

Glutathione S-Transferase M1 and Multidrug Resistance Protein 1 Act in Synergy to Protect Melanoma Cells from Vincristine Effects

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ABSTRACT

Previous studies have shown that glutathione S-transferases (GSTs) can operate in synergy with efflux transporters, multidrug resistance proteins (MRPs), to confer resistance to several carcinogens, mutagens and anticancer drugs. To address the poorly documented role of the GSTM1 in cancer chemoresistance, we used CAL1 human melanoma cells expressing no endogenous GSTM1 and a high level of MRP1. Cells were transfected with an expression vector containing the GSTM1 cDNA, and different clones were selected expressing different levels of GSTM1 (RT-PCR, Western blot, and enzyme activity). Cells overexpressing GSTM1 displayed a 3- to 4-fold increase in resistance to anticancer drugs vincristine (VCR) and chloram-

bucil (CHB) in proliferation, cytotoxic, and clonogenic survival assays. Inhibitors of MRP1 (sulfapyrazone, verapamil) and GST (dicumarol, curcumin) completely reversed the GSTM1-associated resistance to VCR, indicating that a MRP efflux function is necessary to potentiate GSTM1-mediated resistance to VCR. Conversely, MRP1 inhibitors had no effect on the sensitivity to CHB. Using immunofluorescence assay, GSTM1 was also shown to protect microtubule network integrity from VCR-induced inhibition of microtubule polymerization. In conclusion, these results show that GSTM1 alone is involved in melanoma resistance to CHB, whereas it can act in synergy with MRP1 to protect cells from toxic effects of VCR.

Resistance to anticancer agents is a major concern of today's chemotherapy. Among the systems involved in cancer cell chemoresistance, xenobiotic detoxification by phase II glutathione conjugation reactions and phase III efflux transport plays a crucial role (Shen et al., 1997). The conjugation of electrophilic molecules, including anticancer drugs but also carcinogens or mutagens, with the cellular tripeptide glutathione (GSH) is catalyzed by a multigene family of enzymes, the glutathione S-transferases (GSTs), consisting of at least six classes in humans (α , μ , π , θ , ω , and ξ) (Salinas and Wong, 1999; Ketterer, 2001; Townsend and Tew, 2003). Most often, the conjugation renders the electrophilic compounds less chemically reactive and the conjugates are less toxic to the cell. Thus, conjugation with glutathione is thought to be involved in the protection of cancer cells against electrophilic antineoplastic agents.

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Four main observations argue for the role of GST and GSH in drug resistance. First, an elevation of the GST level in tumors is detected after development of a clinical drug resistance (Schisselbauer et al., 1990). Second, tumor cell lines selected for acquired resistance show an increase of different isoforms of GSTs (Tew, 1994; Horton et al., 1999). Third, cancer cells transfected with different isozymes of GST can express drug resistance (Manoharan et al., 1991). Fourth, the inhibition by antisense gene of endogenous GST expression reveals the involvement of GST π in drug resistance (Ban et al., 1996). However, some other investigators have not found associations between cellular resistance to anticancer agents and expression of GSTs (Townsend et al., 1992). Thus, the role of GSTs in the protection of cells against anticancer drugs remains equivocal.

One hypothesis is that overexpression of GSTs is not always sufficient to confer significant protection from the electrophiles. Thus, it was reported that coexpression with GSTs of the glutathione conjugate efflux transporters multidrug resistance proteins 1 and 2 (MRP1 and MRP2) is necessary to

ABBREVIATIONS: GSH, reduced glutathione; GST, glutathione S-transferase; MRP, multidrug resistance protein; ABC, ATP-binding cassette; VCR, vincristine; CDNB, 1-chloro-2,4-dinitrobenzene; PBS, phosphate-buffered saline; BSO, D,L-buthionine-[S,R]-sulfoximine; FCS, fetal calf serum; wt, wild type; RT-PCR, reverse transcription-polymerase chain reaction.

potentiate GST-mediated protection from anticancer agents (Morrow et al., 1998; Harbottle et al., 2001). MRP1 and MRP2 belong to the superfamily of ATP-binding cassette (ABC) transporters (Keppler et al., 2000). ABC proteins are responsible for the active transport across biological membrane (phase III) of drugs and other xenobiotics but also of phospholipids, ions, peptides, steroids, polysaccharides, amino acids, and organic anions (Borst et al., 1999). MRP1, MRP2, and MRP3 have overlapping substrate specificity, including glutathione conjugates (Keppler et al., 2000). MRP4 and MRP5 are involved in nucleotide efflux (Wijnholds et al., 2000). Initially, MRP1 and MRP2 have been shown to confer resistance to various drugs of natural origin, including anthracyclines, vinca alkaloids and epipodophyllotoxins (Borst et al., 1999). Then, the requirement of GSH for MRP1-mediated cellular efflux of some natural products was demonstrated (Rappa et al., 1997). Finally, it was reported that detoxification of various xenobiotics, including the anticancer agents chlorambucil (Morrow et al., 1998), etoposide (O'Brien et al., 2000), and doxorubicin (Harbottle et al., 2001), the carcinogen 4-nitroquinoline 1-oxide (Morrow et al., 2000), or the metalloid arsenic (Liu et al., 2001), implicated a coordinated action of GSTs and MRPs.

The aim of this work was to study the role of GST μ 1 (GSTM1) and MRPs, poorly documented, in the drug resistance of cancer cells. To address this question, we developed a human cancer cell line expressing GSTM1 by gene transfer from the melanoma CAL1 cells expressing the glutathione conjugate efflux transporter MRP1. Both GST and MRP activities were quantified in the different cell lines. Then, viability, proliferation, and clonogenic assays were designed to study the effects of GSTM1/MRP1 expression on drug sensitivity. Finally, the effects of vincristine on microtubule network integrity were evaluated.

