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Glutathione S-Transferase Does Not Play a Role in Multidrug Resistance of L1210/VCR Cell Line

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Summary

Multidrug resistance of cancer cells is often accompanied by the (over)expression of integral plasma membrane P-glycoprotein, an ATP-dependent transport pump for diverse unrelated compounds. The glutathione detoxification system represents another mechanism that may be involved in multidrug resistance. In the multidrug-resistant L1210/VCR cell line obtained by long-term adaptation of parental L1210 cells to vincristine, an increased expression of P-glycoprotein has previously been established. In this paper, we investigated if the glutathione detoxification system is also involved in the multidrug resistance of these cells. L1210/VCR cells with resistance induced by adaptation to vincristine were also found to be cross-resistant to vinblastine, actinomycin D, mitomycin C, doxorubicin and cyclophosphamide. The resistance of the above cells to vincristine and doxorubicin was accompanied by a depression of drug accumulation (which has not yet been established for other drug). L1210/VCR cells are able to survive better than sensitive cells under conditions when glutathione was depleted by L-buthionine sulfoximine. Nevertheless, L-buthionine sulfoximine did not influence the resistance of L1210/VCR cells to vincristine. Moreover, the presence of sublethal concentrations of cytostatics neither changed the IC_{50} value of resistant cells to L-buthionine sulfoximine nor the cytoplasmic activity of glutathione S-transferase, the crucial enzyme of glutathione detoxification system. All the above findings indicate that the glutathione detoxification system is not involved in the mechanisms that ensure the multidrug resistance phenotype of L1210/VCR cells.

Key words

P-glycoprotein • Multidrug resistance • L1210 leukemic cell lines • Cytostatic drugs

Introduction

Multidrug resistance (MDR) of neoplastic cells is manifested by a specific phenotype when cells become resistant not only to agents applied in a selective treatment, but also by massive cross-resistance to a diverse structurally unrelated group of drugs. The overexpression of a drug-transporting glycoprotein of the plasma membrane (P-glycoprotein – PGP) was often observed in MDR cells (for review see Juranka *et al.* 1989, Vendrik *et al.* 1992). PGP, the product of *mdr1* gene (Roninson *et al.* 1986), is a member of the large superfamily of proteins that mediate the transport of substances across plasma membranes (Juranka *et al.* 1989). Another mechanism that may be responsible for MDR resistance is connected with an overexpression of

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glutathione S-transferase (GST) as well as with the reduced level of topoisomerase II expression. An example of this are the Chinese hamster ovarian cells adapted to doxorubicin (Hoban *et al.* 1992). This cell line exhibits cross-resistance not only to several topoisomerase II inhibitors such as mitoxantron, daunomycin and etoposide but also to vincristine, colchicine and mitomycin C. The resistance of the above cells was found to be connected with increased levels of PGP and GST (class π and class α) as well as with decreased levels of topoisomerase II expression. A similar pattern of coexpression of MDR markers was found in cells originating from human kidney and breast carcinomas (Efferth *et al.* 1992). In contrast, hematological malignancies did not show such coexpression of resistance markers (Efferth *et al.* 1992). Hence, cells from murine leukemia (L1210) tumors, grown intraperitoneally and treated *in vivo* by doxorubicin exert an overexpression of PGP only. In contrast, cells from murine sarcoma (Sa 180) tumors (grown and treated similarly) exert a coexpression of PGP and GST π (Volm *et al.* 1992).

The L1210/VCR cell line, obtained by exposure of parental L1210 cells to progressively increasing concentrations of vincristine, was characterized in previous papers (Poleková *et al.* 1992, Barančík *et al* 1993, 1994, 1995, Uhrík *et al.* 1994, Breier *et al.* 1994a,b, Štefanková *et al.* 1996, Drobná *et al.* 1996). These cells exhibit an overexpression of mRNA encoding PGP and plasma membrane P-glycoprotein (Poleková *et al.* 1992). The MDR phenotype of the L1210/VCR cell line was also documented by its cross-resistance to actinomycin D and doxorubicin (Drobná *et al.* 1996). Any information about the involvement of GST in the resistance of this cell line has not yet been described.

The aim of the present paper was to investigate whether the glutathione detoxification system, particularly glutathione S-transferases, are involved in the multidrug resistance of L1210/VCR cells.

Material and Methods

Drugs and chemicals

[^{3}H]-vincristine (1.85 MBq; 10.0 Ci/mmol) was obtained from Amersham Corp. (UK). Other drugs were purchased from: actinomycin D – Merck (USA), cyclophosphamide – Orion Corp., Farmos (Finland), doxorubicin (adriamycin, adriablastin) – Pharmacia, Freiburg (FRG), 5-fluorouracil – Roche (Switzerland), mitomycin C – Kyowa Hakko Kogyo Corp. (Japan), vinblastine and vincristine – Gedeon Richter Corp. (Hungary). L-buthionine sulfoximine (LBSO). Other chemicals were obtained from Sigma (USA).

