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ROLE OF GLUTATHIONE *S*-TRANSFERASE P1, P-GLYCOPROTEIN AND MULTIDRUG RESISTANCE-ASSOCIATED PROTEIN 1 IN ACQUIRED DOXORUBICIN RESISTANCE

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While P-glycoprotein (Pgp) and multidrug resistance-associated protein 1 (MRP1) are known to be important in acquired doxorubicin resistance, the role of glutathione *S*-transferases (GST) remains unclear. Our study assessed roles of these 3 factors in a human drug-sensitive carcinoma cell line (HEp2), a subclone made resistant by prolonged incubation in doxorubicin (HEp2A), and HEp2 cells stably transfected with human GSTP1. Drug-resistant HEp2A cells showed greater total GST activity, GSTP class enzyme expression, Pgp expression, MRP1 transcript expression, drug efflux and at least 13-fold greater resistance to doxorubicin than the parent HEp2 cell line. GSTM class enzyme expression was similar in both cell types, while GSTA class enzymes were not detected. In the resistant HEp2A cells, cytotoxicity was markedly enhanced by the Pgp/MRP inhibitor verapamil at low doxorubicin concentrations. The GST inhibitor curcumin also enhanced cytotoxicity in HEp2A cells when the Pgp/MRP efflux barrier had been reversed by verapamil or overcome by high doxorubicin concentrations. In addition, curcumin had a chemosensitising effect at low doxorubicin concentrations in HEp2 cells. Stable transfection of HEp2 cells with human GSTP1 increases doxorubicin resistance 3-fold over control cells. Our study indicates involvement of GSTP enzymes as well as efflux mechanisms in the acquired doxorubicin-resistance phenotype.

Key words: *acquired resistance; doxorubicin; glutathione* S-*transferases; transporters*

Doxorubicin is an anthracycline antibiotic, useful in the treatment of diverse malignancies, which exerts cytotoxic effects by interaction with topoisomerase II[1] and production of reactive oxygen species,[2] through the redox cycling of its quinone group. Doxorubicin preferentially partitions to membranes,[3] where it may cause lipid peroxidation through reactive oxygen species generated by redox cycling.[1] In certain tumors, however, the ATP-binding cassette transmembrane molecules P-glycoprotein (Pgp), which is encoded by the *MDR1* gene, and multidrug resistance-associated protein (MRP1) may cause rapid drug efflux and diminished cytotoxicity.[4,5] Glutathione *S*-transferases (GSTs) are multifunctional proteins categorised in alpha (GSTA), mu (GSTM), pi (GSTP1), and theta classes, which detoxicate various substrates.[6] Human GSTP1 reduces cytotoxic effects of doxorubicin when transfected into *S. cervisiae*[7] or into NIH3T3 cells.[8] Peroxidase activity of GST could serve to reduce doxorubicin-induced peroxides and conjugate metabolites.[9] Glutathione-mediated resistance may also be linked to other mechanisms. MRP1 is associated with anthracycline resistance[10] and mediates efflux of glutathione conjugates.[11] Glutathione conjugates of doxorubicin show high affinities toward the MRP1 pump, while unmodified doxorubicin is a poor substrate.[12]

Evolution of drug resistance remains a principal, unresolved cause of cancer treatment failure.[13] While Pgp has a well-established role in acquired doxorubicin resistance,[14] the contribution of GST enzymes is less clear. Moscow *et al.*[15] found that stable transformation of the MCF-7 cell line by a GSTP1 expression vector gave no protection against doxorubicin, while in another study GSTP1 was found to give no additional protection against doxorubicin over Pgp alone.[16] More recently, Beaumont *et al.*[17] investigated the role of glutathione *S*-transferases in doxorubicin resistance of 4 human colorectal cancer cell lines. Higher total GST activity and a unique GSTA band were found in doxorubicin-resistant $CaCo_2$ cells in comparison with drug-sensitive cell lines, but GST inhibitors did not potentiate doxorubicin cytotoxity.[17] Using a more selective approach by retroviral transfer of MDR1 alone or MDR1 and GSTP1 together to a single cell line, GSTP1 was found to confer no additional doxorubicin resistance over Pgp alone.[18] Conversely, doxorubicin cytotoxicity was enhanced by transfection of cells with an antisense vector against GSTP1.[19] The interaction between GST expression, conjugation and MRP1-mediated efflux of drug conjugates may be important in determination of drug resistance.[12] However, information relating to MRP1 expression has been lacking in previous studies.[15–19]

Our study has used a representative model to investigate the relative roles of GST enzymes, Pgp and MRP1 in acquired doxorubicin resistance. GST, Pgp and MRP1 expression and function have been studied in a drug-sensitive human laryngeal carcinoma cell line (HEp2) and a subclone made resistant by prolonged culture in doxorubicin (HEp2A), together with HEp2 subclones stably transfected with GSTP1. The contribution of both GST and the transporter proteins to doxorubicin resistance has been assessed by determining the effects of inhibitors of GST and efflux, alone and in combination, on doxorubicin cytotoxicity.

