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MRP1-Transfected Cells do not Show Increased Resistance Against Oxidative Stress

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Sensitivity of V79 Chinese hamster cells and V79 cells transfected with human *MRP1* gene to several agents inducing oxidative stress was compared. Cells overexpressing MRP1 did not show increased resistance to *tert*-butyl hydroperoxide, diamide, paraquat, menadione, dichromate and carmustine as estimated by cell survival and DNA damage assessed by comet assay. These findings suggest that overexpression of MRP1 does not confer increased resistance to oxidative stress.

Keywords: MRP1; Multidrug resistance protein; Glutathione; Oxidative stress

Abbreviations: tBOH, *tert*-butyl alcohol; tBOOH, *tert*-butyl hydroperoxide; DMSO, dimethylsulfoxide; EDTA, ethylenediaminetetraacetate; GSH, glutathione; GSSG, glutathione disulfide; MOPS, 3-[*N*-morpholino]propanesulfonic acid; MRP, multidrug resistance(-associated) protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide); PBS, phosphate-buffered saline

INTRODUCTION

The subfamily of human multidrug resistance (-associated) proteins (MRPs) comprises at least 9 members [1–4]. As ABC (ATP-Binding Cassette) proteins, they possess nucleotide binding folds with conservative amino acid motifs, typical for this superfamily. The amino acid sequence identity among the members of this group varies from 30 to 60%. Probably all MRPs are glycosylated plasma membrane transporters extruding their substrates outside the cytoplasm with the expenditure of energy from ATP hydrolysis. The representatives of the group are found in almost every tissue and organ tested, with MRP1 and MRP5 the most ubiquitously distributed (for a review see Refs. [5–8]). The substrate spectrum of MRPs is broad and covers leukotriene C_4, other glutathione S-conjugates, glutathione disulfide, glucuronates, sulphates, short chain lipid analogues and large anionic molecules like fluorescent dyes calcein or Fluo-3 (for a review of well defined substrates of MRP1, MRP2 and MRP3, see Refs. [6,9]). The reduced glutathione has been proven to be co-transported with some natural substrates [10] but there is also a possibility that GSH can be a substrate itself, at least for some MRPs [11]. The latter is confirmed by the observation that in cells overexpressing MRP1, MRP2 or MRP5, the intracellular level of GSH is diminished (for a discussion see Ref. [5]). The fact that GSSG is a substrate for MRP1, MRP2, MRP3 and MRP5 [7,8,12,13] and that those proteins are identified as the "glutathione *S*-conjugate pumps" [14,15] suggests the role of MRPs in cell protection against oxidative stress. This suggestion has been strengthened by demonstration that oxidative stress induces expression of MRP1 [16,17] and of other members of the MRP family [18]. Furthermore, transport of GSSG mediated by the rat orthologue of MRP1 was observed in cultured astrocytes exposed to hydrogen peroxide [19].

Formation of glutathione disulfide (GSSG) is one of the consequences of oxidative stress. Low levels of

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GSSG formed are reduced back by glutathione reductase but, when the cellular reducing capacity is exceeded, MRP1 or MRP2 (and apparently also other MRPs like MRP3 and MRP5) actively export GSSG. The export is believed to be of considerable physiological importance since accumulation of GSSG has deleterious effects on cellular metabolism [20–22]. All these data suggest that MRP1 (as well as some other members of the MRP family) may play a role in cellular protection against oxidative stress. If so, cells overexpressing this protein should exhibit increased resistance to various oxidative stress-inducing agents. The aim of this study was to compare the sensitivity of two cell lines to various types of oxidants: V79 Chinese hamster cells and MRP1-transfected V79MRP1 cells.