Materials and Methods

Drugs and Chemicals

Geneticin was from Invitrogen (Cergy Pontoise, France). [3 H]Vincristine sulfate (6.6 Ci/mmol) was purchased from Amersham Biosciences (Orsay, France). All other drugs were from Sigma (St. Quen-

tin-Fallavier, France). Stock solutions were vincristine (VCR) (100 μ M in H₂O), vinblastine (100 μ M in H₂O), doxorubicin (3.7 mM in H₂O), mitomycin C (10 mM in H₂O), chlorambucil (100 mM in ethanol), melphalan (100 mM in acidified ethanol), verapamil (5 mM in H₂O), sulfapyrazone (200 mM in dimethyl sulfoxide), dicumarol (50 mM in 0.1 N NaOH), 1-chloro-2,4-dinitrobenzene (CDNB) (100 mM in PBS), glutathione (100 mM in H₂O), curcumin (2.5 mM in ethanol), D,L-buthionine-[S,R]-sulfoximine (BSO) (5 mM in H₂O), 5-fluorouracil (0.1 M in H₂O), cisplatin (5 mM in 0.9% NaCl), methotrexate (50 mM in 0.9% NaCl), mitoxantrone (5 mM in dimethyl sulfoxide).

The cDNA encoding on one hand human GSTA1, GSTP1, and GSTM1 and on the other hand human MRP1 and MRP2 were kindly provided by Dr. C. S. Morrow (Wake Forest University School of Medicine, Winston-Salem, NC) and Pr. D. Keppler (Deutsches Krebsforschungszentrum, Heidelberg, Germany), respectively.

Cell Lines. CAL1 human malignant melanoma cells (Olivier et al., 1990) and Caco-2 cells (American Type Culture Collection, Manassas, VA) were grown at 37°C in a fully humidified 5% CO₂ atmosphere in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS). Parental CAL1 cells (CAL1-wt) expressed one isoform of GSTs (GSTP1) and two isoforms of ABC transporters (MRP1 and MRP5). CAL1 cells expressing human GST μ 1 were established by transfection of CAL1-wt cells with a pcDNA3 expression vector containing the cDNA encoding the human GSTM1 (ppGST μ) using LipofectAMINE (Invitrogen) according to manufacturer's instruction. Stable transfectants were selected for 1 mg/ml G-418 resistance. A transfected clone (CAL1- μ 0) expressing G-418 resistance but not GSTM1 was used as negative control. A second control cell line, called CAL1-mock, was generated by stable transfection of CAL1-wt cells with empty pcDNA3 vector.

Analysis of GST and MRP Expression

RT-PCR. Extraction of total cellular RNA was carried out using TRIzol reagent (Invitrogen) and expression was analyzed by RT-PCR using standard procedures and specific primers (Wang et al., 2000; Decleves et al., 2002) (Table 1). Positive controls were either plasmids containing the coding sequences for GSTs (A1, P1, and M1), MRP1 and MRP2, or cDNA from Caco-2 cells for MRP3, MRP4, MRP5, and MDR1 (Taipalensuu et al., 2001). PCR products were run on agarose gels supplemented with ethidium bromide and visualized by ultraviolet illumination. Band intensities were quantified by densitometry analysis using Bio1D software (Vilber Lourmat, Marne La Vallée, France).

TABLE 1
PCR primers, amplicon sizes, and number of amplification cycles

Genes	Direction	Primer Sequences (5'→3')	Amplicon <i>base pairs</i>	Number of Cycles
β -Actin	Forward	GGCATCGTGATGGACTCCG	650	23
	Reverse	GCTGGAAGGTGGACAGCGA		
GSTA1	Forward	CCTGCCCTTGAAAAAGTCTTAAAG	90	30
	Reverse	AAGTTCACCACAGATGAATGTCA		
GSTP1	Forward	CTCACTCAAAGCCTCCTGCCTAT	85	27
	Reverse	CAGGATGGTATTGGACTGGTACAG		
GSTM1	Forward	CTGGGCATGATCTGCTACAATC	136	32
	Reverse	CAAAAGTGATCTTGTTCCTGCAA		
MRP1	Forward	GGACCTGGACTTCGTCTCA	292	35
	Reverse	CGTCCAGACTTCTTCATCCG		
MRP2	Forward	CTGCCTCTTCAGAATCTTAG	241	35
	Reverse	ATAACCCAAAGTTGCAGGCT		
MRP3	Forward	CTCAATGTGGCAGACATCGG	178	35
	Reverse	GGGAGCTCACAAACGTGTGC		
MRP4	Forward	CCATTGAAGATCTTCTCTGG	240	35
	Reverse	GGTGTTCATCTGTGTGCA		
MRP5	Forward	GGGATAACTTCTCAGTGGGG	383	35
	Reverse	GGAATGGCAATGCTCTAAAG		
MDR1	Forward	GGTGCTGGTTGCTGCTTACA	291	35
	Reverse	TGGCCAAAATCACAGGGT		

Western Blot Analysis. The expression of GSTs in CAL1 cells was investigated as described previously (Evrard et al., 1999). Cells were homogenized on ice in 100 μ l of a lysis buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 100 μ g/ml phenylmethylsulfonyl fluoride, 1 μ g/ml aprotinin, and 1% Triton X-100. The lysates were centrifuged at 20,000g for 10 min at 4°C, and protein levels of the supernatants were determined according to Bradford (1976). Cytosolic proteins (20 μ g) were separated by 12% SDS-polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membrane. The membranes were blocked overnight in 10% milk in PBS-Tween followed by incubation with rabbit polyclonal primary antibodies anti-GST α , π , or μ 1:500 (Novocastra-Tebu, Le Perray en Yvelines, France), or mouse monoclonal primary antibody anti- β -actin 1:2000 (Sigma). Then, membranes were incubated with anti-rabbit or anti-mouse peroxidase-conjugated secondary antibodies 1:5000 for GSTs and β -actin, respectively. Specific proteins were visualized using X-ray films after incubation with enhanced chemiluminescence reagents (Amersham Biosciences).

To study MRP expression, cells were lysed on ice in radioimmunoprecipitation assay buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium desoxycholate, and 0.1% SDS) supplemented with fresh 1 mM dithiothreitol, 2 mM NaF, and protease inhibitor cocktail (Sigma). The lysates were centrifuged and supernatant proteins (100 μ g) were separated by 7.5% SDS-polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membrane. MRP1, MRP2, and MRP5 were visualized by using specific monoclonal primary antibodies (Monosan-Tebu), anti-species peroxidase-conjugated secondary antibodies (Sigma), and enhanced chemiluminescence detection as described above.