MATERIAL AND METHODS

Cell culture

HEp2A cells had been previously isolated by culture of the parent drug-sensitive HEp2 human laryngeal carcinoma line in incremental concentrations of doxorubicin for 15 months.[20] For the studies described in this manuscript, HEp2 and HEp2A cells were grown in DMEM, nutrition mix F-12 (Life Technologies, Paisley, UK), supplemented with 10% FCS (Life Technologies), penicillin 50 units/ml, streptomycin 50 mg/ml, 2 mM L-glutamine, 1% (v/v) nonessential amino acids and 10 IU/500 ml bovine insulin and maintained in humidified 5% CO_2 at 37°C, as previously described.[20] Cells were grown in 75 cm^2 flasks for Western blotting and transport studies and in 96 multiwell plates for toxicity studies.

Cytotoxicity assay

Doxorubicin cytotoxicity was assessed by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide) assay, which measures the ability of viable cells to reduce a soluble yellow tetrazolium salt (MTT) to insoluble purple formazan crys-

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tals.[21] Cells grown in 75 cm^2 tissue culture flasks were trypsinised, seeded into flat-bottom 96-well tissue culture plates and incubated overnight. Cells were then treated with doxorubicin (Sigma, Poole, UK) (1 nM to 100 μM) alone or in combination with the Pgp and GST inhibitors, verapamil (Sigma) (5 μM), curcumin (Sigma) (25 μM) and ethacrynic acid (Sigma) (25 μM). Inhibitors were dissolved in DMSO, then added to media. Control cells were incubated with either medium alone, verapamil, curcumin, ethacrynic acid or dimethyl sulphoxide (DMSO). Cells were incubated for 24 hr, fresh medium containing MTT (0.5 mg/ml) was substituted and cells were then incubated for 4 hr at 37°C. Medium was removed and the purple formazan crystals dissolved in DMSO. The plates were examined on a plate reader (Dynatech MR5000 Sussex, UK) with a test wavelength of 570 nm against a DMSO blank. Each assay was carried out in triplicate and compared with a standard growth curve.

Efflux transport studies in HEp2 and HEp2A cells

Efflux transport of doxorubicin, which is a fluorescent P-glycoprotein (Pgp) substrate, was assessed by flow cytometry.[22] Studies were carried out with and without the Pgp/MRP inhibitors, verapamil and cyclosporine A (Sigma). Briefly, HEp2 and HEp2A cells were grown to 80% confluence in 75 cm^2 flasks, trypsinized, resuspended in 6 × 1.5 ml microfuge tubes/flask in growth medium only, or doxorubicin (20 μM) in medium, either alone or with verapamil (5 μM) or cyclosporine (3 μg/ml). Cells were preloaded by incubation in substrate, with or without inhibitor, at 37°C for 30 min. Cells were then pelleted by centrifugation, resuspended in growth medium and maintained at 4°C for 1 hr (accumulation) or in growth medium with or without inhibitors at 37°C in 5% CO_2 for 1 hr (efflux). At the end of the accumulation or efflux periods, cells were washed and placed in cold Hank's buffered saline solution (HBSS) with 10% FCS on ice and kept in the dark until flow cytometric analysis. Samples were analysed on a FACSscan flow cytometer (Becton Dickinson, Oxford, UK) equipped with a 488 nm argon laser. The orange fluorescence of doxorubicin was assessed on fluorescence channel 2 at 575 nm wavelength. Samples were gated to exclude cell debris and clumps. A minimum of 10,000 events were assayed for each sample. Experiments were carried out in triplicate.

Glutathione S*-transferase activity*

Cells were harvested from confluent 75 cm^2 tissue culture flasks, washed and resuspended in PBS. Cytosolic protein extracts were prepared following cell lysis by sonication by centrifugation at 15,000 rpm at 4°C for 30 min to yield cytosol in the supernatant. Total glutathione *S*-transferase activity was assessed by spectrophotometric assay of glutathione conjugation of 1-chloro-2,4-dinitrobenzene (CDNB).[23]

Immunoblotting

Crude membrane and cytosolic fractions were prepared as described by Germann *et al.*[24] for analysis of P-glycoprotein and glutathione *S*-transferase expression, respectively. Cells were mechanically disaggregated, washed twice in cold PBS, resuspended in cold hypotonic lysis buffer (10 mM Tris-HCl pH 7.5, 10 mM NaCl, 1 mM $MgCl_2$), and incubated on ice for 15 min. The swollen cells were mechanically disrupted in a glass homogenizer and the nuclei removed by centrifugation at 400 × g for 10 min at 4°C. The pellet obtained by subsequent centrifugation at 30,000 × g for 30 min at 4°C was used as the crude membrane fraction. The supernatant from this process yielded the cytosolic fraction. Protein concentrations were determined using the Bradford assay, using bovine serum albumin as a standard.

