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Regulation of cellular glutathione modulates nuclear accumulation of daunorubicin in human MCF7 cells overexpressing multidrug resistance associated protein

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Abstract

Multidrug resistance (MDR) is frequently associated with the overexpression of P-glycoprotein (Pgp) and/or multidrug resistance associated protein (MRP1), both members of the ABC superfamily of transporters. Pgp and MRP1 function as ATP-dependent efflux pumps that extrude cytotoxic drugs from tumour cells. Glutathione (GSH) has been considered to play an important role in the MRP1-mediated MDR. In our study, we examined the effects of buthionine sulphoximine (BSO), an inhibitor of GSH biosynthesis, on the nuclear accumulation of daunorubicin (DNR), in etoposide (VP16) and doxorubicin (ADR) resistant MCF7 cell lines, overexpressing respectively MRP1 (MCF7/VP) and Pgp (MCF7/ADR). The study of DNR transport was carried out using scanning confocal microspectrofluorometry. This technique allows the determination of the nuclear accumulation of anthracyclines in single living tumour cells. Treatment of MCF7/VP cells with BSO increased the sensitivity of these cells to DNR whilst the cytotoxicity of the drug in MCF7/ADR cells remained unchanged. In MCF7 resistant cells treated with BSO, their GSH level decreased as observed by confocal microscopy. DNR nuclear accumulation in MCF7/VP cells was increased by BSO whereas in MCF7/ADR cells BSO was unable to significantly increase the DNR nuclear accumulation. These data suggest a requirement for GSH in MRP1-mediated resistance whilst the nuclear efflux of GSH conjugates is probably not the primary mechanism of Pgp-mediated MDR. Finally, BSO might be a useful agent in clinical assays for facilitating detection of MRP1 expression.

Keywords: MRP1; P-glycoprotein; Glutathione; Buthionine sulphoximine; Anthracyclines; Microspectrofluorometry; Confocal microscopy; RT-PCR; Flow cytometry

1. Introduction

A major obstacle impeding the success of chemotherapy is multidrug resistance (MDR). MDR is caused in part by overexpression of the *mdr1* gene [1], which codes for P-glycoprotein (Pgp), a 170 kDa transmembrane protein, which may function as an energy-dependent drug efflux pump [2]. Another MDR mechanism involves overexpression of the multidrug resistance associated protein (MRP1), which has been identified as a 190 kDa glycoprotein [3]. The mechanism of action of MRP1 is less well defined than that of Pgp, although overexpression of MRP1 has been associated with decreased drug cellular accumulation [4]. In recent studies, we have shown that inhibition of H^+-ATPase function induces a sensitisation of cells overexpressing MRP1 to daunorubicin (DNR) but not those overexpressing Pgp [5,6]. Detoxification of the products of xenobiotic metabolism involves a specific transport system that functions to protect the body from harmful compounds. The glutathione (GSH) and glutathione-S transferase (GST_s) play a primary role in cellular detoxification [7]. Many studies indicate that GSH is a critical determinant in tumour cell resistance [8] by interacting with a wide range of drugs which become conjugated to glutathione S-conjugates [9]. However, several studies have shown that treatment of tumour cells with buthionine sulphoximine (BSO), an inhibitor of GSH biosynthesis, resulted in a complete reversal of resistance to anticancer drugs of some cell lines overexpressing MRP1, but has no effect on Pgp-mediated MDR [8,10].

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In order to understand the mechanisms of DNR transport by MRP1 and Pgp proteins, we have examined the effects of BSO on the cytotoxicity and nuclear accumulation of DNR using scanning confocal microspectofluorometry. Anthracycline distribution in resistant tumour cells has been reported in several studies using confocal microscopy [11]. However, distinction between cytoplasmic and bound nuclear anthracycline can only be made by using techniques like microspectrofluorometry or spectrofluorometry [12,13].