Cells and culture conditions

The sensitive cell line L1210 and multidrug resistant cell subline L1210/VCR were used in our experiments. The resistant cell subline was obtained by long-term adaptation of cells to vincristine and was finally cultivated in a medium containing 1 mg/l of vincristine (L1210/VCR). Cell cultivation was carried out in standard RPMI 1640 medium supplemented with 4 % heat inactivated fetal bovine serum and gentamicin in a humidified atmosphere of 5 % CO_2 at 37 °C.

RPMI 1640 medium, fetal bovine serum and gentamicin were obtained from Gibco (BRL).

Effects of LBSO on the viability of sensitive and resistant cells

The sensitive L1210 and MDR L1210/VCR cells were cultivated in the presence or absence of LBSO (0-1000 μmol/l). LBSO was added directly to the cultivation medium with an inoculum of cells (final concentration 10^4 cells/ml) in 96-well tissue culture plates. After a cultivation period of 3 days, the cells were stained with methylene blue, counted in a hemocytometer and the IC_{50} values of cells sensitive and resistant to LBSO were determined.

Effects of LBSO on the resistance of L1210/VCR cells to vincristine

The L1210/VCR cells were cultivated in the presence of 25 μmol/l LBSO and in the presence or absence of 0-3 mg/l vincristine. After a cultivation period of 3 days, the cells were stained with methylene blue and counted in a hemocytometer. The resistance of L1210/VCR cells was expressed as IC_{50} values to vincristine.

Effects of several cytostatics on the sensitivity of L1210/VCR cells to LBSO

The L1210/VCR cells were cultivated in the presence of 0.2 mg/l of vincristine, vinblastine, mitomycin C, doxorubicin and actinomycin D, and in the presence or absence of 2.5-120 μmol/l LBSO. After a cultivation period of 3 days, the cells were stained with methylene blue, counted in a hemocytometer and IC_{50} values of L1210/VCR cells to LBSO were determined.

Estimation of glutathione S-transferase (GST) activity

GST activities were determined in soluble fractions prepared from sensitive and resistant cells cultivated in the presence of the tested drugs. GST activity was determined colorimetrically by means of a diode-array spectrophotometer (Hewlett-Packard 8452 A) using a single substrate 1-chloro-2,4-dinitrobenzene (CDNB), directly in the photometric cuvette. The enzyme reaction was carried out in 2 ml of the reaction medium containing: 0.1 mol/l potassium phosphate buffer (pH 6.5), 100 mmol/l CDNB, 100 mmol/l reduced glutathione and 20-50 µl enzyme solution at room temperature. The reaction was started by adding reduced glutathione, after stirring, and the change of absorbance was recorded at 340 nm. The rate of conjugation can be calculated using an extinction coefficient of 9.6 l/mmol.cm at 340 nm for the CDNB-glutathione conjugate. Protein concentrations were determined according to Bradford. CDNB was dissolved in dioxane.

Table 1. Characterization of L1210/VCR cells

Overexpression of PGP	• mRNA in slot blot using cDNA probe 5A • mRNA in RT-PCR • 170 kDa protein of plasma membrane using C219 monoclonal antibody	• Poleková *et al.* 1992 • Breier *et al.* 1998a • Poleková *et al.* 1992
Cross-resistance to	Actinomycin D, Dexamethasone, Cyclophosphamide, Vinblastine, Vincristine, Mitomycin C and Doxorubicin	• Fig. 1 (this paper) • Drobná *et al.* 1996 • Breier *et al.* 1998b
Reversal of MDR by	• Calcium entry blockers: Verapamil, Galopamil, Flunarizine, Diltiazem, Nimodipine, Nifedipine, • Neuroleptics: Trifluoperazine, Chlorpromazine, Thioridazine, Perphenazine • Local anesthetics: Cinchocaine, Articaine, Lidocaine • Xanthines: Pentoxiphylline,	• Barančík *et al.* 1994 • Barančík *et al.* 1994 • Barančík *et al.* 1994 • Breier *et al.* 1994a, • Štefanková *et al.* 1995
Reduced level of drug accumulation	• Vincristine • Doxorubicin	• Breier *et al.* 1994b • Breier *et al.* 1998b
Involvement of phosphorylation in MDR regulation	• By PKC • By mitogen activated protein kinases	• Barančík *et al.* 1995 • Barančík *et al.* 1998 • Barančík *et al.* 1999