For Pgp expression studies, crude membrane preparations (20 μg/well) were applied to a 6% SDS-polyacrylamide minigel, separated by electrophoresis, then transferred to a 0.45 μm nitrocellulose membrane (Hybond-C, Amersham-Pharmacia, Bucks, UK) at 40 mA for 16 hr at 4°C. Nonspecific binding was blocked by incubation of the nitrocellulose for 1 hr in Tris buffered saline (pH 7.5) with 0.5% tween (TBST) + 5% w/v, dried milk (Marvel, Premier Beverages, Stafford, UK). The membrane was then incubated with the C219 monoclonal antibody (MAb) (Centocor Diagnostics, Malvern, PA) for 2 hr, followed by 3 washes for 15 min each in TBST. Membranes were then incubated for 60 min with a biotin-conjugated goat anti-mouse secondary antibody (Dako, Glostrup, Denmark). After 3 further washes in TBST, the nitrocellulose was finally incubated with a StreptAvidin Biotin-horseradish peroxidase complex for 60 min (Dako). Following further washing, protein expression was visualised using an enhanced chemoluminescence system according to the manufacturer's instructions (ECL-Plus, Amersham-Pharmacia). Intensity was assessed semiquantitatively by densitometry by capturing the images using a Kodak DC40 digital camera and analysing band intensity using Kodak 1D digital software (Kodak Scientific Imaging Systems, Rochester, NY). All values were compared between HEp2 and HEp2A cells and were calculated as the average of 3 separate experiments.

For studies on GST expression, cytosol proteins (100 μg/well) were applied to a 12% SDS-polyacrylamide gel and separated overnight at 45 V, using rat liver cytosol as a positive control. Transfer to a 0.45 μm nitrocellulose membrane (Hybond C, Amersham Pharmacia) was carried out at 60 V for 4 hr at 4°C. After blocking nonspecific protein binding overnight in TBST +5% w/v dried milk (Marvel), the nitrocellulose was incubated for 1 hr with the primary antibodies against GSTA, GSTM and GSTP classes (raised against GSTA1, GSTM1b, GSTP1, respectively, and supplied by Prof. J.D. Hayes, University of Dundee, Scotland). Antibody binding was detected by use of Biotin-conjugated antibody and ECL detection as described for Pgp.

Reverse transcriptase polymerase chain reaction (RT-PCR) assay of MDR1 and MRP1

Total RNA was extracted from HEp2A and HEp2 cells by use of an RNAzol kit (Biogenesis, Poole, UK) according to the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed in the presence of random hexamers (final concentration 2.5 μM), using MuLV reverse transcriptase (2.5 U/μl) in a total volume of 20 μl per sample. To ensure that amplification was occurring in the linear phase, samples of 3 μl and 5 μl, which represented 6% and 10% of reverse transcribed cDNA mix, respectively, from each of the cell lines were analysed. Samples were added to Gene Amp PCR buffer II (Perkin Elmer, Santa Clara, CA), 2.5 mM $MgCl_2$, 0.25 mM DTNP, 1.25 units Taq polymerase (Amplitaq Gold, Perkin Elmer) primers and DEPC-treated H_2O, to provide a final total volume of 50 μl. For the *GAPDH* control, the primers were 5′ AGTCAACGGATTTGGTCGTA 3′ and 5′ AAATGAGCCCCAGCCTTCT 3′ at a final concentration of 0.0216 μM. A product of 310 bp was obtained. For *MDR1*, the primers were 5′ GAGGAAAGCACACATCTTTGG 3′ and 5′ TGTGGGCTGCTGATATTTTGG 3′ at a final concentration of 0.125 μM. A product of 220 bp was obtained. For *MRP1*, the primers were 5′ CTGTTTTGTTTTCGGGTTCC 3′ and 5′ GATGGTGGACTGGATGAGGT 3′ at a final concentration of 0.125 μM. A product of 287 bp was obtained.

Primer specificities for MDR1 and MRP1 were assessed against known gene sequences. MDR1 primers were complementary to MDR1 but not MRP1 gene sequences. Similarly, MRP1 primers were complementary to MRP1 but not MDR1 and other MRP gene sequences.

For MDR1 analysis, the master mix was optimised to give co-amplification of GAPDH and MDR1. The annealing temperature was 55°C for 40 cycles after activating the Amplitaq Gold at 95°C for 12 min, as recommended by the supplier. For MRP1 analysis, the master mix was optimised to give amplification of GAPDH and MRP1 in separate tubes. The annealing temperature was 50°C for 30 cycles after activating the Amplitaq Gold at 95°C for 12 min.

