as xenobiotic detoxification. In the present study, we analyzed five human melanoma cell lines with 1- to 6-fold doxorubicin resistance for doxorubicin retention and *MDR-1* and GST π gene expression. All the cell lines had high doxorubicin retention, and efflux blockers such as trifluoperazine and verapamil did not have a major effect on drug retention or cytotoxicity. Even though all the cell lines carried the *MDR-1* and GST π genes, gene amplification was not associated with drug resistance. Both laser flow cytometry and immunoperoxidase staining showed high expression of C-219 reactive P-glycoprotein in some of the resistant cells which was not accompanied by either high drug efflux or sensitivity to doxorubicin efflux blockers.

Acquired or intrinsic tumor cell resistance to chemotherapeutic drugs may be one of the major reasons for failure of cancer chemotherapy in refractory disease. Several mechanisms, either alone or in combination, have been shown to be involved in cellular drug resistance. Overexpression of multidrug resistance (MDR)† related 170 kDa P-glycoprotein [1–3], increased glutathione content [4–6], enhanced expression of glutathione *S*-transferase (GST) [7–9] and changes in topoisomerase II [10, 11] have been implicated in cellular drug resistance.

P-glycoprotein acts as an energy-dependent efflux pump, resulting in decreased cellular drug accumulation. Enhanced P-glycoprotein expression may be accomplished by amplification and/or increased transcription of the *MDR-1* gene. Transfection of the *MDR-1* gene has been shown to confer resistance in drug-sensitive cells [12, 13]. Although several reports on the increased P-glycoprotein expression have been published [14–17], the precise role of P-glycoprotein and drug efflux in resistance of refractory tumor cells remains to be elucidated. In human lung tumor cells, Lai *et al.* [18] have reported expression of the MDR phenotype without changes in cellular drug accumulation or P-glycoprotein expression.

GSTs represent a family of isoenzymes that catalyze the conjugation of glutathione to a wide variety of electrophilic substances [19, 20] and metabolize lipophilic xenobiotics including anticancer drugs [21, 22]. On the basis of their primary structure, soluble human GSTs have been grouped into four classes: alpha, mu, pi (π), and theta [23]. GST π is particularly interesting due to its association with malignancy [24, 25] and drug resistance [7, 26]. Cowan and co-workers [27, 18] have reported that the GST π gene is overexpressed in resistant tumor cells as compared to normal cells and its activity correlates with drug resistance. In human melanoma cells two reports on *MDR-1* gene expression are available [29, 30]. However, no studies have been reported on the expression of GST π gene in drug resistance of human melanoma cells.

Most of the studies published on doxorubicin resistance have focused on murine and human cell lines which were made resistant *in vitro* by exposure to high levels of doxorubicin. These resistant cell lines have doxorubicin IC_{50} values several orders of magnitude higher than those of the parental drug-sensitive cell lines. Although these studies have been valuable in our understanding of the phenomenon of multiple drug resistance, their relevance in the study and management of clinical drug resistance needs to be determined. It may be more relevant from a clinical standpoint to study tumor cells and cell lines showing intrinsic resistance without selection pressures caused by exposure to high drug concentrations *in vitro*. Even though intrinsic resistance of these cell lines is low (IC_{50} values up to 10-fold higher than that of the most drug-sensitive cell lines), in-depth studies on these refractory tumor cells may provide us with a better understanding of the refractory disease.

MATERIALS AND METHODS

Drugs and reagents. Doxorubicin hydrochloride (Adriamycin®, NSC-123127) was purchased from

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† Abbreviations: MDR, multidrug resistance; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; FBS, fetal bovine serum; SDS, sodium dodecyl sulfate; FITC, fluorescein isothiocyanate; and SSC, 0.15 M sodium chloride + 0.015 M sodium citrate.

Adria Laboratories, Inc. (Columbus, OH). Stock solutions of doxorubicin were prepared in Ca^{2+} and Mg^{2+} free phosphate-buffered saline (PBS). Fresh serial dilutions were prepared in normal saline before use.

Cell lines. Human melanoma cell lines, FCCM-2 and FCCM-9, were established in our laboratory from residual biopsy material obtained for diagnostic purposes from two different patients. Three other human melanoma cell lines (HM-1, NH, and G361) were obtained from Dr. Michael Wick, Dana Farber Cancer Center, Boston, MA. Cells were maintained in RPMI-1640 medium containing 25 mM HEPES buffer, 10% fetal bovine serum (FBS), penicillin (100 IU/mL), streptomycin (100 μg/mL) and growth factors, 10 nM bombesin and SGF (Scott Laboratories, Fiskeville, RI). All cell lines were melanin positive and tumorigenic in nude mice.