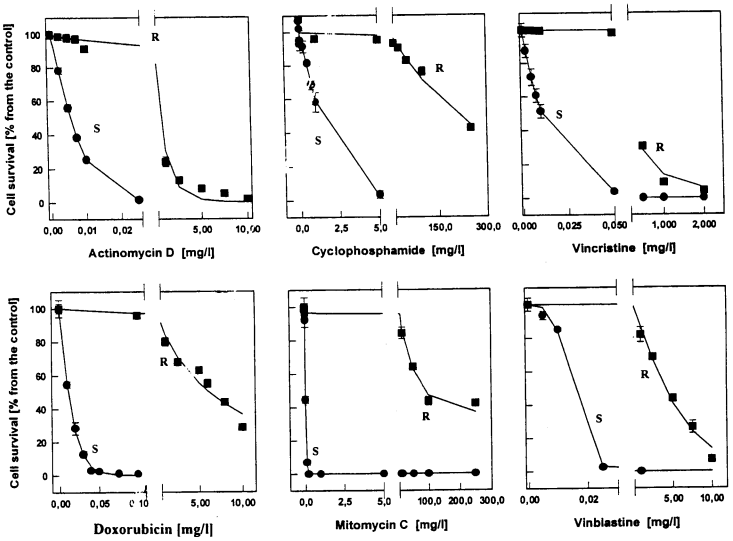

Fig. 1. *Resistance of L1210/VCR cells to several cytostatic agents. Symbols: ● – sensitive L1210 cell line; ■ – resistant L1210/VCR cell line. Data are expressed in % from the control, i.e. when drugs were not present and represent mean values ± S.D. from 9 independent values.*

Results

The L1210/VCR cell line exhibited a high resistance to vincristine (340-fold in comparison with parental L1210 cells) and also cross-resistance to vinblastine, doxorubicin, mitomycin C, actinomycin D and cyclophosphamide (Table 1, Fig.1). The resistance of L1210/VCR cells to vincristine and doxorubicin was accompanied by reduced drug accumulation in comparison with sensitive L1210 cells (Table 1). These cells also exerted an overexpression of mRNA encoding PGP and the plasma membrane P-glycoprotein (Table 1).

Resistant cells were considerably less sensitive to L-buthionine sulfoximine (LBSO, a substance depleting glutathione) as the sensitive cells (Fig. 2). Nevertheless, LBSO was not able to depress the resistance of L1210/VCR cells to vincristine significantly (Fig. 2). Vincristine, vinblastine, mitomycin C, doxorubicin and actinomycin D did not significantly change the IC_{50} values of L1210/VCR cells for LBSO (Fig. 3). No significant changes were observed in soluble GST activities when the sensitive L1210 and resistant L1210/VCR cells were compared (Fig. 4). The activities of this enzyme did not depend on the presence of vincristine, vinblastine, mitomycin C, doxorubicin and actinomycin D in the medium during cultivation (Fig. 4).

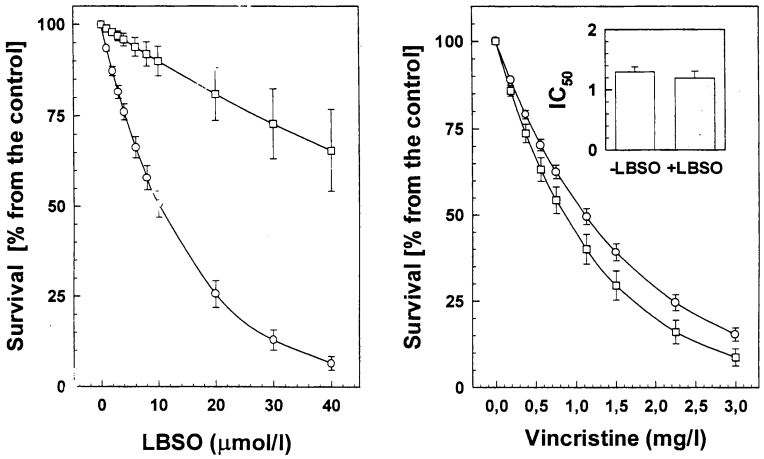

Fig. 2. *Effect LBSO on sensitive L1210 and resistant L1210/VCR cells. Left panel: Cytotoxicity of LBSO on sensitive (○) and resistant (□) cells. Right panel: Cytotoxicity of vincristine on L1210/VCR cell line in the absence (○; –LBSO) or presence (□; +LBSO) of LBSO in concentration 25 μmol/l. Data are expressed in % from the control, i.e., when drugs were not present and represent mean values ± S.D. from 9 independent values. IC_{50} – inhibitory concentration of vincristine (mg/l), survival of 50 % of cells.*