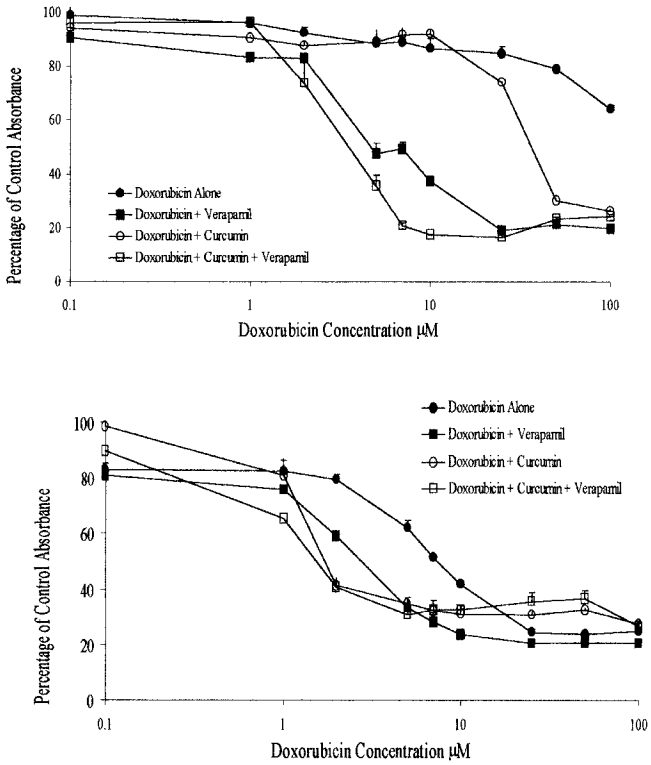

FIGURE 1 – Effects of Pgp and GST inhibitors on doxorubicin-induced cytotoxicity in *(a)* HEp2A cells and *(b)* HEp2 cells. The percentage of surviving cells is indicated by the percentage of control absorbance. The effects of verapamil (5 μM), curcumin (25 μM) and verapamil (5 μM) combined with curcumin (25 μM) were determined. Each point is the mean of triplicate determinations.

TABLE I – DOXORUBICIN IC50 VALUES IN HEp AND HEp2A CELLS CHEMOSENSITIZED BY INHIBITORS OF PGP (VERAPAMIL) AND/OR GST (CURCUMIN)

	HEp2 cells μM (mean ± SD)	HEp2A cells μM (mean ± SD)
Doxorubicin alone	7.29 ± 0.3	>100
Doxorubicin + verapamil	2.79 ± 0.2	5.28 ± 0.82
Doxorubicin + curcumin	2.46 ± 1.11	36.22 ± 0.88
Doxorubicin + verapamil + curcumin	1.52 ± 0.10	3.54 ± 0.45
Doxorubicin + ethacrynic acid	0.79 ± 0.40	20.23 ± 0.73

Verapamil concentration was 5 μM, curcumin 25 μM and ethacrynic acid 25 μM.

Construction of GSTP1 expression vector

A 635 bp human GSTP1 cDNA (supplied by Dr. C. Henderson, University of Dundee, Scotland) was inserted into pcDNA3.1Zeo+ (Invitrogen, Groningen, the Netherlands) at the *Eco*RI site in the multiple cloning site. Orientation of the cDNA was checked by sequencing.

Stable transfection of HEp2

Cells were grown in 25 cm^2 tissue culture flasks until 60% confluent. An amount of 3 μg of pcDNA3.1Zeo+HGSTP1 was then transfected into cells using lipofectin reagent as recommended by the supplier (Life Technologies). Cells were then grown under normal growth conditions for 48 hr, after which time cells were trypsinised and seeded onto a 120 mm cell culture dish and grown in normal growth medium containing 400 μg/ml Zeocin (Invitrogen) for selection. Colonies were grown for 2 weeks, replacing the selective medium every 3 days, after which time colonies were large enough to trypsinise and transfer to 48-well culture plates. Cells continued to be grown in selective medium and expanded until stable cultures were large enough to be grown in 75 cm^2 flasks. At this point, cells were maintained in medium containing 200 μg/ml zeocin and expression and function of GSTP1 assessed.

TABLE II – EFFECT OF CURCUMIN ON GST ACTIVITY IN HEp2, HEp2A, RAT LIVER CYTOSOL AND PURIFIED GSTP1-1

	GST activity (mmol/min/mg protein)	
	Control	Curcumin (25 μM)
Rat liver cytosol	0.163 ± 0.059	0.089 ± 0.049
HEp2	0.010 ± 0.004	0.003 ± 0.006
HEp2A	0.016 ± 0.003	0.008 ± 0.003
GSTP1-1	85.1 ± 14.1	14.6 ± 2.1

GST activity was measured with CDNB. GSTP1-1 was supplied by Calbiochem (Nottingham, UK).

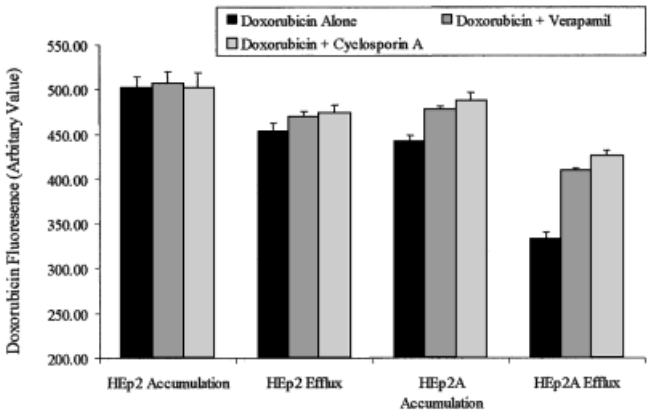

FIGURE 2 – Doxorubicin efflux in HEp2 and HEp2A cells. Doxorubicin accumulation, efflux after 1 hr, effects of cyclosporine (3 μg/ml) and verapamil (5 μM) are shown. Measurements are given in arbitrary units of fluorescence. Each point represents the mean of triplicate determinations.