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MATERIALS AND METHODS

Reagents

Cell culture media and fetal calf serum were from Gibco™ (Invitrogen, Carlsbad, USA). Anti-MRP1 antibody MRPr1 was obtained from Alexis (Läufelfingen, Switzerland). Zeocin® was from Invitrogen (Carlsbad, USA). [^{3}H]-labeled glutathione was obtained from NEN (Boston, USA). [^{3}H]-labeled glutathione disulfide was synthesized according to Akerboom and Sies [23]. All other reagents were from Sigma (Deisenhofen, Germany).

Cell Culture Conditions

V79MRP1 transfectant cell line was developed from the V79 Chinese hamster lung fibroblast cell line as previously described [24]. The cells were grown in Dulbecco's modified Eagle medium (DMEM) containing glucose (4500 mg/l), sodium pyruvate and pyridoxine, supplemented with 10% (v/v) heat-inactivated bovine calf serum, penicillin (10 U/l) and streptomycin (10 mg/l). MRP1-transfected cells were grown in the presence of Zeocin® (0.25 mg/ml) as a selecting agent. Cells were cultured at 37°C in the atmosphere of 5% CO_2.

Evaluation of Drug Sensitivity

Drug sensitivity of cells was determined using the microtiter plate MTT assay [25]. Cells were seeded on 96-well plates at the concentration of 10^3 cells per well in 100 μl volume of complete medium. After 24-h incubation at 37°C, the tested compound was added and the incubation was continued for 72 h under the same conditions. The cell sensitivity factor (IC_{50}) was defined as the concentration of drug required to reduce cell survival down to 50%. As in the presence of some redox-active agents (e.g. superoxide anion produced by menadione) MTT may be reduced excessively, we compared the survival of cells treated with menadione evaluated both with MTT and by acid phosphatase release, finding no significant differences in IC_{50} values (data not shown). Therefore, the MTT method was used throughout this study.

Alkaline Comet Assay

For the alkaline assay the cells were processed as described previously [26]. Briefly, equal volumes of cell suspension (4×10^5 cells/ml) were mixed with low melting point agarose Type VII at a final concentration of 1%. The suspensions were cast on microscope slides pre-coated with 0.5% regular agarose Type IA and allowed to set under cover slips on an ice-cooled metal plate. After solidification, the cover slips were removed and the slides placed in the lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10 and 1% Triton X-100) for 1 h at 4°C. Thereafter, the slides were placed in a horizontal gel electrophoresis unit filled with fresh electrophoretic buffer (1 mM Na_2EDTA and 300 mM NaOH, pH $>$ 13) and left in this buffer for 40 min for DNA unwinding. The slides were then electrophoresed for 30 min at 25 V (1.2 V/cm, 42–44 mA) at 8°C without changing the alkali solution. Pictures of 50 randomly selected comets per slide, from 2 slides in 3 separate experiments were captured at 200 × magnification using an epifluorescence microscope (Labophot-2, Nikon) equipped with an UV-1A filter block (excitation filter of 365/10 nm and a barrier filter of 435 nm). Image analysis of the data was done using the Comet v.3.1 (Kinetic Imaging Ltd., Liverpool, UK). The measure of damage was the tail moment (fraction of DNA in the tail multiplied by tail length).

Measurement of Bimane-*S*-glutathione Transport

The transport of bimane-*S*-glutathione was measured according to Pułaski and Bartosz [27], with minor changes. V79 and V79MRP1 cells were seeded on 6-well plates at a cell concentration of 10^6/well in 1 ml volume of complete medium. After 4–5 h (time allowing the cells to attach), the medium was removed and cells were washed with incubation buffer (138 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose buffered with 8 mM phosphate buffer, pH 7.4). One millilitre of ice-cold incubation buffer supplemented with 1 μM monochlorobimane was added and the cells were incubated for 10 min on ice to allow the dye precursor to enter the cytoplasm. The buffer was replaced with a fresh portion and the cells were

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placed at 37°C. Aliquots of 1 ml were taken at different time intervals, spun down to sediment possible cell residues and fluorescence of the supernatant was measured (excitation: $\lambda = 386$ nm, emission: $\lambda = 476$ nm).