This work was carried out on MCF7 cells overexpressing MRP1 (MCF7/VP) and Pgp (MCF7/ADR) [14–16]. These cells are resistant to many structurally and functionally dissimilar cytotoxic agents, such as mitoxantrone (MTX), doxorubicin (ADR), teniposide (VM26) and daunorubicin (DNR) [5].

2. Materials and methods

2.1. Drugs and chemicals

DNR, BSO and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma (St Quentin Fallavier, France). ADR and etoposide (VP16) were respectively purchased from Farmitalia (Milan, Italy) and Sanofi Winthrop (Gentilly, France).

2.2. Cell lines

The parental MCF7 (MCF7/WT) is a human breast cancer cell line. The multidrug-resistant subclones MCF7/ADR and MCF7/VP cells were kindly supplied by J. Robert (University of Bordeaux, France).

2.3. Culture conditions

MCF7/VP and MCF7/ADR cells were isolated by stepwise selection in increasing concentrations of VP16 and ADR, respectively. MCF7/VP and MCF7/ADR cells were maintained by exposure to 1 μM VP16 and ADR, respectively [14,16].

MCF7 cells were grown in a 5% CO_2 atmosphere at 37°C, and RPMI-1640 medium (Gibco, Paris, France) supplemented with 10% fetal calf serum (Gibco) and 2 mM L-glutamine (Sigma).

2.4. MTT cytotoxicity assay

Cytotoxicity experiments were performed using the 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyl-tetrazolium bromide (MTT) assay [17]. Cells (25 000/ml) were treated for 2 h with non-toxic concentrations of BSO, washed and left for 24 h in fresh medium before treatment with DNR (0.01–50 μM) at 37°C for 2 h. Cells were washed and seeded in 96-well microplates for 72 h. A solution of MTT (20 μl, 2.5 mg/ml) was added to each well. After 3 h, the medium was then discarded and 200 μl of DMSO were added to each well. Optical densities were measured at 540 nm using a 'series 750 microplates reader' (Cambridge Technology, Watertown, MA, USA). The 50% inhibitory concentration (IC_{50}) was determined as the drug concentration which resulted in a 50% reduction in cell viability. Resistance index (RI) was calculated by dividing the IC_{50} obtained for the resistant subline by the IC_{50} obtained for the parental cell line.

2.5. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total cellular RNA was prepared using the guanidine isothiocyanate/acid/phenol method [18]. mdr1, mrp1 and gapdh transcripts were analysed by semi-quantitative RT-PCR using the following gene-specific oligonucleotide primers: MDR1 forward primer, 5′-CCCATCATTGCAATAGCAGG-3′; MDR1 reverse primer, 5′-GTTCAAACTTCTGCTCCTGA-3′; MRP1 forward primer, 5′-TCTCTCCCGACATGACCGAGG-3′; MRP1 reverse primer, 5′-CCAGGAATATGCCCC-GACTTC-3′; GAPDH forward primer, 5′-TGG-GGAAGGTGAAGGTCGGA-3′; GAPDH reverse primer, 5′-GAAGGGGTCATTGATGGCAA-3′ [19]. The *gapdh* gene was used as an internal control. In brief, reverse transcription (RT) was performed using 1 μg of total RNA. Total cytoplasmic RNA was reverse transcribed using Moloney Murine Leukemia Virus reverse transcriptase (M-MLV reverse transcriptase, Gibco) according to the manufacturer's instructions. Aliquots representing 1/50 of cDNA template were diluted to 100 μl in Taq polymerase buffer containing 0.25 mM dNTP, 100 pmoles of each primers, 1.5 U of DNA polymerase (Goldstar, Eurogentec Seraing, Belgium). PCR conditions were 94°C for 3 min followed by 32 cycles of 94°C for 45 sec, 61°C for 45 sec and 72°C for 90 sec then 1 cycle of 72°C for 10 min. Following PCR, aliquots (10 μl) were subjected to electrophoresis on 2% agarose gels and bands were visualised by ultraviolet (UV) transillumination using ethidium bromide staining prior to photography. Densitometry was performed using IMAGER of APPLIGEN ONCOR and the ratio between the target and control PCR products, for each cDNA sample, was determined by dividing the densitometric volume of the target electrophoretic band by that of the control band.