Functional Analysis of GST and MRP Activities. Evaluation of GST activity was performed as follows. Cells were lysed in PBS, pH 6.5, by three freezing (in liquid nitrogen)/defrosting (37°C) cycles, and cytosols were recovered by centrifugation for 10 min at 4000g and 4°C. The total GST activity was measured using CDNB as substrate (Habig et al., 1974). Conjugation of reduced GSH to CDNB at 25°C and pH 6.5 was monitored spectrophotometrically at 340 nm. When used, curcumin (30 μ M) was added to cell culture medium 15 min at 37°C before cell lysis and to reaction mixture during GSH conjugation determination.

Evaluation of MRP functional activity was assessed by studying [3 H]vincristine accumulation as described previously (Decleves et al., 2002). Cells, plated onto 12-microwell plates, were incubated for 1 h at 37°C with 100 nM [3 H]vincristine in culture medium. After washing with PBS, cells were incubated at 37°C for 3 h in the absence (control) or presence of inhibitors (2 mM sulfinpyrazone, 0.4 mM dicumarol). Then, cells were washed with ice-cold PBS to eliminate the extracellular tritiated drug and lysed with 500 μ l of 0.1 N NaOH. Intracellular [3 H]vincristine concentration was determined by β -scintillation counting and normalized to protein counting.

Cytotoxicity Assays. The effect of anticancer agents on cell viability was assessed using the neutral red assay as described previously (Evrard et al., 1999). Briefly, aliquots of cell suspension (10^4 cells/well) were seeded in 96-well microtiter plates and incubated for 24 h at 37°C. Cells were exposed to mitomycin, chlorambucil, cisplatin, mitoxantrone, and melphalan for 1 h at 37°C and for 72 h at 37°C to 5-fluorouracil and methotrexate (150 μ l in fresh medium per well, eight wells per agent concentration). For doxorubicin, vincristine and vinblastine, cells were exposed to drug for 4 h at 37°C in fresh medium supplemented with 1% FCS. When used, GST or MRP inhibitors were added 15 min before exposure to anticancer drugs. After 72-h incubation, cells were washed with PBS and 150 μ l of a neutral red solution (33 μ g/ml) was added. After 3 h at 37°C, 5% CO₂, cells were washed with PBS and destained with 150 μ l of glacial acetic acid (1%)/ethanol (50%) (v/v). Absorbance was measured at 540 nm using a microplate reader (Labsystems Multiscan MS; PAA, Farnborough, UK). The effect of the drugs on cell survival was expressed as a percentage of viability of treated-cells compared with control cells.

Cell Survival Assay. The cytotoxicity of vincristine was determined by loss of colony-forming ability as described previously (Cai et al., 1999). Cells were plated at the density of 1.4×10^6 /T75 flask. On the following day, cells were treated with 2 nM vincristine for 4 h at 37°C and the replaced for 16 h in fresh medium. When used, GST or MRP inhibitors were added 15 min before exposure to vincristine. Then cells were replated at a density of 1000 cells/100-mm dish. Fifteen days later, cell colonies were stained with Giemsa (Sigma) and counted using Bio1D software (Vilbert Lourmat, Marne la Vallée, France). Survival was expressed as a percentage of the colony-forming efficiency of appropriate control cells exposed to vehicle alone.

Proliferation Assay. The effect of vincristine on cell proliferation was assessed as follows. Cells were plated at the density of 0.5 10^6 /T25 flask. Twenty-four hours later, cells were treated with 10 nM vincristine for 4 h at 37°C and then replaced in fresh medium. Cells were trypsinized and counted using a Casy1 Scharfe System Counter beginning 24 h after treatment and every day thereafter.

Immunofluorescence Microscopy. Cells were seeded onto glass coverslips, incubated in 600 nM vincristine at 37°C in Dulbecco's modified Eagle's medium for 20 min, fixed in methanol (−20°C) for 5 min and permeabilized with 0.1% Triton X-100 (Sigma). A mouse monoclonal antibody against fluorescein isothiocyanate-conjugated β -tubulin (Sigma) diluted 1:25 in 3% PBS-bovine serum albumin and incubated 1 h at room temperature in dark. Cells were washed with water and mounted in Mowiol (Aldrich). Cells were observed with a 63 \times oil immersion objective (numerical aperture 1.3; Leica, Wetzlar, Germany). Images were captured with a MicroMax 1300 charge-coupled device camera (Princeton Instruments, Trenton, NJ) driven by MetaMorph (version 4.11; Universal Imaging, Westchester, PA) software. Images were processed using Adobe Photoshop and Adobe Illustrator (Adobe Systems, Mountain View, CA).

Results

Characterization of Cell Lines. CAL1 malignant melanoma cells were chosen to study the effects of expression of GSTM1 in combination with MRP1 on drug resistance. This cell line, which was established from metastasis of human melanoma (Olivier et al., 1990), expressed specific melanoma markers such as MelanA, tyrosinase, MAGE1, and NA17 (RT-PCR analysis, data not shown). RT-PCR experiments were designed to study the expression of GSTs and ABC transporters in CAL1 cells. As shown in Fig. 1, the only GST isoform expressed in these cells was GSTP1. Additionally, two MRP were evidenced: MRP1 involved in glutathione conjugate efflux (Keppler et al., 2000) and MRP5, which preferentially transports nucleotides (Wijnholds et al., 2000) (Fig. 2). On the contrary, neither GSTM1, GST α 1 (GSTA1), MRP2, MRP3, nor MRP4 were detected. The multidrug resistance P-glycoprotein MDR1, belonging to the ABCB family and also responsible for drug detoxification (Gottesman et al., 2002), was not detected in these cells. Thus, CAL1 cells are a good model to study, by gene transfer, the combined effect of GSTM1 and MRP1 in melanoma chemoresistance.