Preparation of Plasma Membrane Vesicles

Plasma membrane vesicles from V79MRP1 and control V79 cells were prepared from hypotonically lysed cells as described by Keppler *et al.* [28] Twenty four hours before harvesting, the cells were cultured in the presence of 2 mM sodium butyrate, an inhibitor of histone deacetylase, to enhance MRP1 expression. Proteinase inhibitors (0.3 μM aprotinin, 0.1 μM leupeptin, 0.1 mM phenylmethylsulfonyl fluoride (PMSF)) were used in all solutions used for membrane preparation. Isolated membrane vesicles were snap-frozen in liquid nitrogen and stored at −70°C.

SDS-polyacrylamide Gel Electrophoresis and Western Blotting

Membrane proteins (10 or 15 μg per well) were separated in polyacrylamide gel (3% stacking gel and 9% separating gel) according to Laemmli [29] in the presence of 0.1% sodium dodecyl sulfate (SDS) and β-mercaptoethanol. Electrophoresis was run at 120 V. For Western blotting, proteins were electro-transferred to Immobilon at 370 mA for 90 min. The blots were incubated with MRPr1 antibody diluted 1:5,000. Anti-rat horseradish peroxidase conjugated-IgG was used as the secondary antibody at a dilution of 1:20,000. The protein was visualized with a standard enhanced chemiluminescence system.

GSSG Accumulation in Plasma Membrane Vesicles

Vesicles (20–30 μg protein) were incubated at 37°C in presence of 100 μM GSSG (including [^{3}H]-labeled GSSG), 250 mM sucrose, 1 mM $MgCl_2$, 10 mM creatine phosphate, 1 U/ml of creatine phosphokinase, 10 mM Tris–HCl pH 7.4 with 1 mM ATP or AMP in control samples. Aliquots were taken at different time intervals (1, 2, 3 and 5 min) and the accumulation of the substrate was stopped with 1 ml of ice-cold 250 mM sucrose, 10 mM Tris–HCl pH 7.4 buffer. Rapid filtration technique was applied to determine the accumulation of GSSG into plasma membrane vesicles [30]. The difference in accumulation of GSSG in presence of ATP and AMP was considered as the ATP-dependent accumulation.

Measurement of Enzyme Activities

The cells cultured in flasks were rinsed with PBS, trypsinized, rinsed three times with PBS and lysed in 1% Triton X-100, 0.25 M sucrose and 10 mM Tris–HCl pH 7.4. Extracts were centrifuged at 1000*g* at 4°C for 5 min. The supernatants were used for enzyme activity assays.

Glutathione peroxidase activity was quantified spectrophotometrically by measuring the loss of NADPH absorbance at 340 nm [31]. The reaction mixture contained 0.15 mM NADPH, 0.24 U/ml glutathione reductase, 1 mM reduced glutathione (GSH) and 100 μl of the supernatant in 1 ml (final volume) of 0.05 M phosphate buffer, pH 7.0, with 0.05 mM EDTA. The reaction was initiated by addition of *tert*-butyl hydroperoxide to a final concentration of 1.2 mM.

Glutathione reductase activity was estimated by measurement of NADPH consumption at 340 nm. The reaction mixture contained 100 μl of the supernatant, 1 mM GSSG, 0.1 mM NADPH and 0.1 M phosphate buffer, pH 7.6, containing 0.5 mM EDTA in a final volume of 1.0 ml. A millimolar extinction coefficient of $\varepsilon_{340} = 6.22$/mM/cm for NADPH was used [32].

Glutathione S-transferase activity was assayed by measuring at 340 nm the rate of GSH conjugation with 1-chloro-2,4-dinitrobenzene at 30°C. The reaction mixture was brought to 0.1 M phosphate buffer, pH 6.5 with 1.0 mM GSH, 1.0 mM 1-chloro-2,4-dinitrobenzene in a final volume of 1.0 ml. A millimolar absorption coefficient of $\varepsilon_{340} = 9.6$/mM/cm was assumed for 2,4-dinitrophenyl-*S*-glutathione [31].