2.6. Pgp and multidrug resistance associated protein expression

2.6.1. Pgp

For flow cytometric determination of cell-surface Pgp, MCF7 cells were washed and incubated for 30 min at

4°C in the presence of 50 μl of 10 μg/ml of the MAb MRK16 (Valbiotech, Paris, France) which recognises an external epitope of Pgp. After washing with ice-cold phosphate buffer solution (PBS) containing 1% bovine serum albumin (BSA) (Sigma), cells were incubated for 30 min at 4°C with the F(ab′) fragment of goat anti-mouse IgG fluorescein-conjugate (Sigma) used at a working dilution of 1:50.

2.6.2. Multidrug-resistance associated protein

MCF7 cells were permeabilised in 15% (v/v) lysing solution G (Becton Dickinson, Mountain View, CA, USA) in H_2O and incubated for 15 min in PBS/BSA containing 1% (v/v) normal goat serum. Cells (5×10^5) were incubated for 1 h at 4°C in 100 μl PBS/BSA 5% containing either the monoclonal antibody MRPm6 at 2 μg/ml (provided by G.J.R. Zaman, University of Amsterdam, The Netherlands) or the mouse isotype-matched control MAbs. Antibody binding was detected with fluorescein-labelled goat anti-mouse immunoglobins (Immunotech, Marseille, France) in accordance with the consensus recommendations of Beck and colleagues [20].

After washing, the cells were immediately analysed with a FACScan flow cytometer (Becton Dickinson, Le Pont de Claix, France). The excitation was an argon ion laser emitting at 488 nm. Fluorescence emission was collected after passage through a 530 nm band pass filter. Data were collected and analysed on a Hewlett-Packard model 310 computer interfaced with the FACScan. The green fluorescence was measured on a logarithmic scale.

2.7. Confocal fluorescence imaging of intracellular glutathione

For evaluation of intracellular GSH, the MCF7 cells were treated with mercury orange (50 μM) at 37°C for 15 min. The problem with mercury orange is that this stain can also label other intracellular thiols, particularly protein sulphhydryls [21]. After 24 h of treatment with 100 μM BSO, cells were exposed to mercury orange, washed and examined at room temperature with a MRC-1024 confocal system (Bio-Rad, Microscience, Hemel Hempstead, UK). The scanning confocal system MRC-1024 was coupled to an Optiphot epifluorescence microscope (Olympus, Tokyo, Japan) equipped with a 60× magnification water immersion objective of numerical aperture N.A. = 1.4. Mercury orange–glutathione fluorescence was excited using the 488 nm argon ion laser line and detected by a long pass filter above 560 nm.

2.8. Confocal laser scanning microspectrofluorometry

Fluorescence emission spectra from a microvolume of living cells were recorded using confocal laser microspectrofluorometry (Dilor, Lille, France). An optical microscope Olympus BX40 equipped with 100× phase contrast water-immersion objective (Olympus UVFL 100PL, Tokyo, Japan) allowed the observation of the sample, the focusing of a 4 μW laser beam emitting at 457.9 nm (2065A model, Spectra Physics, Les Ulis, France) and the collection of the fluorescence emission in the 500–700 nm range through the same optics. The emission from a point is deflected onto the entrance slit of the spectrograph where the emission signal is projected onto a CCD detector (Wright, Stonehouse, UK).