As described under *Materials and Methods*, CAL1-wt cells were transfected with ppGST μ , a pcDNA3-derived expression vector containing the cDNA encoding human GST μ 1, and stable transfectants were selected for G-418 resistance. Then, stable clones were analyzed for GSTM1 expression by RT-PCR, Western blot, and GST activity assay. Two clones were selected for further analysis, CAL1- μ 0 and CAL1- μ 1. The clone CAL1- μ 1 expressed high levels of GSTM1 mRNA and protein (Fig. 1); the total GST activity in CAL1- μ 1 cells was significantly increased (about 2-fold) compared with that

in parental cells (Fig. 3). In contrast, neither the expression of the other GSTs (α and π) nor that of the MRPs was affected by GSTM1 expression in these cells (Figs. 1 and 2). The clone CAL1- μ 0, which expressed G-418 resistance but not GSTM1 (Fig. 1), was selected as negative control. Additionally, control cells (CAL1-mock) were generated by stable transfection of parental CAL1 cells with empty expression vector encoding antibiotic resistance to G-418 but not GSTM1: transfection of empty vector had no effect on GST levels or MRP expression (data not shown).

Effect of GSTM1 Expression on the Drug Sensitivity of CAL1 Cells. Chemotherapeutic drugs can affect different cellular functions including cell viability, cell cycle and survival. We first studied the effects of GSTM1 overexpression on the drug sensitivity of CAL1 cells with the use of the neutral red uptake assay. Anticancer drugs belonging to different therapeutic classes were tested: antimetabolites (5-fluorouracil, methotrexate), alkylating agents (chlorambucil, melphalan, mitomycin C, cisplatin), intercalating agents (mitoxantrone), topoisomerase II inhibitors (doxorubicin), and vinca alkaloids (vincristine, vinblastine). The corresponding data are summarized in Table 2. The expression of GSTM1 significantly increased the resistance of CAL1 melanoma cells to chlorambucil and vincristine (about 4- and 3-fold,

respectively) but had no significant effect on relative resistance to the other agents tested, including the vinca alkaloid vinblastine. Moreover, the cytotoxicity profiles of parental CAL1-wt cells and of control cells CAL1- μ 0 (Table 2) and CAL1-mock (data not shown) were similar. Thus, data reported below will not differentiate between parental cells and their corresponding control derivatives.

Requirement of GSTM1 Activity for the Resistance of CAL1- μ 1 Cells to Vincristine and Chlorambucil. To make sure of the requirement of functional GSTM1 for CAL1- μ 1 resistance to vincristine and chlorambucil, we tested whether inhibition, by the GST inhibitor curcumin (Harbottle et al., 2001), of GST catalytic activity would reverse resistance in these cells. As shown in Fig. 3, curcumin significantly reduced the total GST activity in CAL1- μ 1 cells. Moreover, it induced a considerable sensitization to vincristine and chlorambucil of CAL1- μ 1 cells, but it did not modify the drug sensitivity of CAL1-wt or CAL1- μ 0 cells (Table 3). Similar results were obtained (Table 3) with dicumarol, another GST inhibitor (Morrow et al., 1998). To confirm the requirement of glutathione conjugation for drug resistance, we used BSO, an inhibitor of glutathione synthesis (Bailey, 1998), to deplete cellular glutathione before exposure to anticancer drugs. As shown in Table 3, glutathione depletion

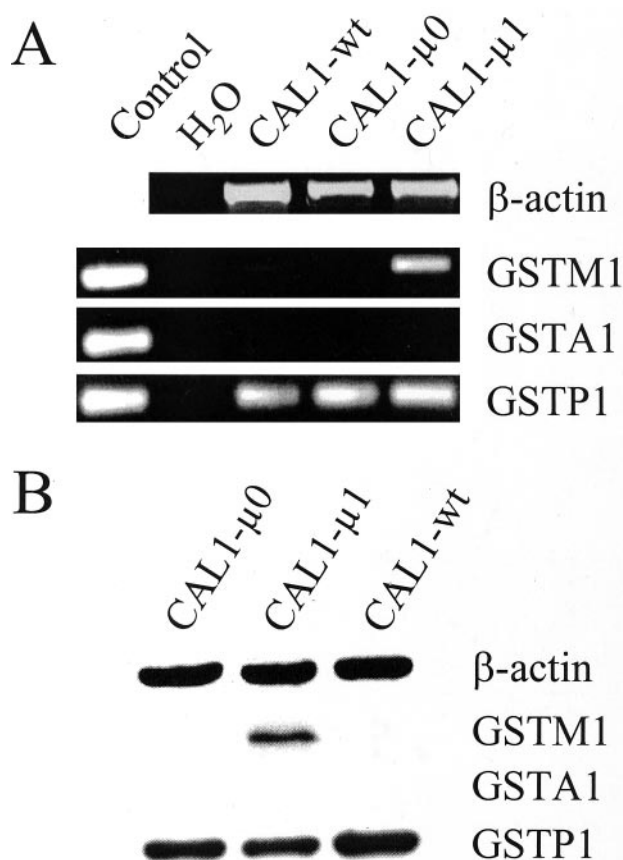


Fig. 1. Expression of GSTs in CAL1 cell lines. Levels of GST mRNA (A) and protein (B) were determined by RT-PCR and Western blot, respectively, as described under *Materials and Methods*. Examined are cytosolic protein (20 μ g in each lane) and cellular mRNA (4 ng in each lane) from parental CAL1 cells (CAL1-wt) and from CAL1 cells transfected with ppGST μ , an expression vector encoding human GSTM1, and selected for G-418 resistance (CAL1- μ 0 and CAL1- μ 1). Positive controls were plasmids encoding GSTA1, M1, and P1 for PCR experiments. β -Actin was used as standardization control in two detection experiments.