Murine leukemic P388 and doxorubicin-resistant P388/R84 cell lines were maintained in RPMI-1640 containing 10% FBS, penicillin (100 IU/mL), and streptomycin (100 μg/mL).

Clonogenic assay. The effect of doxorubicin on cell growth was determined by a soft agar colony forming assay. Cells from log phase cultures exposed to various doxorubicin concentrations (0.01 to 1.0 μM for 1 hr at 37°) were washed (2×) with cold PBS, resuspended in medium containing 0.3% agar and layered onto a previously prepared underlayer of 0.5% agar in RPMI-1640 medium containing 10% FBS, 1.0 mM sodium pyruvate, 10 μM mercaptoethanol, 100 IU/mL penicillin and 100 μg/mL streptomycin. Multi-well plates with the double layer of soft agar were incubated at 37° in a humidified atmosphere of 5% CO_2 and 95% air for 12–15 days. The IC_{50} values (mean ± SD) were determined by counting the colonies from three different experiments performed over a period of 2 months, using three wells for each drug concentration tested. To determine the effect of doxorubicin efflux blockers, tumor cells were incubated with doxorubicin (0.1 μM) and trifluoperazine (10 μM) or verapamil (15 μM) for 1 hr at 37°, washed (2×) with cold PBS, and plated in soft agar.

DNA index. Cells were stained by the propidium iodide/hypotonic citrate method for quantitation of cellular DNA content. Details of our staining method and analytical procedures have been reported earlier [31].

Preparation of DNA probe. The recombinant plasmid p683 carrying human multidrug-resistant gene (*MDR-1*) was obtained from Dr. A. T. Fojo, National Cancer Institute, Bethesda, MD [14]. The *MDR-1* cDNA is a 683 bp fragment covering the residues from −27 up to +656 bp of the coding region, cloned onto the *Sma* I site of pGEM 3Z (Promega) plasmid vector. GST π-1 plasmid carrying human GST π gene was purchased from the human and mouse DNA probe repository maintained at the American Type Culture Collection (ATCC), Rockville, MD [32]. GST π cDNA is a 725 bp insert cloned onto the *Eco* RI site of pGEM-4 (Promega) plasmid vector. Human β-actin gene (clone HHC189 from ATCC) was used as a control in the hybridization experiments. To prepare ^{32}P-labeled DNA probes, plasmid p683 was digested with *Eco* RI and *Bam* H1, and GST π-1 and HCC189 with *Eco* RI. The inserted genes were electroeluted from the gel after electrophoresis, purified by passage through Elutip-D column (Schleicher & Schuell, Keene, NH) and precipitated by ethanol. DNA fragments were labeled with [^{32}P]dCTP (3000 Ci/mol; New England Nuclear, Boston, MA) by the random prime method [33] using hexadeoxyribonucleotides (Pharmacia, Piscataway, NJ) and Klenow fragment enzyme (Bethesda Research Laboratories, Bethesda, MD).

Southern blot hybridization. Genomic DNA extracted from the cell lines was digested with *Eco* RI and spermidine (Boehringer Mannheim Biochemicals, Indianapolis, IN) [34]. The digested DNA (10 μg) was fractionated on 1% agarose gel, stained with ethidium bromide and photographed. DNA in the gel was denatured with a solution containing 0.2 N NaOH and 0.6 M NaCl for 30 min and then neutralized with 0.025 phosphate buffer (pH 6.6) for 1 hr. The denatured DNA was transferred to a nitrocellulose filter using 0.025 phosphate buffer (pH 6.6) as solvent [35]. The filter after baking at 80° for 2 hr was prehybridized in 6 mL of hybridization buffer [50% formamide, 0.75 M NaCl, 0.15 mM Tris–HCl (pH 8.0), 10 mM phosphate buffer (pH 6.8), 1× Denhardt's solution, 10% dextran sulfate, 0.1% sodium dodecyl sulfate (SDS)] for 4–6 hr. The buffer was drained and replaced with fresh hybridization buffer containing 2×10^7 cpm ^{32}P-labeled *MDR-1* or GST π gene probe. After incubation at 42° for 24 hr, the filter was washed twice with 2× SSC (SSC = 0.15 M sodium chloride + 0.015 M sodium citrate) containing 0.1% SDS at 25° for 15 min, followed by three more washes with 2× SSC at 50° for 2 hr and another wash at 25° for 10 min. The washed filter was air dried and exposed to Kodak X-omat film at −70° for 24 hr using an intensifying screen. The film was developed using Kodak GBX developer.