2.9. Nuclear drug concentration determination

The fluorescence emission spectrum from nuclei of treated cells $F(\lambda)$, can be expressed as a sum of the spectral contributions of free DNR, DNA-bound DNR and signal of nuclear autofluorescence [22], according to the following equation:

$$F(\lambda) = C_f \cdot F_f(\lambda) + C_b F_b(\lambda) + C_n \cdot F_n(\lambda)$$

where F_f and F_b are the fluorescence spectra of free and bound drug referred to a unitary concentration. Taking this concentration into account, C_f and C_b represent intranuclear concentration of free and bound drug, respectively, C_n is the contribution of autofluorescence responsible for the intrinsic nuclear spectrum F_n. In aqueous solution, each of these contributions has a characteristic spectral shape. The fluorescence yield in the free form was 40 times higher than that of the bound DNA form. These spectral contributions lead to the concentrations of free and DNA-bound DNR. The sum of the values obtained gives the total nuclear concentration of DNR [22–25]. The validity of the method has been established with ^{14}[C]-doxorubicin [22]. A mapping of the fluorescence response of DNR inside the cell nucleus was obtained by focusing the laser beam in different microvolumes of the same nucleus. The drug concentration was constant within 10%. When the spectra from each sample (30–50 cell nuclei) were analysed, the standard deviation was calculated to be 20% of the mean value. Data presented are the mean ± standard deviation of at least three independent experiments. Mean values of nuclear DNR concentrations between different cell treatments were tested by a paired student *t*-test.

3. Results

3.1. RT-PCR analysis

In order to determine whether resistance of MCF7/VP and MCF7/ADR cells was mediated by Pgp and/or MRP1, expression of *mdr1* and *mrp1* genes was studied using the RT-PCR technique (Fig. 1). The ratio between

the target electrophoretic band and the control band measured by densitometry shows that MCF7/VP and MCF7/ADR cells expressed high and moderate amounts of the *mrp1* gene (0.85 and 0.5, respectively). The *mdr1* gene was highly expressed in MCF7/ADR cells (1.1). MCF7/WT and MCF7/VP cells did not express the *mdr1* gene.

3.2. Flow cytometry analysis

Results of MRP1 and Pgp protein expression are shown in Table 1. Flow cytometry determination of MRP1 and Pgp shows relatively high (MCF7/VP cells) and moderate (MCF7/ADR cells) expression of MRP1 (3.5 ± 0.3 and 1.5 ± 0.2, respectively). MCF7/ADR cells expressed much higher amounts of Pgp (12.9 ± 2.5) whilst this protein was not expressed in MCF7/VP cells. However, we were unable to detect Pgp and MRP1 proteins in MCF7/WT cells (Table 1).

3.3. Effects of BSO on DNR cytotoxicity in MCF7 cells

The effects of BSO on DNR cytotoxicity in MCF7/VP- and MCF7/ADR-resistant cells were also investigated and results are shown in Table 2. MCF7/VP and MCF7/ADR cells were 3.1 ± 0.9 and 25 ± 0.3 fold resistant to DNR, respectively. Incubation of MCF7/VP cells with 10 μM BSO, caused a significant decrease in the resistance index value for DNR in these cells (1.5 ± 0.2). 50 and 100 μM BSO decreased the level of DNR cytotoxicity in MCF7/VP cells to that in the MCF7/WT cells. BSO at 10, 50 and 100 μM, had no effect on DNR cytotoxicity in MCF7/ADR cells (Table 2). Treatment of MCF7/VP cells with BSO completely sensitised these cells to VP16 (data not shown).