Superoxide dismutase activity was assayed by the epinephrine method [33]. The reaction mixture contained 0.33 mM epinephrine in 50 mM carbonate buffer, pH 10.2, with 100 μM EDTA. Absorbance was read at 480 nm.

The total *glutathione content* was measured with glutathione reductase and 5,5′-di(thionitrobenzoic acid) (DTNB) [34]. Briefly, cells were extracted with 2 M $HClO_4$ with 4 mM EDTA. Extracts were centrifuged at 5000*g* for 5 min, the supernatants were neutralized with 1 M KOH contained 0.3 M MOPS. The samples contained 50 μl of NADPH (4 mg/ml in 0.5% (w/v) $NaHCO_3$), 20 μl of DTNB (1.5 mg/ml in 0.5% (w/v) $NaHCO_3$), 20 μl of glutathione reductase (6 U/ml) and the cell extract containing 0.5–1 nmole of glutathione in a total volume of 1 ml made up with 0.1 M phosphate buffer containing 0.1 mM EDTA, pH 7.0. Absorbance increase at 412 nm was monitored.

The results are presented as mean ± SD of at least three independent experiments. Statistical analysis was performed using the paired Student's "*t*" test.

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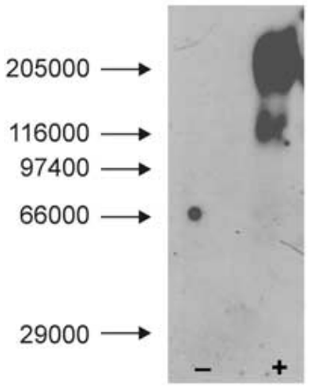

FIGURE 1 Western blots of membranes of V79 parental cell line (−) and transfected V79MRP1 cells (+) incubated with MRPr1 antibodies. 10 μg of protein applied per lane. The arrows point at the respective molecular weight markers positions.

RESULTS AND DISCUSSION

Figure 1 documents expression of MRP1 in the cellular membranes of transfected V79MRP1 cells and the lack of this protein in the parental cell line V79. It is in line with observations of Cuff *et al.* for cellular localization of MRP1 protein in several MRP1-transfected V79 clones [24].

In order to check the functionality of MRP1 in V79MRP1 cells, we compared the rate of release of bimane-*S*-glutathione from parental and transfected cells. A 4-fold difference in the rate of transport was observed between cell lines (see Table I). ATP-dependent GSSG accumulation was also considerably higher in inside-out vesicles derived from plasma membranes of V79 and V79MRP1 cells (Table I). Etoposide and sodium arsenite were significantly less toxic to MRP1 transfected cells, confirming their increased resistance to drugs extruded by MRP1 (Table I). These results confirm the proper localization and function of MRP1 protein in the plasma membrane of V79MRP1 cells.

The survival of V79 and V79MRP1 cells subjected to several oxidative stress-inducing agents was compared (Fig. 2). Some of them (*tert*-butyl hydroperoxide, diamide) induce rapid stoichiometric oxidation of cellular glutathione. In order to test whether the toxicity of tBOOH (especially in the higher concentration range) is due to the hydroxide and not to the *tert*-butyl moiety, the effect of *tert*-butyl alcohol (tBOH) was also studied in the same concentration range. No toxicity of tBOH to the cells was found at concentrations up to 100 μM. Other compounds used, viz. paraquat and menadione, are redox-cycling agents and can induce a more prolonged oxidative stress. Chromate and carmustine are inhibitors of glutathione reductase and can be expected to impose chronic oxidative stress. Carmustine strongly increased the toxicity of tBOOH. However, the MRP1 transfected cells did not show increased resistance to any of the compounds studied. A slight (though not statistically significant) rise of resistance of V79 cells to low concentrations of tBOOH was noted.