RNA extraction and slot blot hybridization. Total RNA was extracted according to the procedure of Chirgwin *et al.* [36]. Before blotting, RNA was examined for any degradation by monitoring the integrity of 18S and 28S rRNA bands in the ethidium bromide stained gels. For RNA slot blot analysis, total RNA from the cell lines was serially diluted (1, 2.5, 5 and 10 μg) in 20× SSC and spotted onto the nitrocellulose filter in a Bio-Dot slot format apparatus (Bio-Rad, Richmond, CA). The filter was dried, baked at 80° for 2 hr, prehybridized with 6 mL of hybridization buffer containing 50% formamide, 0.75 M NaCl, 0.15 mM Tris–HCl (pH 8.0), 10 mM phosphate buffer (ph 6.8), 1× Denhardt's solution, 10% dextran sulfate, 0.1% SDS at 42° for 4–6 hr. The buffer was drained and replaced with fresh hybridization buffer containing ^{32}P-labeled random prime probe of *MDR-1*, GST π or β-actin gene. After incubation at 42° for 24 hr, the filter was washed once with 2× SSC containing 0.1% SDS at 25° for 15 min, followed by three more washes at 50° for 2 hr and one wash at 25° for 15 min. The blots were dried and autoradiographed. Slot blot experiments were repeated several times for confirmatory results. Autoradiographs were scanned in a Zeineh soft laser scanning densitometer under

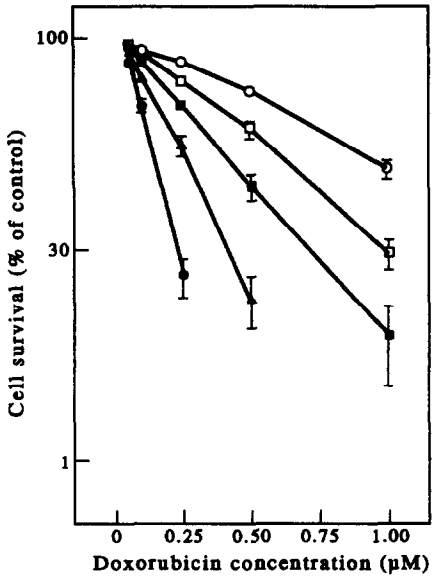


Fig. 1. Soft agar clonogenic assay data of melanoma cell lines after a 1-hr incubation with different doxorubicin concentrations. Data are presented as means ± SD based on a counting of the total number of surviving colonies from triplicate plates in three separate experiments. Key: HM-1 (●), G361 (▲), FCCM-2 (■), NH (□) and FCCM-9 (○).

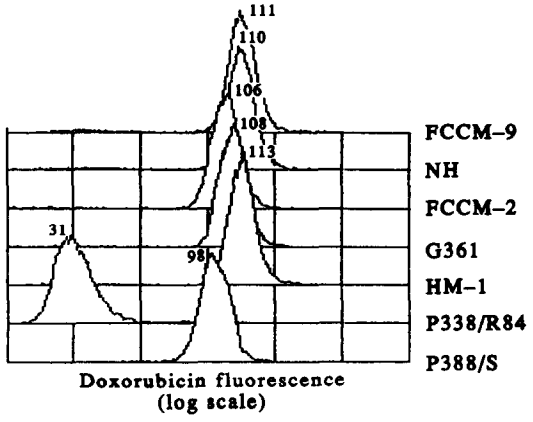


Fig. 2. Doxorubicin retention in melanoma cell lines compared to that of murine P388 and doxorubicin-resistant P388/R84 cells. Cellular doxorubicin fluorescence was analyzed in a FACScan flow cytometer after incubating cells in doxorubicin (2 μg/mL) for 1 hr at 37°.

tungsten light and values were expressed in arbitrary units. At least three blots of each cell lines were read in the densitometer and mean values calculated.

P-glycoprotein detection by flow cytometry and immunoperoxidase staining. To determine P-glycoprotein expression, cells were fixed in 100% methanol, incubated first with C-219 antibody (Centocor, Inc., Malvern, PA) and then in fluorescein isothiocyanate (FITC) labeled goat antimouse IgG. FITC-labeled cells were analysed on a Becton & Dickinson FACScan flow cytometer. P-glycoprotein positive cells were also microscopically examined after staining by the immunoperoxidase technique. Our flow cytometric and immunoperoxidase staining procedures for P-glycoprotein detection have been published recently [37].