Data analysis

Differences in fluorescence values in efflux experiments and cytotoxicity were assessed by the 2-sample *t*-test and by Anova. Results were considered to be statistically significant at $p < 0.05$.

RESULTS

Drug sensitivity

Using the MTT test, the sensitivity of the wild-type and resistant cells to doxorubicin and the effects of inhibitors of GST and drug transporters on this sensitivity was compared to determine the contribution of metabolism and drug efflux to resistance. The highest concentration of doxorubicin used (100 μM) produced 30% cell death in untreated drug-resistant HEp2A cells. IC50 value was therefore defined as >100 μM (Fig. 1*a*; Table I). In HEp2 cells, the doxorubicin IC50 value was 7.29 μM (Fig. 1*b*; Table I). HEp2A cells were thus >13-fold more resistant to doxorubicin than HEp2 cells. Treatment of HEp2A cells by verapamil reduced the doxorubicin IC50 from >100 μM to 5.28 μM ($p < 0.001$). Inhibition of GST alone by curcumin, which has been previously shown to be a GST inhibitor with selectivity toward GSTP1[25,26] and was a potent inhibitor of both total GST activity in the cell lines and purified GSTP1 activity at the concentration of 25 μM used (Table II), reduced doxorubicin IC50 from >100 μM to 36.22 μM in HEp2A cells ($p < 0.001$). Curcumin alone chemosensitised drug-sensitive HEp2 cells and reduced doxorubicin IC50 from 7.29 μM to 2.46 μM ($p < 0.001$) (Fig. 1*b*; Table I).

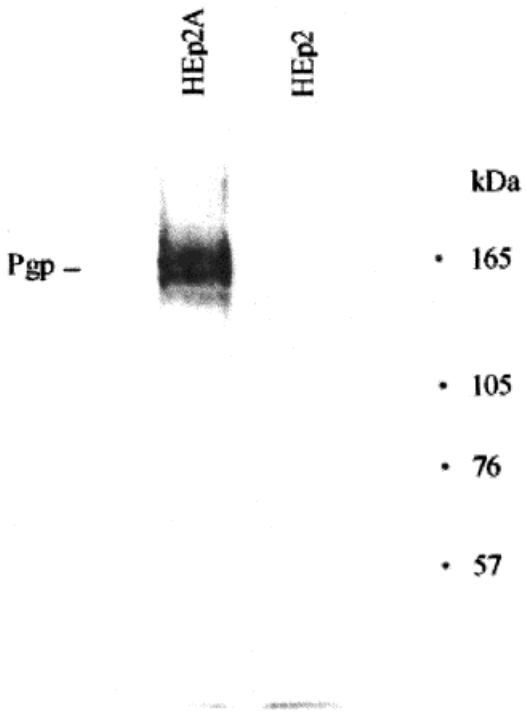

FIGURE 3 – Immunoblot for P-glycoprotein (Pgp) in HEp2A and HEp2 cells. The position of Pgp is indicated.