We also compared the genotoxicity of some oxidants (paraquat, *tert*-butyl hydroperoxide and potassium dichromate) to the Chinese hamster fibroblasts V79 and V79MRP1, using a single cell electrophoresis (comet) assay (Fig. 3). The comet assay allows measurement of DNA damage in single cells with a high degree of sensitivity. There were no significant differences between fragmentation of DNA induced by cytotoxic compounds in parental cell line V79 and in the cell line containing MRP1 protein in the plasma membrane.

The resistance to oxidative stress is known to be dependent on several factors such as activities of antioxidant enzymes and the levels of low-molecular weight antioxidants. Therefore, the level of glutathione and of activities of superoxide dismutase and of key enzymes of glutathione metabolism was compared in the cell lines studied. We found a decreased glutathione level in the transfected cells. This result is consistent with the findings of other authors demonstrating lower GSH concentrations in MRP1 overexpressing cells and augmented GSH levels in cells of MRP1-knockout mice, probably due to the MRP1 mediated co-transport of glutathione with some endogenous substrate of MRP1 [10,35]. The activity of total superoxide dismutase was higher in V79MRP1 cells while that of glutathione peroxidase was decreased. The activity of glutathione reductase did not differ between the parent and transfected cell lines while glutathione *S*-transferase activity was about 40% higher in the parental cell line cells (Table II). The augmented

TABLE I The characteristics of MRP1 function in V79MRP1 cells (mean ± SD)

Parameter	V79	V79MRP1	*N*
Bimane-*S*-glutathione transport rate (pmole/min × 10^6 cells)	1.6 ± 0.2	6.5 ± 0.2***	3
ATP-dependent glutathione disulfide accumulation rate (pmole/min × mg protein)	4 ± 2	61 ± 13**	3
IC_{50} value for etoposide (nM)	300 ± 140	460 ± 90*	3
IC_{50} value for sodium arsenite (μM)	3.3 ± 0.3	6.2 ± 1.5*	3

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ with respect to V79 cells.