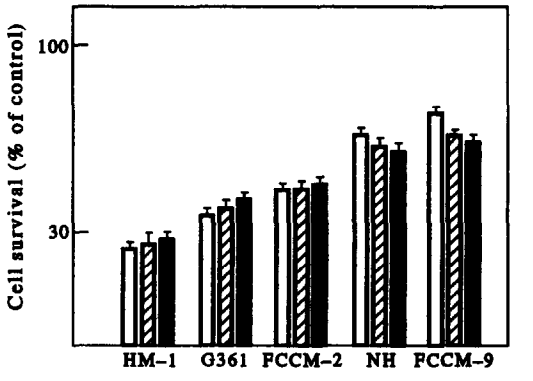


Fig. 3. Soft agar clonogenic assay of melanoma cell lines after a 1-hr incubation with 0.1 μM doxorubicin (open bars) in the absence and presence of 10 μM trifluoperazine (lined bars) or 15 μM verapamil (solid bars). Data presented are the means with SD based on a counting of the total number of surviving colonies from triplicate plates in three separate experiments.

RESULTS

Doxorubicin retention and cytotoxicity. Four melanoma cell lines used in the present study (NH, HM-1, G361, and FCCM-2) had a cell number doubling time of 16–19 hr in monolayer cultures. The fifth cell line, FCCM-9, had a much longer doubling time of 26.7 ± 2.6 hr. In soft agar assays, the IC_{50} for doxorubicin (1 hr of incubation) varied from 0.09 (HM-1) to 0.55 μM (FCCM-9) (Fig. 1).

Flow histograms in Fig. 2 show doxorubicin fluorescence (log scale) of cells incubated with doxorubicin (2 μg/mL) for 1 hr. Cellular doxorubicin retention in the five cell lines was high as compared to that of the doxorubicin-resistant P388/R84 cells. Co-incubation with efflux blockers (verapamil, trifluoperazine) did not have a major effect on cellular doxorubicin content (data not shown). Similarly, as shown in Fig. 3, doxorubicin efflux blockers, verapamil or trifluoperazine, did not alter cytotoxicity significantly in soft agar assays.

DNA analysis. The top panel of Fig. 4 shows the ethidium bromide stained DNA gel of murine leukemic and human melanoma cell lines. Southern blots of melanoma DNAs probed with a *MDR-1* gene hybridized to 3.1 kb DNA fragment and with a GST π probe to 5.0 kb DNA fragment are presented in the center and bottom panels of Fig. 4, respectively. No major amplification of either the *MDR-1* or GST π genes was observed in the seven (five human melanoma and two murine) cell lines

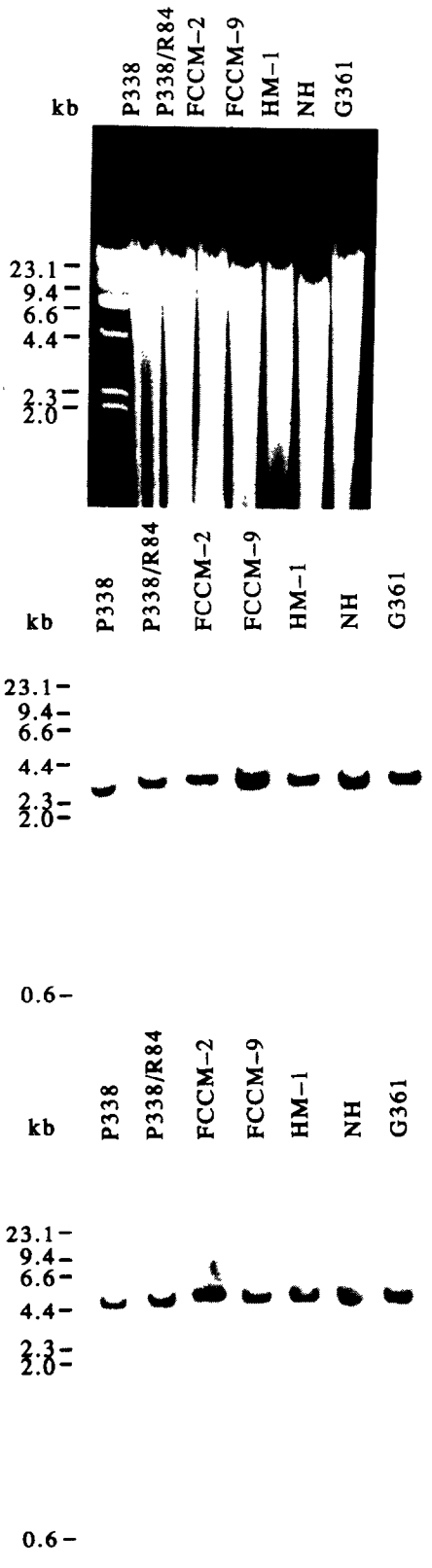


Fig. 4. Southern blot hybridization of genomic DNA from melanoma, murine P388 and P388/R84 cells. (Top panel) Ethidium bromide stained gel of genomic DNA. (Center panel) Hybridization with ^{32}P-labeled *MDR-1* probe. (Bottom panel) Hybridization with ^{32}P-labeled GST π probe.

examined, except for a small increase in the intensity of hybridization in FCCM-9 with the *MDR-1* probe.