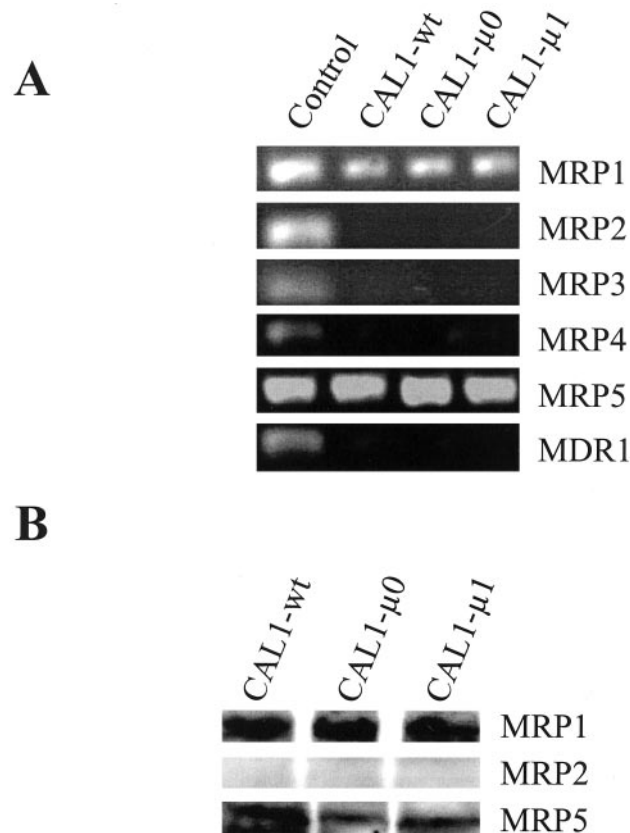


Fig. 2. Expression of MRPs and MDR1 in CAL1 cell lines. Levels of MRP mRNA (A) and protein (B) were determined by RT-PCR and Western blot, respectively, as described under *Materials and Methods*. Examined are protein (100 μ g in each lane) and cellular mRNA (4 ng in each lane) from parental CAL1 cells (CAL1-wt) and from CAL1 cells transfected with ppGST μ , an expression vector encoding human GSTM1, and selected for G-418 resistance (CAL1- μ 0 and CAL1- μ 1). Positive controls were plasmids containing the coding sequences for MRP1 and MRP2, or cDNA from Caco-2 cells for MRP3, MRP4, MRP5, and MDR1 for PCR experiments.

reversed the resistance of the CAL1- μ 1 cells to chlorambucil and vincristine. None of the inhibitors used (dicumarol, curcumin, and BSO) had any effect on cell viability when used alone.

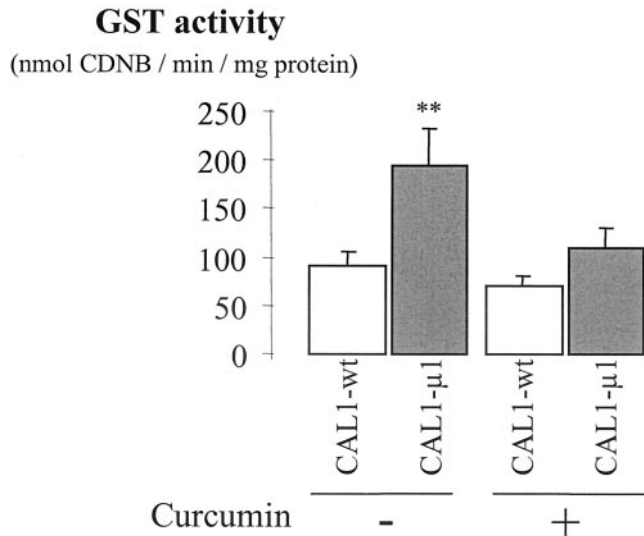


Fig. 3. GST activities in CAL1 cell lines. Cytosolic protein were extracted from parental (CAL1-wt) or GSTM1-overexpressing (CAL1- μ 1) cells and assessed for their ability to conjugate CDNB to GSH as described under *Materials and Methods*. GST activities, expressed as nanomoles of CDNB conjugated with GSH (per minute) per milligram of cytosolic protein, are means \pm S.E.M. of at least three separate experiments. **, $p < 0.01$ according to Student's *t* test comparing values obtained in studied cells with those obtained in CAL1-wt cells in the absence of GST inhibitor (30 μ M curcumin).

TABLE 2
Effects of GSTM1 expression on cellular sensitivities to anticancer drugs

Relative resistance is IC_{50} of cell line/ IC_{50} of CAL1-wt cell line. Data are means from at least three separate experiments.

Drug	Relative Resistance		
	CAL1-wt	CAL1- μ 0	CAL1- μ 1
Melphalan	1.0	1.3	1.6
Chlorambucil	1.0	1.1	4.5**
Mitomycin C	1.0	1.0	1.0
Cisplatin	1.0	0.6	1.1
5-Fluorouracil	1.0	0.6	0.6
Methotrexate	1.0	1.2	1.1
Mitoxantrone	1.0	1.3	1.1
Doxorubicin	1.0	0.9	1.3
Vincristine	1.0	1.3	3.2**
Vinblastine	1.0	N.D.	1.8

N.D., not determined.

** $p < 0.01$ (Student's *t* test).

TABLE 3
Effects of inhibitors of MRP (sulfapyrazone, verapamil), GST (curcumin, dicumarol), and GSH (BSO) on cellular sensitivities to vincristine and chlorambucil

Fold sensitization = IC_{50} of anticancer agent in the absence of inhibitor/ IC_{50} of anticancer agent in the presence of inhibitor. Data are means from at least three separate experiments.

Inhibitor	Fold Sensitization to Vincristine			Fold Sensitization to Chlorambucil		
	CAL1-wt	CAL1- μ 0	CAL1- μ 1	CAL1-wt	CAL1- μ 0	CAL1- μ 1
Curcumin	1.3	N.D.	2.6**	0.9	N.D.	1.9*
Dicumarol	1.7	1.1	3.7**	N.D.	N.D.	N.D.
BSO	0.71	1.3	2.1*	1.9	N.D.	4*
Sulfapyrazone	1.4	1.3	3.0**	1.5	1.4	1.4
Verapamil	1.0	1.2	3.0**	1.2	1.2	1.2

N.D., not determined.

* $p < 0.05$ (Student's *t* test).