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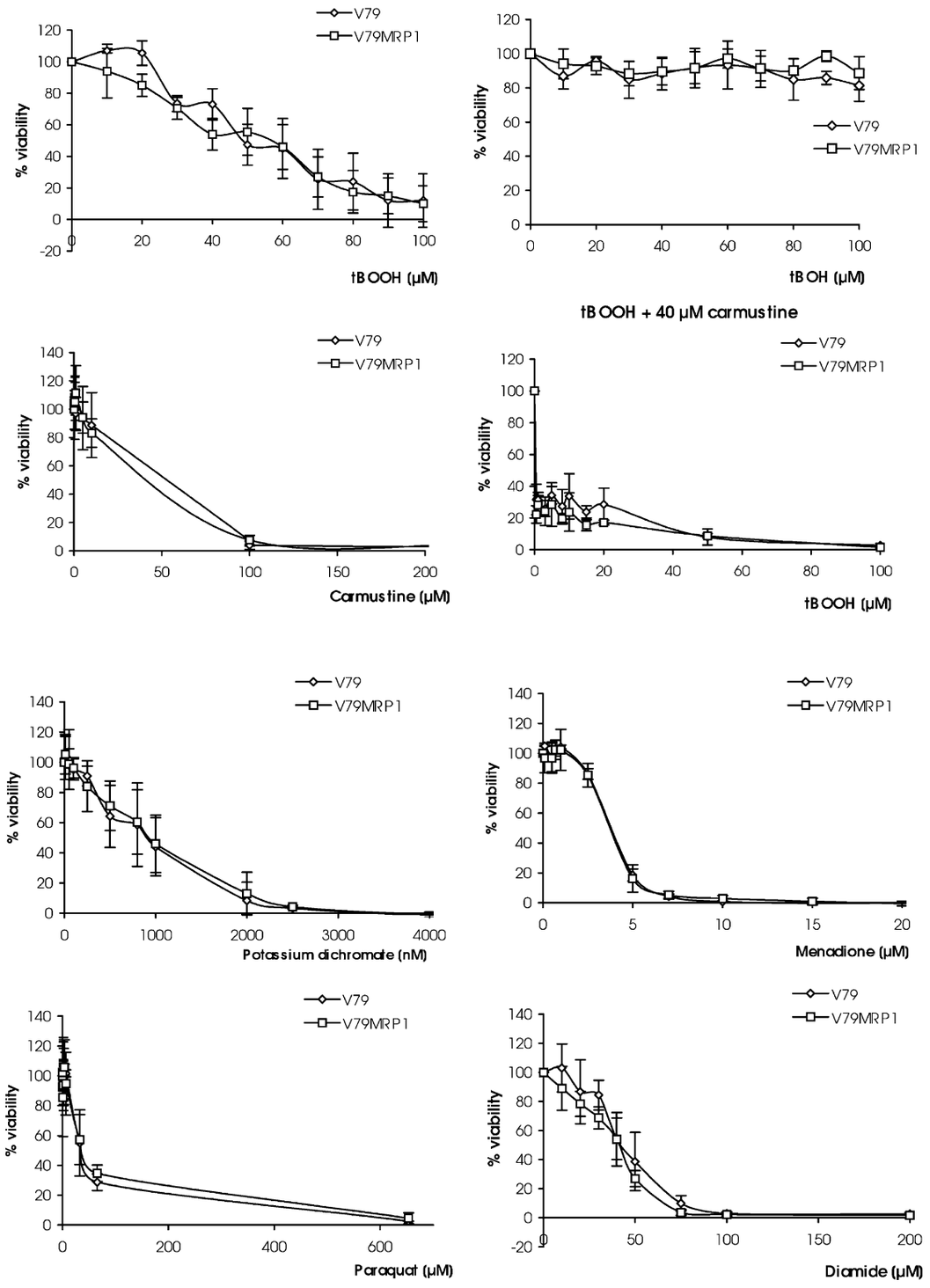

FIGURE 2 The effect of various compounds inducing oxidative stress on the viability of V79 and V79MRP1 cells. No significant differences in IC_{50} between V79 and V79MRP1 cells were found for any compound studied.

superoxide dismutase activity might have been of importance in exposure to menadione and paraquat that are superoxide-generating agents; however, V79MRP1 cells were not more resistant to these agents. The lack of differences in the activity of glutathione reductase makes the comparison of the sensitivities of both cell lines to agents inducing glutathione oxidation a valid measure of the importance of MRP1 in response to oxidative stress.