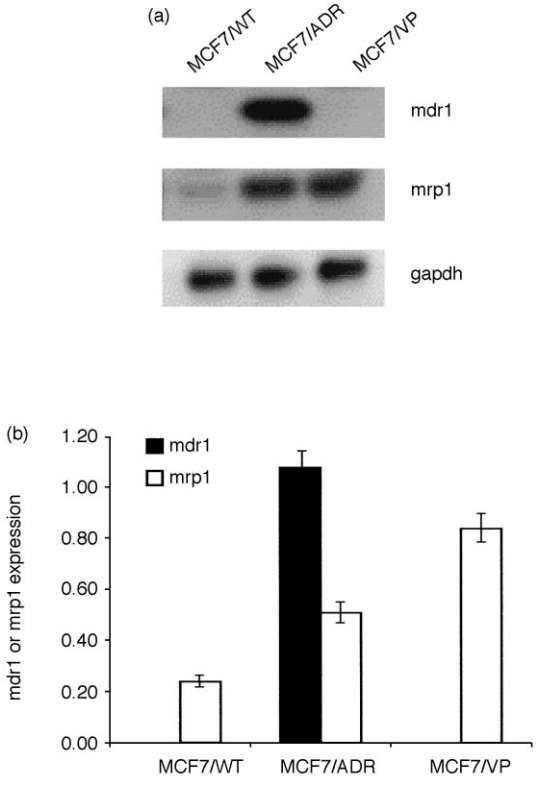

Fig. 1. (a) Quantitative PCR assay for mdr1 and mrp1 in sensitive parental cells (MCF7/WT) and resistant cells overexpressing Pgp (MCF7/ADR) and MRP1 (MCF7/VP). (b) Quantification of PCR (arbitrary unit). The ratio between mdr1, mrp1 and gapdh mRNA expression represents the mean of at least three experiments.

Table 1
Analysis of MRP1 and Pgp protein expression in sensitive and resistant MCF7 cells by flow cytometry[a]

Cell lines	Protein expression measured by flow cytometry	
	MRP1	Pgp
MCF7/WT	1.1 ± 0.2	1 ± 0.2
MCF7/ADR	1.5 ± 0.2	12.9 ± 2.5
MCF7/VP	3.5 ± 0.3	1.2 ± 0.2

[a] Protein values were expressed after adjustment for control values and are represented as the ratio of mean fluorescence of MAb MRK16/IgG2a control or MRPm6/IgG1 control. Data represent the mean ± standard deviation (S.D.) of at least three experiments.

3.4. Confocal fluorescence imaging of intracellular glutathione

Treatment of MCF7 cells with mercury orange shows that in MCF7/ADR cells, a high fluorescence intensity of mercury orange–glutathione complex in the nuclei (Fig. 2c) whilst the fluorescence is localised mainly in the cytoplasm of MCF7/WT and MCF7/VP cells (Fig. 2a, b). Incubation of MCF7/VP (Fig. 2e) and MCF7/ADR (Fig. 2f) cells with 100 μM BSO for 24 h reduced mercury orange–glutathione fluorescence intensity in these cells when compared with the control cells (Fig. 2b and c, respectively). 100 μM BSO had no effect on mercury orange–glutathione fluorescence in MCF7/WT cells (Fig. 2d).

Table 2
Effect of BSO on cytotoxicity of DNR in MCF7/VP and MCF7/ADR cells[a]

BSO concentration (μM)	0	10	50	100
MCF7/VP	3.1 ± 0.9	1.5 ± 0.2	1.1 ± 0.1	1 ± 0.1
MCF7/ADR	25 ± 0.3	26.5 ± 0.1	25.8 ± 0.3	24.9 ± 0.2

[a] The three-step MTT cytotoxicity assay was carried out. Values for resistance indices (RI) represent the mean ± standard deviation (S.D.) of at least three experiments.