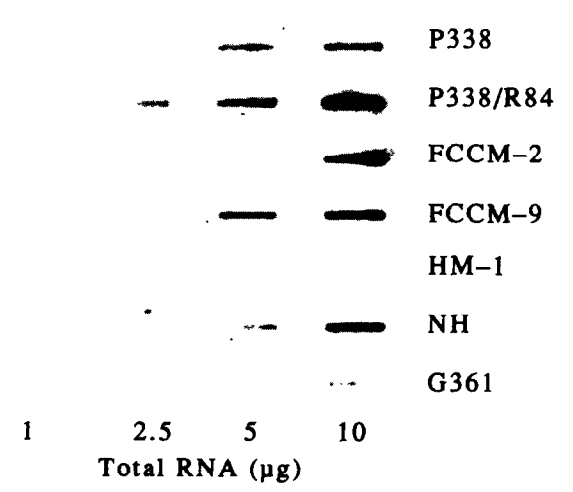
Analysis of RNA for MDR-1 *and GST* π *expression.* Slot blots of RNAs from the melanoma cells hybridized with *MDR-1* or GST π probes are shown in the top and center panels of Fig. 5, respectively. The slot blot hybridized with human β-actin gene was used as an internal control (Fig. 5, bottom panel). Of the five human melanoma cell lines examined, FCCM-9 (6-fold resistant) showed the highest *MDR-1* (26.4 units) and GST π (38 units) mRNA levels (Table 1). The doxorubicin-sensitive cell lines, HM-1 and G361, with 1- and 1.7-fold resistance showed comparatively lower levels of *MDR-1* and GST π RNA expression.

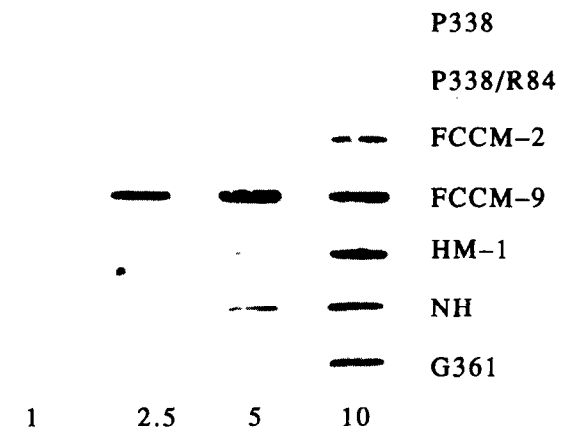
In Fig. 6, we have plotted the correlation between doxorubicin IC_{50} values and *MDR-1* as well as GST π gene expression (mRNA level) of the five melanoma cell lines examined. The correlation coefficients for GST π and *MDR-1* mRNA levels with doxorubicin IC_{50} values in soft agar were 0.94 and 0.83, respectively.

P-glycoprotein expression. Two parameter flow cytometric histograms in Fig. 7 show the DNA content (linear fluorescence, PI) and P-glycoprotein expression (log fluorescence, FITC) of the five melanoma cell lines (A to E) and of the doxorubicin-resistant Chinese hamster CH^RC5 cells (F). Insets in the upper right corner of each panel show single parameter overlay histograms of FITC fluorescence of the isotype controls and the C-219 reacted cells. Flow cytometric measurement of P-glycoprotein with C-219 monoclonal antibody showed that in the FCCM-2 (C, 2.7-fold resistant) and FCCM-9 (E, 6.1-fold resistant) cell lines more than 50% of cells were P-glycoprotein positive. The three other cell lines, HM-1, G361 and NH (A, B and D), with 1- to 3.9-fold resistance had 7, 24 and 27% P-glycoprotein positive cells, respectively. The peak fluorescence channel values ranged from 7 to 23 in isotype controls and from 24 to 54 in the C-219 reacted human melanoma cell lines (Fig. 7, A–E). In contrast, the peak channel value for C-219-reacted FITC fluorescence in the drug-resistant Chinese hamster cell line, CH^RC5, was 69 with 68% of the population having positive P-glycoprotein expression (Fig. 7F). Both the number of positive cells (derived after electronic exclusion of 99% of fluorescent cells from the isotype controls) and the difference between the peak channel value of the isotype control and C-219 reacted cells were higher in FCCM-2 (C) than in HM-1 (A) or G361 (B) cells. P-glycoprotein expression was also determined by immunoperoxidase staining of cells using C-219 antibody and the intensity of staining correlated with flow cytometric analysis (data not shown).