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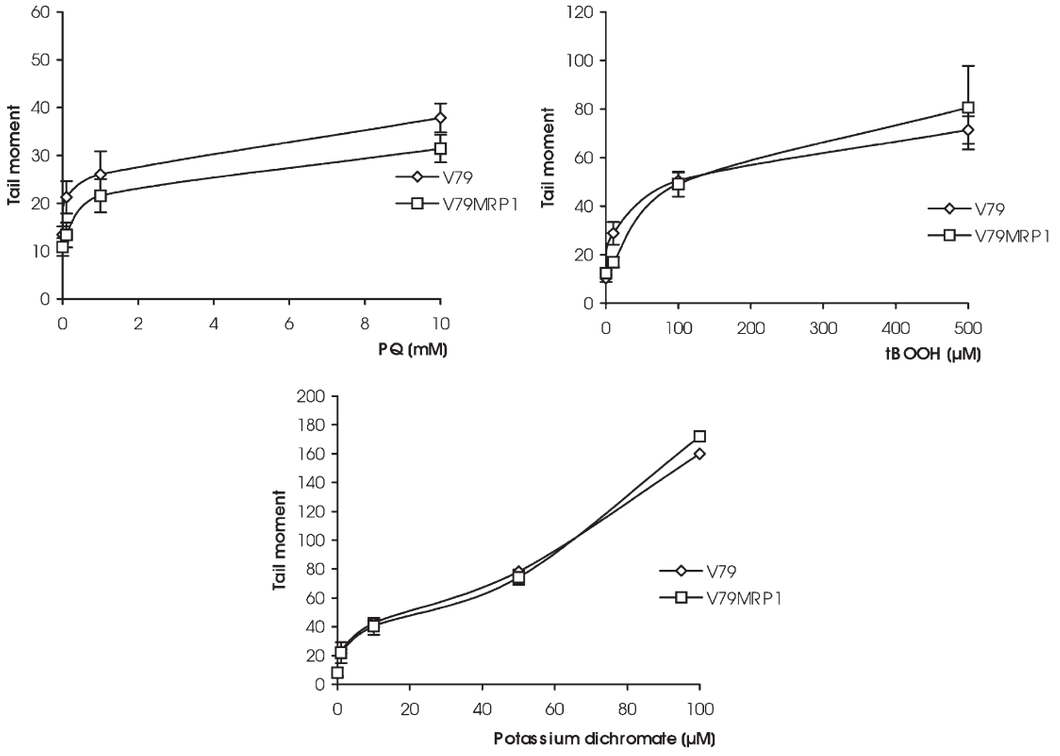

FIGURE 3 The effect of oxidants on DNA damage in V79 and V79MRP1 cells. The differences are not significant statistically ($P > 0.05$).

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These data indicate that, at least in V79 cells, overexpression of MRP1 does not confer resistance to oxidative stress. Recently, Hirrlinger and co-workers reported that rat astrocytes exposed to hydrogen peroxide generated in xanthine oxidase/superoxide dismutase system exhibit MRP1-dependent GSSG transport into extracellular medium [19]. However, the time of cell exposure to oxidative agent was rather short (not exceeding 60 min) and thus should not be considered "chronic" as the authors stated. The results obtained in their study confirm the ability of MRP1 to transport GSSG rather than speak for the major role of this protein in the anti-oxidative stress protection. We did not observe any increased survival of V79MRP1 cells after exposure to glutathione reductase inhibitors in the absence and in the presence of exogenous oxidant (tBOOH). Beside using the alkaline comet assay to measure extent of DNA damage in cells induced by oxidants, we found no differences between the parental and MRP1-transfected cell lines.

The data presented are surprising in view of the induction of MRP1 and other MRPs by prooxidants [16,18]. However, this induction depends on many factors, e.g. p53 status [18] and is not always effective, being subject to suppression by elevated intracellular glutathione [16]. Our results suggest that the role of MRP1 in cellular protection is due to the transport of conjugated xenobiotics or their co-transport with glutathione and that this protein, at least in some cell types, does not have any important role in long term protection against oxidative stress. One cannot exclude such a role for other MRPs able to transport glutathione disulfide (MRP2, MRP3 or MRP5). However, the broad tissue distribution of MRP1 and its relatively high affinity

TABLE II Comparison of glutathione concentration and enzymatic activities in parental cell line and MRP1-transfected cells (mean ± SD)

Parameter	V79	V79MRP1	*N*
Glutathione (nmol/mg protein)	3.51 ± 0.14	2.41 ± 0.21*	4
Superoxide dismutase (units/mg protein)	54.0 ± 5.6	75.8 ± 7.1*	3
Glutathione peroxidase (U/g protein)	6.9 ± 0.9	3.4 ± 0.4*	4
Glutathione reductase (U/g protein)	21.6 ± 0.5	23.1 ± 0.7	6
Glutathione *S*-transferase (U/g protein)	143 ± 11	104 ± 8*	7

*$P < 0.05$ with respect to V79 cells.

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for glutathione disulfide suggests that the role of other MRPs in the defense against oxidative stress may be also not important.

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