** $p < 0.01$ (Student's *t* test).

Effect of MRP1 Inhibitors on the Resistance of CAL1- μ 1 Cells to Vincristine and Chlorambucil. To investigate the involvement of endogenous MRP1 in the drug resistance, cells were pretreated with MRP1 inhibitors before exposure to antineoplastic agents. As shown in Fig. 4 and Table 3, sulfapyrazone, an MRP1- and MRP2- selective inhibitor (Morrow et al., 1998, 2000; Evers et al., 2000), completely reversed the vincristine resistance of GSTM1-overexpressing CAL1- μ 1 cells, whereas it had no significant effect on the vincristine sensitivity of parental or control cells. On the contrary, sulfapyrazone could not diminish the resistance of CAL1- μ 1 cells to chlorambucil (Table 3) or melphalan (data not shown), and it had no significant effect on the nitrogen mustard sensitivity of CAL1-wt and CAL1- μ 0 cells. Similar results were obtained with verapamil, an MDR and MRP1 inhibitor (Barrand et al., 1993). Indeed, verapamil completely reversed the resistance of CAL1- μ 1 cells to vincristine but not to chlorambucil (Table 3), whereas it had no significant effect on the drug sensitivity of both parental and control CAL1- μ 0 cells. Neither sulfapyrazone nor verapamil had any effect on cell viability when used alone. Taken together, these data suggested a combined implication of GSTM1 and MRP1 in the detoxification process of vincristine and the implication of GSTM1 alone in chlorambucil resistance.

Requirement of MRP1 Activity for the Resistance of CAL1- μ 1 Cells to Vincristine. To confirm the requirement of functional MRP1 in the observed resistance of CAL1- μ 1 melanoma cell to vincristine, we studied the accumulation of [3 H]vincristine in the presence or absence of the MRP1 inhibitor sulfapyrazone. As shown in Fig. 5, sulfapyrazone increased the accumulation of tritiated vincristine both in parental and clone- μ 1 CAL1 cells. The increase of the accumulation of vincristine was significantly higher in CAL1- μ 1 cells (about 19%) than in parental cells (about 10% increase). Moreover, inhibiting the cellular GST activity by dicumarol also significantly increased the accumulation of the vinca alkaloid in CAL1- μ 1 cells (Fig. 5).

GSTM1 and MRP1 Decrease the Effects of Vincristine on Cell Proliferation and Cell Survival. To ascertain whether GSTM1 influenced cell proliferation, the growth rates of CAL cell lines were determined. GSTM1 gene transfer did not modify the growth rate of CAL1 cells. The doubling times of CAL1-wt and CAL1- μ 1 were similar (41.5 and 39.4 h, respectively). On the other hand, CAL1-wt cells were found to be about 3-fold more sensitive to vincristine than CAL1- μ 1 cells. Indeed, the vincristine-induced lowering

Fraction surviving (%)

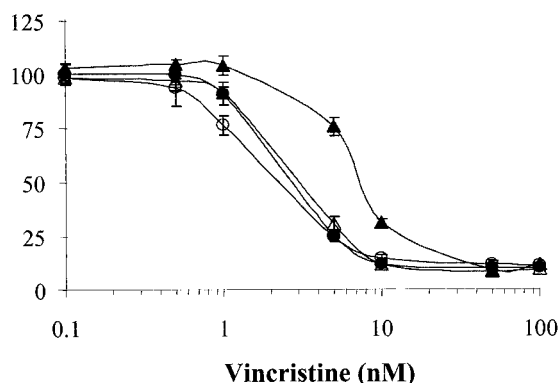


Fig. 4. Effect of sulfinpyrazone on the vincristine sensitivity of CAL1 cell lines. Shown are representative cytotoxicity profiles of parental CAL1-wt (circles) and GSTM1 overexpressing CAL1- μ 1 cells (triangles) treated with vincristine for 4 h in the absence (closed symbols) or presence (open symbols) of 2 mM sulfinpyrazone as described under *Materials and Methods*. Data are mean \pm S.E.M. of three independent determinations.

of the proliferation rate was 3-fold higher in CAL1-wt cells than in CAL1- μ 1 cells.

Perhaps the most important parameter for the efficacy of an anticancer drug is its long-term effect on cancer cell viability. We used clonogenic assays to test whether the inhibition of GSTM1 or MRP1 during a pulse of vincristine treatment would have a bearing on the outcome in a long-term assay that challenges the capacity of a cell to survive. As shown in Fig. 6, the colony-forming ability was more reduced in CAL1-wt than CAL1- μ 1 cells by 2 nM vincristine treatment (54% and 24% decrease of cell survival, respectively). In contrast, the relative resistance of CAL1- μ 1 cells was reversed when cells were pretreated with either sulfinpyrazone

[³H]-vincristine concentration

(% of control)

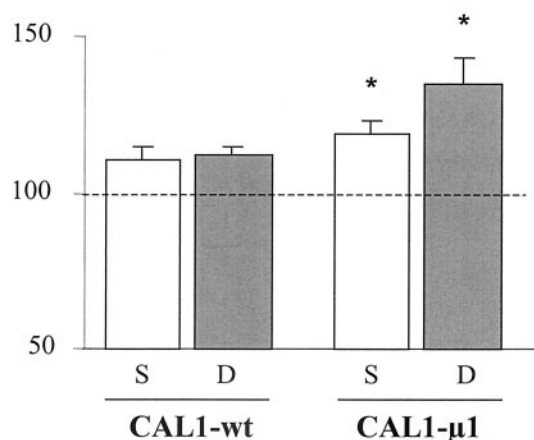


Fig. 5. Functional activity of MRP1 in CAL1 cell lines. Cells were incubated for 1 h at 37°C with 100 nM [³H]vincristine in culture medium. After washing with PBS, cells were incubated at 37°C for 3 h in the absence (control) or presence of inhibitors [2 mM sulfinpyrazone (S), 0.4 mM dicumarol (D)] and intracellular [³H]vincristine concentration by β -scintillation counting as described under *Materials and Methods*. Data, expressed as percentage of [³H]VCR concentration of control cells unexposed to inhibitors, are means \pm S.E.M. of three separate experiments. *, $p < 0.05$ according to Student's t test comparing values obtained with inhibitor in cells with those obtained without inhibitor.

or dicumarol (Fig. 6). Neither inhibitor had any effect on cell survival when used alone.

GSTM1 and MRP1 Decrease the Effects of Vincristine on Microtubule Network. Microtubules are an integral part of the cytoskeleton and are molecular target of several currently used anticancer drugs such as vinca alkaloids which inhibit microtubule polymerization. To study the effects of GSTM1 on the vincristine-mediated microtubule network alteration, immunohistochemistry experiments were performed as described above. As shown in Fig. 7, the microtubule network was more affected by vincristine treatment (600 nM, 20 min) in CAL1-wt than in CAL1- μ 1 cells, confirming a protective effect of GSTM1 in transfected cells.