DISCUSSION

Several investigators have implicated the role of *MDR-1* [38, 39] and GST π gene expression [8, 9] in drug resistance. However, only a few published reports are available on the expression of *MDR-1* gene in human melanoma cells. Increased *MDR-1* gene expression and decreased drug accumulation have been observed by Lemontt *et al.* [29] in 4- to





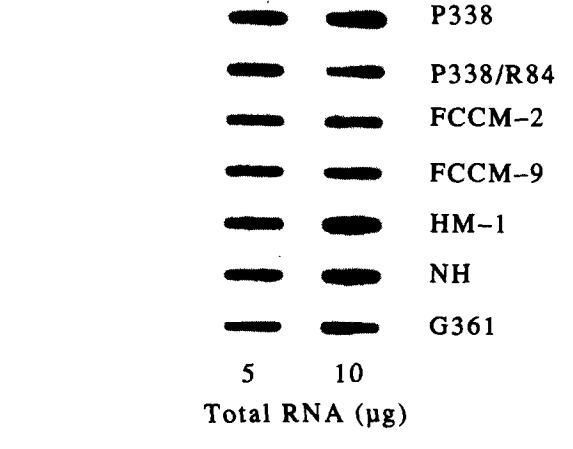


Fig. 5. Slot blot analysis of *MDR-1* and GST π RNA levels in melanoma cells. Total RNA of murine P388 and P388/R-84 cells were also blotted as controls. (Top panel) Total RNA at serial dilutions (1, 2.5, 5 and 10 μg) blotted and hybridized with *MDR-1* probe. (Center panel) Total RNA at serial dilutions (1, 2.5, 5 and 10 μg) blotted and hybridized with GST π probe. (Bottom panel) Total RNA (5 and 10 μg) blotted and hybridized with β-actin probe.

Table 1. *MDR-1* and GST π mRNA expression in human melanoma cell lines

Cell line	Relative resistance*	*MDR-1* mRNA†	GST π mRNA†
HM-1	1.0	8.57 ± 4.20	18.25 ± 2.02
G361	1.7	8.73 ± 4.02	20.20 ± 3.74
FCCM-2	2.7	20.00 ± 3.21	18.05 ± 1.29
NH	3.9	19.93 ± 5.96	26.75 ± 2.84
FCCM-9	6.1	26.40 ± 2.87	38.00 ± 4.69

Data are based on densitometric scanning of RNA slot blot autoradiographs and values are expressed as arbitrary units.

* Based on the IC_{50} of a 1-hr doxorubicin exposure in soft agar assays.

† Values are means ± SD (N = 3).

24-fold *in vitro* selected multidrug-resistant human melanoma cell lines. Goldstein *et al.* [40] could not detect *MDR-1* mRNA in three human melanomas by slot blot analysis. Fuchs *et al.* [30] reported that drug resistance in human melanomas does not usually depend on P-glycoprotein-dependent drug efflux as they found P-glycoprotein expression in only 1/37 primary melanomas and 1/27 melanoma metastases. In the present investigation, we found high *MDR-1* mRNA levels in the resistant melanoma cell lines (FCCM-9, NH, and FCCM-2) and more than 50% P-glycoprotein positive cells in the FCCM-2 and FCCM-9 cell lines. All of the cell lines had high doxorubicin retention, and efflux blockers (trifluoperazine and verapamil) did not alter either drug retention or cytotoxicity in a major way. In contrast, in the doxorubicin-resistant murine leukemic P388/R84 cells (84-fold), we have demonstrated previously a significant effect of efflux blockers on doxorubicin retention and cytotoxicity [41]. In other words, despite the high expression of *MDR-1* mRNA and P-glycoprotein in some of these human melanoma cell lines examined, the functional effect of P-glycoprotein, namely that of drug efflux and enhanced sensitivity to efflux blockers, was not a major factor. Perhaps the difference in the isoform nature or defects in glycosylation and phosphorylation of P-glycoprotein may be responsible for the lack of efflux function of P-glycoprotein in those human melanoma cell lines. P-glycoprotein isoforms with distinct properties, electrophoretic mobilities and functions encoded by *MDR* genes have ben reported in the mouse [42, 43]. Further enhanced P-glycoprotein phosphorylation has been reported after treatment of human Adriamycin resistant cells with phorbol esters [44], thereby suggesting a possible role of protein kinase C (PKC) in the regulation of P-glycoprotein function. PKC isolated from KB-3 cells can phosphorylate P-170 [45] and differential N-linked glycosylation has also been reported in P-glycoprotein processing [46]. Hence, it is quite possible that a defective step in any of the aforementioned processes may affect functionality of the P-glycoprotein efflux pump in tumor cells. It has been reported that as the phosphorylation level of P-glycoprotein is decreased,