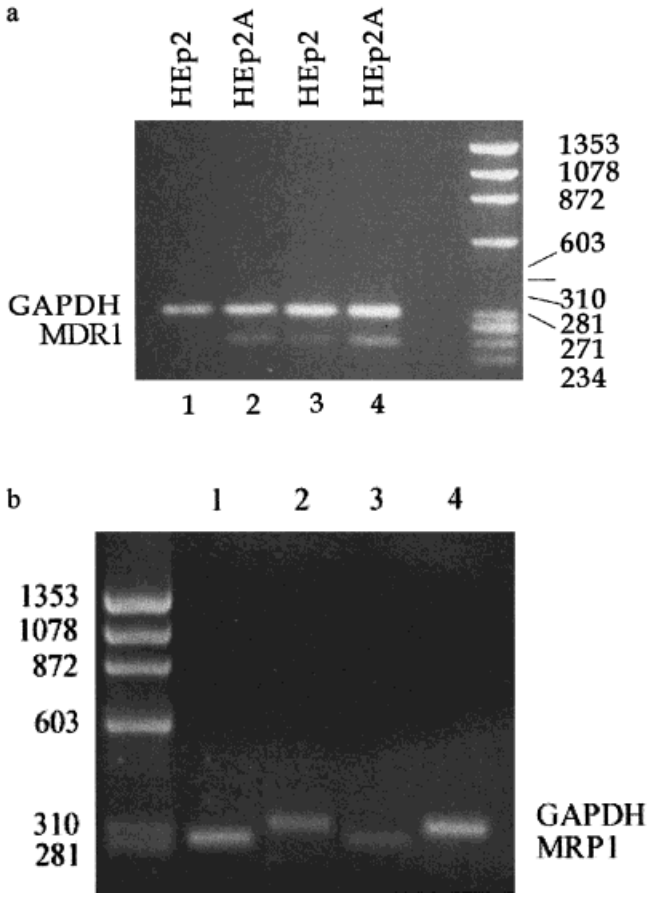

FIGURE 4 – Pgp and MRP1 mRNA expression in HEp2A and HEp2 cells. Ethidium bromide-stained agarose gel electrophoresis of the products of RT-PCR analysis for *(a)* Pgp and *(b)* MRP1 transcript expression. GAPDH is internal control. In *(a)*, lanes 1 and 2 represent 3 μl samples (6% total cDNA) reverse transcribed cDNA mix from HEp2 and HEp2A cells, while lanes 3 and 4 represent 5 μl (10% total cDNA) samples. In *(b)*, lanes 1 and 2 represent HEp2A MRP1 and GAPDH, respectively, and lanes 3 and 4 represent HEp2 MRP1 and GAPDH, respectively. Molecular weight markers are also shown.

There was no effect on levels of GSTP1 protein in HEp2A cells treated with 25 μM curcumin, suggesting that the effect of curcumin was to inhibit enzyme activity and was not on gene expression. Another GST inhibitor, ethacrynic acid, was slightly cytotoxic to cells in the absence of doxorubicin but gave broadly similar IC50 values when combined with doxorubicin to those for curcumin combined with doxorubicin of 0.79 μM for HEp2 and 20.23 μM for Hep2A, confirming the effect of GST inhibition on resistance (Table I). Verapamil and curcumin administered together had an additive chemosensitising effect in HEp2A cells at doxorubicin concentrations between 5 μM and 10 μM ($p < 0.05$) (Fig. 1*a*).

Doxorubicin efflux

Doxorubicin accumulation and efflux in the sensitive and resistant cells were assessed by flow cytometry. Doxorubicin accumulation was greater in HEp2 (501 ± 12.5 arbitrary units) than HEp2A cells (441 ± 6.8 units $p < 0.001$). After 1 hr, fluorescence declined in HEp2A cells to 331 ± 8.9 units (25% loss) due to efflux. At this interval, fluorescence in HEp2 cells was 453 ± 9.4 units ($p < 0.001$) representing 10% loss. Both verapamil and cyclosporine inhibited efflux in HEp2A cells. Fluorescence values after 1 hr were 408.9 ± 2.5 (7.5% loss verapamil) and 425.2 ± 6.1 (4% loss cyclosporine) units, respectively ($p < 0.001$ vs. untreated HEp2A cells). Efflux in HEp2 cells was not significantly affected by verapamil or cyclosporine (Fig. 2).

P-glycoprotein and MRP1 expression

To determine whether the differences between the Hep2 and Hep2A cells with respect to doxorubicin cytotoxicity and efflux were due to Pgp and/or MRP1 overexpression, levels of mRNA and protein for both were analysed by immunoblotting and RT-PCR. Both Pgp protein and mRNA were expressed in HEp2A cells. Pgp protein was undetectable in HEp2 cells by immunoblotting, although the transcript was detected at low level by RT-PCR (Figs. 3, 4*a*). Pgp transcript expression was 2.9-fold greater in HEp2A than in HEp2 cells (Fig. 4*a*). Expression of MRP1 was also greater in HEp2A cells. RT-PCR experiments showed that HEp2A cells expressed 3.2-fold higher levels of MRP1 transcript than HEp2 cells (Fig. 4*b*).

Glutathione S*-transferase expression and activity*

To determine whether GST isoforms might also contribute to doxorubicin resistance in Hep2A cells, immunoblotting experiments were performed using a range of antisera specific for individual GST classes. Total GST activity was also assessed by enzyme assay. Densitometry analysis of immunoblots demonstrated 5.7-fold greater expression of GSTP1 in HEp2A cells than in the HEp2 line (Fig. 5*a*). GSTM class levels were similar with densitometry ratios of 1:1.1 (Fig. 5*b*). GST alpha class enzymes were undetectable in either HEp2A or HEp2 cells (data not shown). Total glutathione *S*-transferase activity measured by CDNB assay was 1.7-fold greater in HEp2A than HEp2 cells.

Stable transfection of glutathione S*-transferase P1*

To further analyse the role of GSTP1 in doxorubicin resistance, HEp2 cells were stably transfected with a full-length cDNA clone for GSTP1. Immunoblot and CDNB activity of clones produced by stable transfection of HEp2 are shown in Figure 6. Clone G3, which showed 1.5-fold greater GST activity than the control clone, was chosen for cytotoxicity assays. In