Discussion

Glutathione *S*-transferases and multidrug resistance proteins can act, sometimes in synergy, to protect tumor cells from the cytotoxicity of anticancer drugs. Thus, a coordinated action of MRPs and GSTP1 (O'Brien et al., 2000; Harbottle et al., 2001) or GSTA1 (Morrow et al., 1998) has been reported. By contrast, the role of GSTM1 in drug resistance is poorly documented. In this article, we examined the effects of *GSTM1* gene transfer on the in vitro drug sensitivity of human melanoma CAL1 cells, in relation with endogenous MRP proteins.

Human melanoma CAL1 cells were chosen because their expression profile of GSTs and MRPs was representative of the expression profile of these detoxifying enzymes in individual tumors. In fact, CAL1 cells expressed GSTP1, a GST isoform found in 100% of melanoma specimens, but not GSTM1, which is detected in only 40% of patients (Moral et al., 1997). CAL1 cells expressed also MRP1, an ABC transporter expressed in about 50% of the primary and metastatic melanoma specimens (Schadendorf et al., 1995) and involved in glutathione conjugate efflux (Keppler et al., 2000). MRP5, another ABC transporter, was also detected by RT-PCR experiments. However, its role in drug resistance seems to be restricted to the efflux of nucleotides (Wielinga et al., 2003). Thus, CAL1 cells were a good model to study the effect of

Cell survival (%)

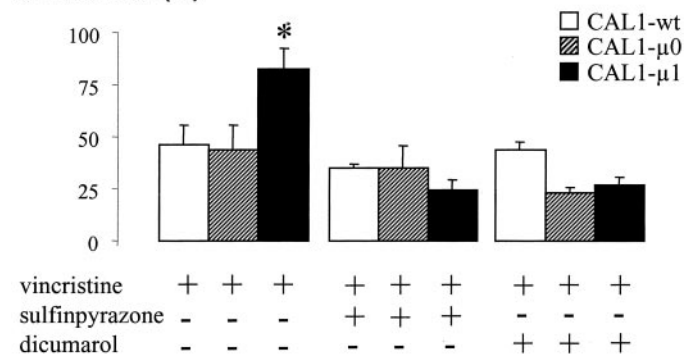


Fig. 6. Effects of GSTM1 expression on cell survival. CAL1-wt, CAL1- μ 0, and CAL1- μ 1 cells were treated with vincristine (2 nM, 4 h, 37°C), in the absence or presence of MRP1 inhibitor (2 mM sulfinpyrazone) or GST inhibitor (0.4 mM dicumarol), and colonies were counted 2 weeks after exposure as described under *Materials and Methods*. Survival was expressed as a percentage of the colony-forming efficiency of appropriate control cells exposed to vehicle alone. Values are means \pm S.E.M. of three independent experiments. *, $p < 0.05$; significantly different from CAL1-wt cells in the same experimental conditions (Student's t test).

GSTM1 overexpression, in relation with endogenous MRP1, in the drug resistance of human melanoma.

From the CAL1 cells transfected with an expression vector containing the GSTM1 coding sequence and selected for G-418 resistance, the clone CAL1- μ 1, which expressed high GSTM1 levels (mRNA and protein) and a significantly increased GST activity, was selected for further analysis. The control cells used were CAL1- μ 0, expressing G-418 resistance but not GSTM1, and CAL1-mock, transfected with empty vector. Previous works have suggested that expression of GSTs may influence jun-N-terminal kinase signaling pathways, resulting in altered gene expression and cell proliferation (Adler et al., 1999). Accordingly, we studied both the growth rates and the expression of GST, MRPs, and MDR1 in the different CAL1 cell lines. Our data showed that the expression of GSTM1 in human CAL1 cells had no effect on either cell proliferation rates or gene expression profiles. This result is in agreement with previous study (Morrow et al., 2000) showing the absence of effect of GSTP1 expression in HepG2 cells on proliferation rates and MRP2 expression. Thus, it seems likely that the effects of GSTs on proliferation and gene expression are very dependent on the cell type.

Nitrogen mustards are known to form glutathione conjugates both spontaneously and in GST-catalyzed reactions (Ciaccio et al., 1991). However, GSTM1 transfection experiments failed to demonstrate any increase in the nitrogen mustard resistance of cancer cells (Townsend et al., 1992; Morrow et al., 1998). In this article, we report for the first time that the overexpression of GSTM1 by gene transfer increases the chlorambucil resistance of human cancer cells. In fact, the GSTM1 overexpressing CAL1- μ 1 cells were found to be 4-fold more resistant to chlorambucil than their control counterparts. The chlorambucil resistance of CAL1- μ 1 cells required the expression of functional GSTM1, as shown by using curcumin or BSO, which significantly reduced GST activity in CAL1- μ 1 cells; these agents specifically reversed the protective effect of GSTM1 overexpression in CAL1- μ 1 cells, whereas they had no effect on control cells. This result is in agreement with previous report showing a correlation between GSTM1 expression level and chlorambucil resistance in ovarian A2780 cells selected by repeated exposure to this alkylating agent (Horton et al., 1999).

Chlorambucil was found to be efficiently protected by

GSTM1 expression in CAL1- μ 1 cells whereas melphalan was not. The explanation for the difference between these closely related nitrogen mustards is unknown. However, a similar discrepancy was previously reported by using MCF7 cells overexpressing GSTA1 (Morrow et al., 1998). Thus, as shown by Paumi et al. (2001) for GSTA1, a poorer catalytic efficiency of monogluthionyl melphalan formation by GSTM1 could explain the absence of effect of GSTM1 on the sensitivity to melphalan of CAL1- μ 1 cells.

The absence of effect of the MRP inhibitors on the chlorambucil sensitivity of either control or GSTM1-overexpressing CAL1 cells indicated that MRP1 was not involved in chlorambucil detoxification. This result may be surprising because the glutathione conjugate of chlorambucil was previously shown to be efficiently exported by MRP1 from GSTA1 overexpressing MCF7 cancer cells (Paumi et al., 2001). However, because the GSTM1 gene transfer failed to protect the same MCF7 cells from chlorambucil cytotoxicity (Morrow et al., 1998), it seems likely that the anticancer drug protection conferred by expression of GSTs and/or MRPs is mainly dependent on the cancer cell type. In agreement with O'Brien et al. (2000), we suggest that GSH conjugation may be sufficient for decreased chlorambucil toxicity, and the presence of the MRP1 affects cell survival only marginally.