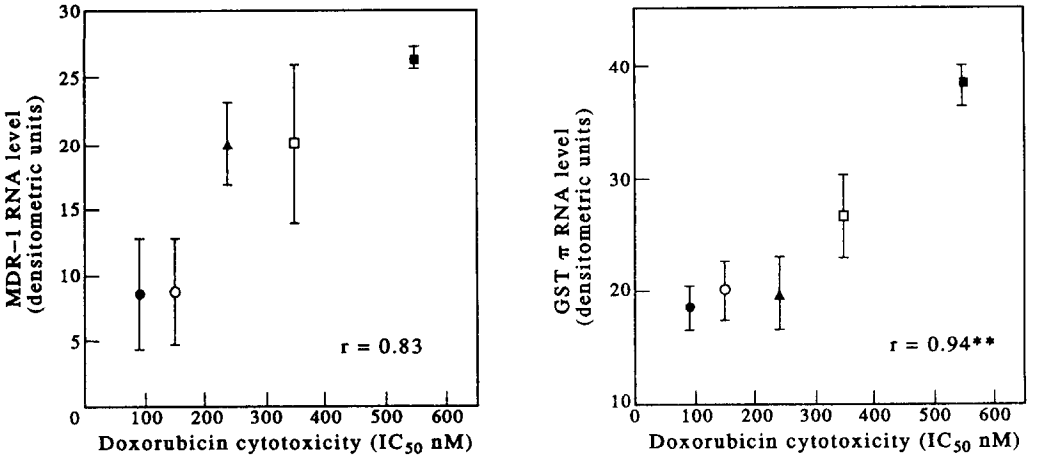


Fig. 6. (Left panel) Mean *MDR-1* RNA levels (arbitrary units obtained by densitometric scanning of slot blot autoradiographs) plotted against IC_{50} values of melanomas cell lines. Vertical lines represent SD (N = 3). Key: HM-1 (●), G361 (○), FCCM-2 (▲), NH (□) and FCCM-9 (■). (Right panel) Mean GST π RNA levels (arbitrary units obtained by densitometric scanning of slot blot autoradiographs) plotted against IC_{50} values of melanomas cell lines. Vertical lines represent SD (N = 3). Key: HM-1 (●), G361 (○), FCCM-2 (▲), NH (□) and FCCM-9 (■). The double asterisk signifies $P < 0.05$.