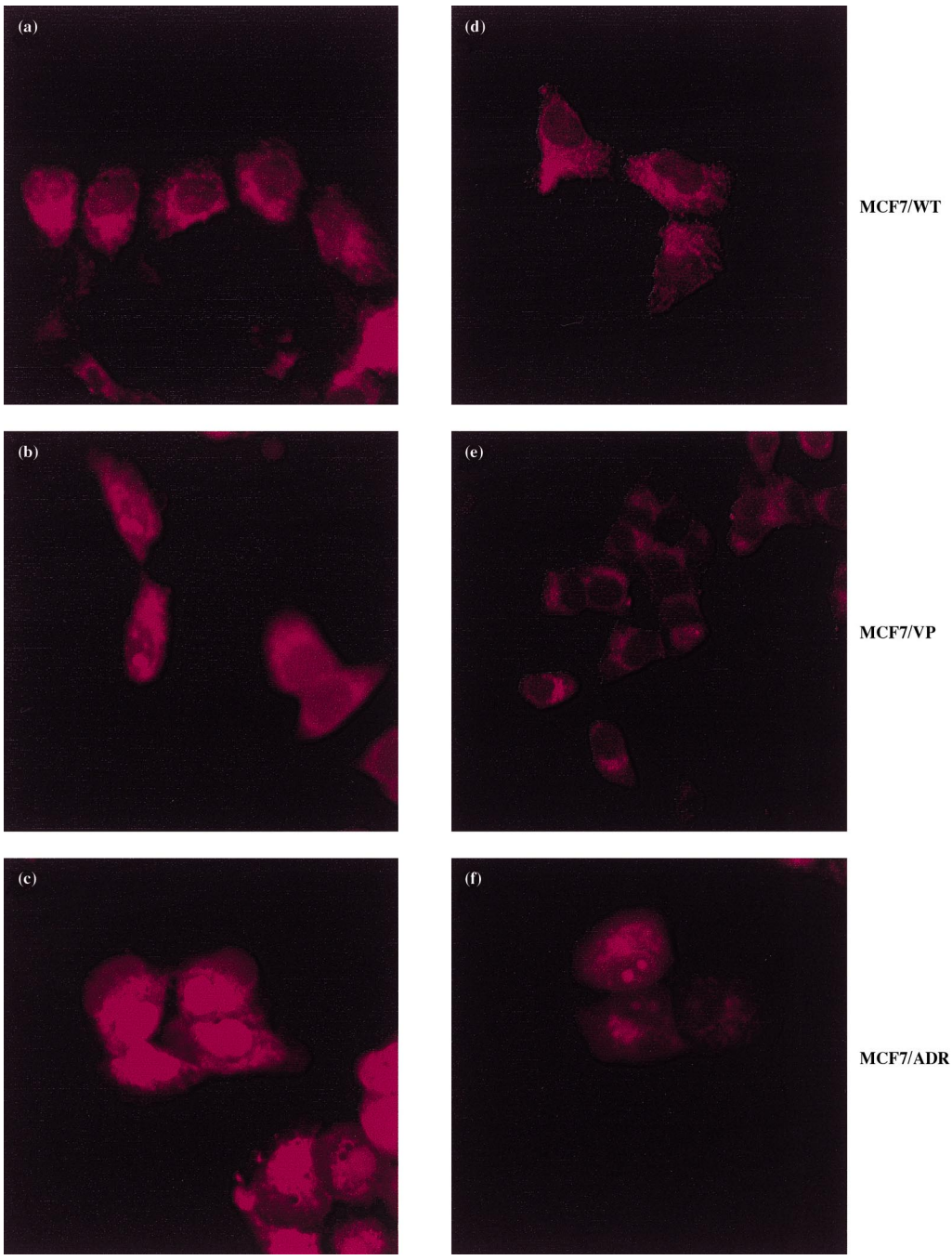

Fig. 2. Effect of BSO on intracellular distribution of mercury orange–glutathione fluorescence in MCF7 cells. Cells treated with BSO and mercury orange were examined by confocal microscopy.

3.5. Effects of various concentrations of BSO on nuclear accumulation of DNR in MCF7 cells

Various concentrations of BSO (3–100 μM) did not increase the nuclear accumulation of DNR in MCF7/WT and MCF7/ADR cells (Fig. 3). Treatment of MCF7/VP cells with BSO at 3 or 10 μM enhanced the nuclear accumulation of DNR in these cells. BSO, at 50 or 100 μM, increased DNR nuclear accumulation in MCF7/VP cells to a level equal to that of MCF7/WT cells (Fig. 3).