A coordinated action of phase II drug conjugation (GSTM1/glutathione) and phase III drug/conjugate efflux (MRP1) in the detoxification process of vincristine has not been described previously. In particular, the data indicating the involvement of functional GST in the detoxification process against vincristine were contradictory. Thus, neither GSTP1 nor GSTM1 or GSTA1 was shown to confer any resistance to vincristine when expressed in the MRP1 expressing MCF7/VP cells (Morrow et al., 1998), whereas the addition of GSTP1 in NIH-3T3 cells was found to confer protection against vincristine (O'Brien et al., 2000). The data obtained from complementary viability, proliferation, survival, and tubulin network visualization assays showed that the overexpression of GSTM1 increased the vincristine resistance of CAL1- μ 1 cells. The specific reversal of the GSTM1-mediated protection in CAL1- μ 1 cells by dicumarol, curcumin and BSO indicated that GSTM1 enzyme activity was necessary for efficiently protecting cells from vincristine cytotoxicity. As suggested previously for GSTP1 (O'Brien et al., 2000), it is less likely that reactive oxygen species play an important role in vincristine toxicity; although glutathione conjugates of vincristine have not been described, GSTM1 could render the cells less sensitive to vincristine cytotoxicity by clearing the oxidized metabolites of the drug that could be generated by monoamine oxidases or cytochrome P450s (Rosazza et al., 1992).

Several studies argued for a potential role of GSH in the MRP-mediated protection against vinca alkaloids. On one hand, the glutathione dependence (cotransport with) of the transport of nonanionic compounds, such as unmodified vincristine, by MRPs was reported (Loe et al., 1998). On the other hand, a glutathione-associated drug export through MRP protein was also shown for another vinca alkaloid, vinblastine (Evers et al., 2000). Thus, a more complete protection against vincristine may be achieved in CAL1- μ 1 cells by a MRP1-mediated efflux of vincristine. To determine the role of endogenous MRP1 in cell resistance to vincristine, the effects of the MRP1 inhibitors sulfinpyrazone and verapamil

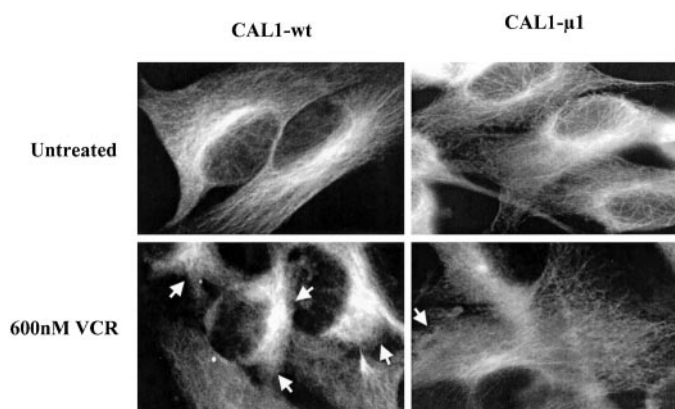


Fig. 7. Effect of GSTM1 on microtubule network alteration by vincristine in CAL1 cell lines. Cells, treated without or with 600 nM VCR for 20 min, were stained with anti- β -tubulin after fixation and microtubule network visualized by immunofluorescence microscopy as described under *Materials and Methods*. Arrows point to microtubule alterations.

were evaluated. As determined by viability and clonogenic assays, both inhibitors completely and specifically abolished the vincristine resistance of CAL1- μ 1 cells, whereas they had no effect on the control cell lines. The involvement of functional MRP1 in vincristine detoxification process was demonstrated by studying the accumulation of tritiated vincristine in CAL1 cell lines. The MRP1 inhibitor sulfinpyrazone strongly increased the accumulation of [3 H]vincristine in CAL1- μ 1 cells. Moreover, the increase of the accumulation by dicumarol indicated that the exclusion of vincristine from CAL1- μ 1 cells through MRP1 transporter was dependent on the presence of functional GSTM1. It can be noticed that the MRP1-mediated vincristine efflux in parental CAL1 cells, evidenced by the small but measurable increase of [3 H]vincristine accumulation by sulfinpyrazone in CAL1-wt cells, was not strong enough to protect cells from the vincristine cytotoxicity. In fact, the inhibition of MRP1 by sulfinpyrazone did not significantly increase the vincristine sensitivity of parental cells. Taken together, these data suggested a coordinated action of GSTM1 and MRP1 in vincristine detoxification in melanoma CAL1- μ 1 cells.

Vincristine, but not vinblastine, was protected by GSTM1 expression in the MRP1 expressing CAL1- μ 1 cells. The reason for this discrepancy is not known. It was reported previously that the efflux of vinca alkaloids was mediated through specific ABC transporters: vincristine was preferentially excluded from cells through MRP1 (Rajagopal et al., 2002) and vinblastine through MRP2 (Evers et al., 2000) transporters. In agreement with these data, we observed that the inhibition of MRP1 by sulfinpyrazone had no effect of the vinblastine sensitivities of the different CAL1 cell lines (data not shown). Thus, the absence of MRP2 in CAL1 cells could be responsible for the lack of effect of GSTM1 overexpression on the sensitivity of CAL1- μ 1 to vinblastine. Alternatively, those compounds cannot be oxidized to metabolites that can be combined to GSH and thus excluded from CAL1 cells by MRP1.

In conclusion, we report that GSTM1 can act in synergy with MRP1 to protect CAL1 cancer cells from vincristine cytotoxicity, whereas GSTM1 alone is sufficient to confer resistance to chlorambucil. The coordinated action of phase II GST-dependent and phase III MRP-dependent detoxification processes seems to be highly specific, on one hand for the drug/GST isozyme/MRP isozyme combination and on the other hand for the cell or tissue concerned. Moreover, these data provide rationale for studying the relationship between the expression levels of GSTM1 and MRP1 in malignant melanoma tumors and the clinical outcome of patients treated with alkylating agents or vinca alkaloids.

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