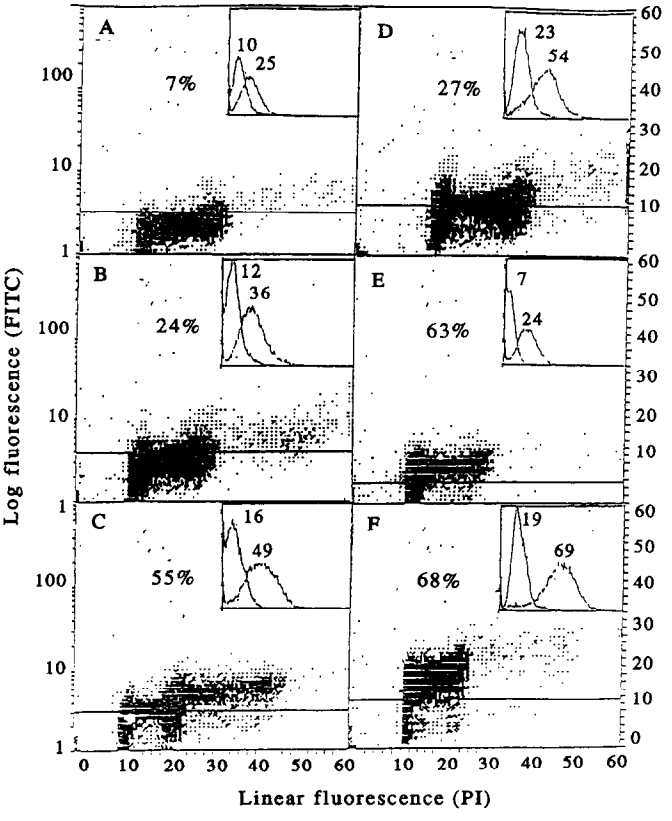


Fig. 7. Two parameter dot plots and single parameter histograms (insets) of melanoma cells stained for cellular P-glycoprotein expression (log FITC fluorescence) after incubation with C-219 P-glycoprotein specific antibody. Horizontal axis records cellular DNA content after propidium iodide staining. Horizontal lines represent electronic gates used to exclude 99% of fluorescent cells in the isotype control. Single parameter insets show FITC fluorescence from the isotype controls. The percentage of P-glycoprotein positive cells and peak fluorescence channel values (in the single parameter insets) are also shown. Key: (A) HM-1, (B) G361, (C) FCCM-2, (D) NH, (E) FCCM-9, and (F) CH^RC5 (doxorubicin-resistant Chinese hamster cell line).

a parallel decrease in the ability of protein to efflux the drugs from the resistant cells can be seen [47, 48]. Furthermore, protein kinase inhibitors, like staurosporine, inhibit phosphorylation of P-glycoprotein resulting in increased cellular daunomycin accumulation [48].

C-219 antibody has been used most widely for the immunocytochemical detection of P-glycoprotein and staining has been observed on the plasma membranes, Golgi membranes, and, possibly, cytoplasmic vesicles [49]. The C-219 has been reported to cross-react with heavy chain of myosin and certain other proteins containing ATP-binding sites [50]. Recently, Battifora [51] also raised concern on the accurate detection of P-glycoprotein by immunochemical staining of histological sections by C-219. In the present study, in addition to the detection of P-glycoprotein with C-219, we have also employed nucleic acid hybridization (Southern and Slot blots) to detect *MDR-1* DNA and mRNA in the cell lines. Our data would suggest that in some of our cell lines, especially FCCM-9, enhanced expression of P-glycoprotein may be correlated with increased content of the mRNA and positive staining with C-219 was not an artifact.

All of the melanoma cell lines examined carry GST π gene and express mRNAs. Further, a significant correlation was found between doxorubicin resistance and GST π gene expression in the melanoma cell lines. In a recent study, we determined that the activity of the GST π and glutathione peroxidase enzymes, and GST π content were significantly higher in our drug-resistant FCCM-2 and FCCM-9 cell lines and low in the most sensitive HM-1 cell line.* Also, depletion of 90% glutathione by incubation with 50 μM buthione-*S,R*-sulfoximine (BSO) significantly increased doxorubicin cytotoxicity in the FCCM-2 and FCCM-9 cell lines.* Based on these observations and results from the present study, it is possible that glutathione and related detoxification pathways involving GST π may play a role in the low level doxorubicin resistance of human melanoma cells even though no conjugation of GSH with doxorubicin has been reported. GST π enzyme is involved in the detoxification of drugs by acting as a catalyst in the conjugation of drugs to glutathione or in the selenium-independent GSH peroxidase activity leading to the detoxification of lipid and nucleic acid hyperperoxides generated by redox cycling [9, 52]. GST π appears to be the major isozyme present in many human tumors [53]. The elevation of GST π gene expression and enzyme activity has been reported in multidrug-resistant Adr^R MCF-7 cells [7, 27] and SCC-25 squamous carcinoma cells resistant to *cis*-diammine-dichloroplatinum [54]. The rat anionic GST, GST-P, an isozyme usually present in low levels in liver, is markedly elevated in xenobiotic resistant rat hyperplastic nodules [55, 56]. Despite all these reports, the precise mechanism and role of GST π overexpression in doxorubicin-resistant cells are not well understood. Even transfection experiments with GST π gene have not fully resolved the precise role of anionic GST π isoenzyme in the drug resistance phenomenon [57, 58].

All human melanoma and murine cells analyzed in the present study carry *MDR-1* and GST π genes. We failed to detect any conspicuous amplification of the GST π gene in human melanoma cell lines responsible for increased message level and drug resistance. Hence, amplification of the GST π gene may not be involved in the enhanced expression of mRNAs in these resistant cell lines. In summary, our data show that both *MDR-1* and GST π genes co-express in the low level drug-resistant human melanoma cells. However, the *MDR-1*/P-glycoprotein associated efflux mechanism did not appear to be a major factor, as there was no detectable drug efflux, and efflux blockers did not enhance doxorubicin retention and cytotoxicity significantly in soft agar assays in resistant melanoma cell lines.

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