4. Discussion

Multidrug resistance (MDR), especially that associated with overexpression of the *mdr1* gene and its product Pgp, is thought to play a role in the outcome of

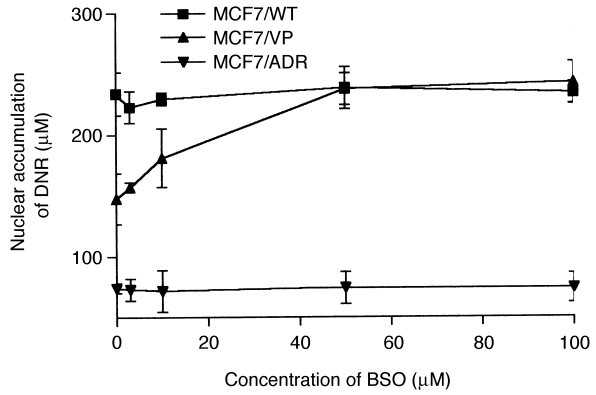

Fig. 3. Effect of BSO (3–100 μM) concentrations on nuclear accumulation of DNR in MCF7/WT, MCF7/VP and MCF7/ADR cells. A two-step procedure consisted of: (a) treatment with BSO for 2 h, cells were washed and left for 24 h in fresh medium; (b) treatment with 1 μM DNR for 2 h, cells were washed in cold PBS before microspectrofluorometric analysis. Data are the mean ± standard deviation (S.D.) of at least three independent experiments.

therapy for some human tumours. More recently, other drug resistance proteins, notably MRP1, have also been implicated in multidrug resistance [3]. The role and function of this protein are still under discussion. Consequently, functional tests play a major part in the understanding of the MDR phenotype.

Several dyes (Rhodamine 123, $DiOC_2$) and drugs (daunorubicin, doxorubicin) may be used to assess Pgp function [26]. Pgp-mediated MDR can be reversed by resistance-modifying agents such as verapamil, cyclosporin A and PSC-833. However, these resistance modifiers are usually less effective in reversal of MRP1-mediated MDR [27].

Different compounds and modulators are available to study MRP1 activity. Calcein acetoxymethyl ester (calcein-AM) is a non-fluorescent compound, which permeates the plasma membrane, and upon cleavage of the ester bonds by intracellular esterases is transformed into a fluorescent compound which might be a substrate for MRP1 but not for Pgp [28]. MK-571, a leukotriene D_4 receptor antagonist [29], probenecid, an inhibitor of organic anion transport [30], genistein, a specific inhibitor of tyrosine kinases [31] and 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole, a H^+-ATPase pump inhibitor [5].

GSH is widely distributed in animals, plants and microorganisms [32]. Cellular GSH plays a primary role in cellular detoxification of electrophilic compounds. Moreover, in previous studies, elevated levels of GSH, together with increased activities of GST_s, may protect cells from cytotoxic drugs [9]. GST_s catalyses the conjugation reaction between reduced GSH and many xenobiotics [33]. Elimination of the GS-X requires a specific ATP-dependent the GS-X export pump termed the GS-X pump [34]. Increased expression of MRP1 is associated with increased ATP-dependent transport of GS-X into isolated membrane vesicles [35]. These data are consistent with MRP1 being a GSH conjugate transporter. In many studies, treatment of cells with BSO, an inhibitor of γ-glutamylcysteine synthetase, resulted in the depletion of GSH and increased sensitivity of cells to several drugs [10].

In our study, in order to understand whether GSH plays an important role in Pgp- and MRP1-mediated MDR, we have examined the effect of BSO on DNR cellular distribution. The results obtained indicate that BSO was able to increase nuclear accumulation of DNR in cells overexpressing MRP1 (MCF7/VP) but had no effect in cells that expressed especially Pgp (MCF7/ADR). Moreover, BSO caused significant sensitisation of MCF7/VP cells to DNR whereas the cytotoxicity of the drug in MCF7/ADR cells remained unchanged. These data suggest a requirement for GSH in MRP1-mediated drug resistance whilst the efflux of GSH conjugates is probably not the primary mechanism of Pgp-mediated MDR. Finally, BSO might be a useful agent in clinical assays for facilitating detection of MRP1 expression [20,36].

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