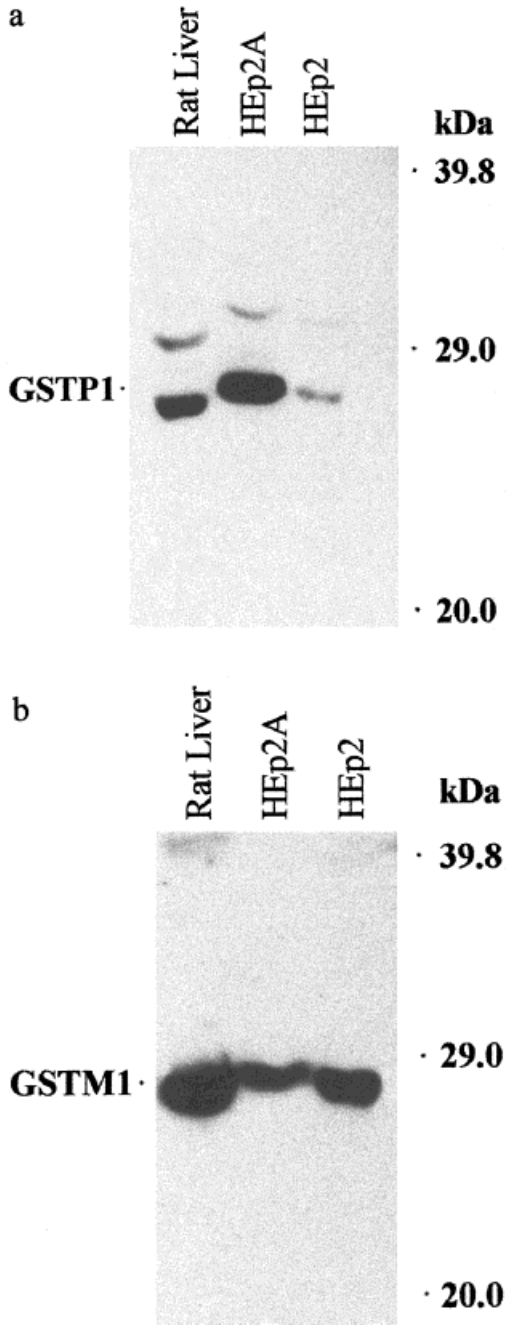

FIGURE 5 – Immunoblot for GST expression in HEp2A and HEp2 cells. *(a)* Shown is a blot incubated with anti-GSTP class antibody and *(b)* a blot incubated with anti-GSTM class. Rat liver cytosol was included as a positive control and the positions of size markers are indicated.

the case of clone G3, the IC50 value for doxorubicin was 15.47 μM as compared with 6.94 μM in the control cells ($p = 0.046$) (Fig. 7). Treatment of the HEp2 clones with curcumin resulted in a increase in doxorubicin cytotoxicity, with the IC50 in control cells being reduced from 6.94 μM to 3.24 μM ($p = 0.03$), while in the GSTP1 expressing G3 cells the IC50 value was reduced from 15.47 μM to 3.99 μM ($p = 0.01$), which would be consistent with the effect of curcumin already observed in HEp2 and HEp2A cells. Similar results were obtained for another clone G4.

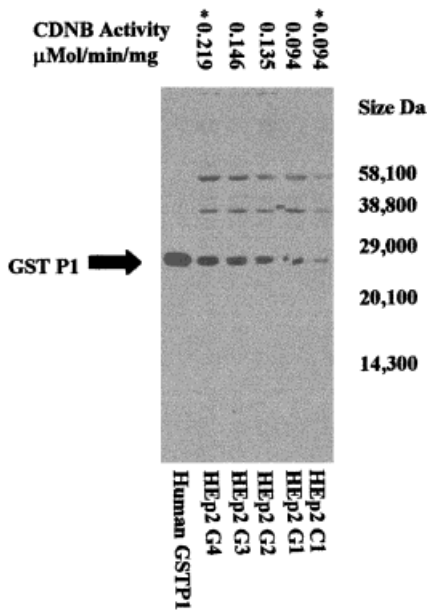

FIGURE 6 – Immunoblot analysis and GST activity of HEp2 clones stably expressing GSTP1. Clones marked * were selected to be used in doxorubicin cytotoxicity assays.

DISCUSSION

Acquired drug resistance follows selection or induction by therapy of diverse molecular events and is a principal unresolved cause of cancer treatment failure. The relationship between efflux mechanisms and drug resistance is well established.[27] Pgp-mediated efflux of unconjugated doxorubicin is proficient, while MRP1 supports energy-dependent efflux of glutathione-drug conjugates.[11] In our study, verapamil, which inhibits both the Pgp- and MRP1-mediated efflux pathways, chemosensitised HEp2A cells to doxorubicin, possibly through effects on both efflux pathways since Pgp and MRP1 were overexpressed in the resistant cell line. By verapamil treatment, doxorubicin IC50 was reduced to levels of <5 μM, which is within the concentration range that may be achieved during clinical therapy.[28]

In addition to the well-established roles for Pgp and MRP1 overexpression, our study indicates a role for GSTP1 in the resistance of HEp2A cells to doxorubicin. The contribution of GSTP1 to resistance is suggested both by the loss of resistance of the HEp2A cells in the presence of GSTP1 inhibitors and by stable expression of GSTP1 in the parental cell line also resulting in decreased cytotoxicity, which is curcumin-sensitive. However, the role of GSTP1 in cellular resistance to doxorubicin remains controversial. In a number of studies, overexpression of this isoform correlates with resistance to doxorubicin,[8,29] but in other studies, which generally involved other cell types, no evidence for a role for GSTP1 in resistance was obtained.[16,30] In particular, there is no clear evidence for the production of a doxorubicin-GSH conjugate *in vivo* though *in vitro* synthetic experiments suggest this would be possible,[31] and the synthetic conjugate is a substrate for MRP1.[12] It has been suggested that since anthracyclines exert some of their toxicity through production of reactive oxygen species and lipid peroxidation, GST overexpression might protect by detoxicating products of oxidative stress rather than direct conjugation.[3] However, it is also clear that MRP1 overexpression can contribute to doxorubicin resistance probably through increased efflux, which would involve either formation of a doxorubicin-GSH conjugate or a cotransport mechanism with GSH.[11,32] A study on doxorubicin resistance using 3T3-fibroblasts transfected with combinations of MRP1, γ-glutamylcysteine synthetase and GSTP1 found little effect by GSTP1 transfection alone, but cotransfection with all 3

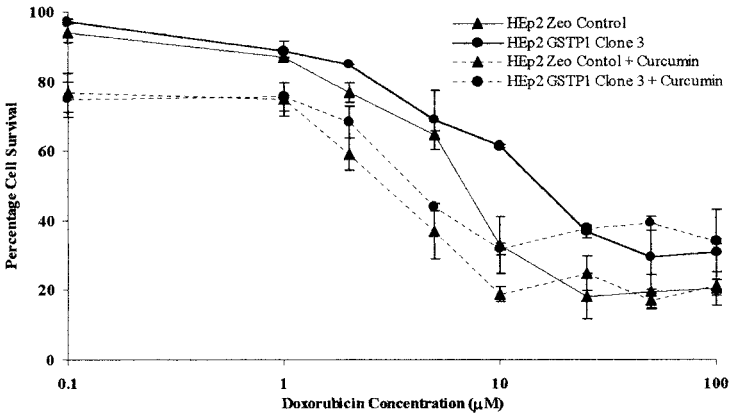

FIGURE 7 – Effect of stable transfection of HEp2 cells with human GSTP1 and the GSTP1 inhibitor curcumin (25 μM) on doxorubicin-induced cytotoxicity. Each point is the mean of triplicate determinations. The experiment shown was performed with clone G3.

cDNAs resulted in the largest resistance to doxorubicin, which would be consistent with a role for both GSTP1 and cellular GSH levels in doxorubicin detoxication in addition to MRP1.[33] The failure to see an effect in 3T3 cells and in earlier studies on MCF-7 cells[34] when GSTP1 only was transfected may reflect a need for some MRP1 expression in the parental cell line to allow efflux of doxorubicin. Though MRP1 levels in HEp2 cells were approx. 3-fold lower than in HEp2A cells, a signal was detectable by RT-PCR, indicating a low level of expression of this protein. In our study, curcumin alone had a small effect in potentiating doxorubicin cytotoxicity in the parent HEp2 cells, which expressed GSTP1 at a low level but had little Pgp and MRP1. In the HEp2A subclone, it had a greater chemosensitising effect at high doxorubicin concentrations, which would be consistent with a greater role for GSTP1 in resistance when there was also overexpression of MRP1.

It has been suggested that alterations in glutathione reductase activity could contribute to doxorubicin resistance in resistant HL-60 human leukemia cells by raising intracellular glutathione levels,[35] but we found no evidence that this was the case in HEp2A cells (data not shown), suggesting again that mechanisms for doxorubicin resistance depend on the type of cell line involved.

Clinical responsiveness to cancer chemotherapy may involve factors of tumor burden, tumor heterogeneity and drug clearance, in addition to mechanisms of drug resistance. Assessment of resistance markers against response to individual drugs such as doxorubicin is complicated by the clinical requirement for combination chemotherapy. Studies of leukemias have shown that Pgp expression correlates with a poor response to multimodal chemotherapy involving the anthracyclines doxorubicin or daunorubicin.[36] Similarly, an inverse relationship has been reported between GSTP1 expression and response to combination chemotherapy with doxorubicin in ovarian cancer.[37] Modulators of Pgp and GST are currently undergoing clinical evaluation, although questions relating to altered pharmacokinetics and effects of therapy on normal tissues have been raised.[38] Preliminary reports indicate mixed success. The use of the Pgp inhibitor cyclosporine has been associated with clinical responses to drug combinations including doxorubicin, to which the disease had previously been unresponsive.[39] GST inhibitors such as sulphasalazine have been used in combination with melphalan in patients with previously treated advanced cancers of different origins. A partial response was observed in 2 outof 4 patients with ovarian cancer.[40]

Our study supports a role for GSTP1 metabolism alongside MRP1- and Pgp-mediated efflux in acquired doxorubicin resistance. Further work may promote development of rational treatment strategies of cytotoxic agents and appropriate resistance modulators aimed at improved outcome in acquired drug resistance.

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