Discussion

L1210/VCR cells exhibit a high level of cross-resistance (at least more than 30 times as compared with sensitive cells) to actinomycin D, cyclophosphamide, vinblastine, mitomycin C and doxorubicin (Fig. 1). The resistance to doxorubicin, actinomycin D, vinblastine, vincristine and mitomycin C, but not to 5-fluorouracil, methotrexate, cytosine arabinoside, was described for cells with the PGP-mediated MDR phenotype (Baguley *et al.* 1992, Volm *et al.* 1992, Fan *et al.* 1994, Adams and Knick 1995). Similarly, our cells were not found to be resistant to 5-fluorouracil, methotrexate, cytosine arabinoside (data not shown). This fact suggested that PGP is predominantly responsible for the MDR character of L1210/VCR cells. The resistance of these cells to vincristine was found to be sensitive (Barančík *et al.* 1994) to several calcium entry blockers (such as verapamil), antagonists of calmodulin (such as trifluoperazine) and local anesthetics (such as cinchocaine). The sensitivity of PGP-mediated MDR to verapamil and trifluoperazine has often been described and may be used as a criterion for this type of resistance (Baguley *et al.* 1992, Hoban *et al.* 1992, Volm *et al.* 1992, Safa *et al.* 1994). Active role of the PGP in MDR phenotype of L1210/VCR cells could also be documented by the significant depression of [^{3}H]-vincristine and doxorubicin accumulation in resistant cells when compared with the accumulation of these drugs in sensitive cells (Table 1). The overexpression of PGP in our cells was documented by the protein and mRNA levels (Poleková *et al.* 1992).

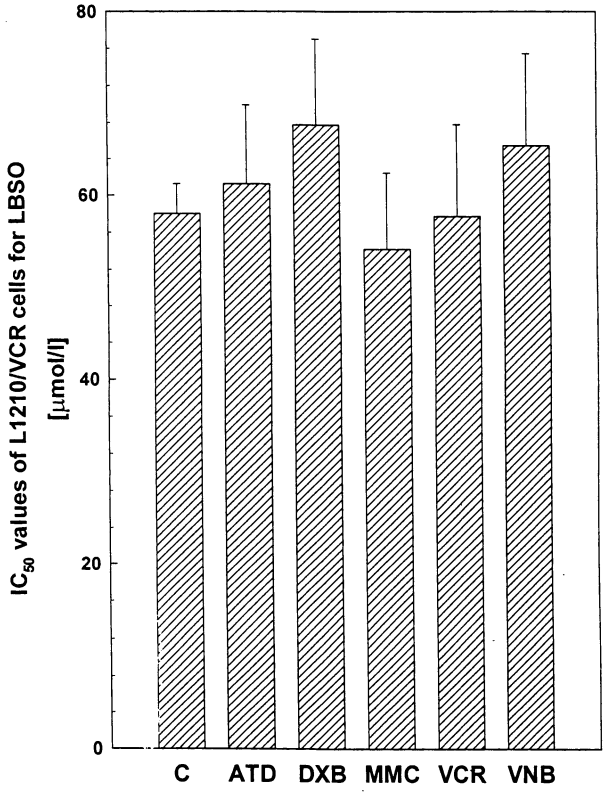

Fig. 3. *Effect of actinomycin D (ATD), doxorubicin (DXB), mitomycin C (MMC), vincristine (VCR) and vinblastine (VBL) on IC_{50} value of L1210/VCR cells for LBSO. C – control situation, cells cultivated in the absence of cytostatics. Data represent mean values ± S.D. from 9 independent values.*

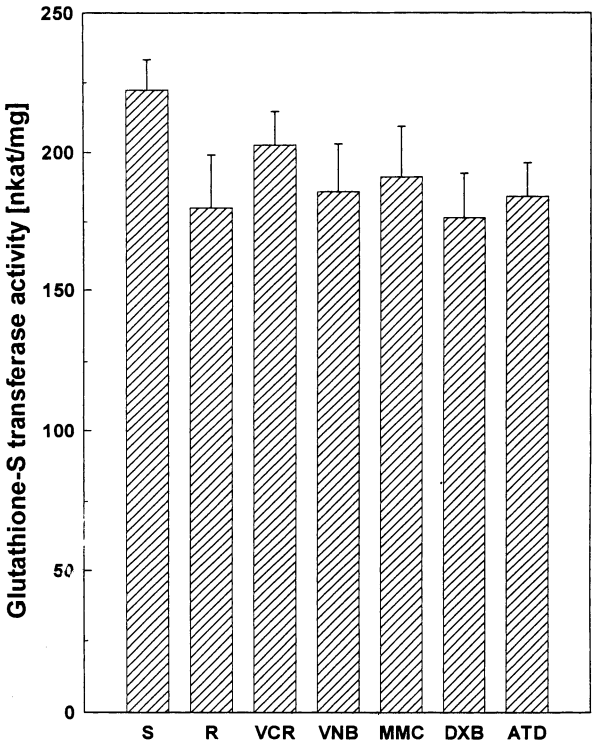

Fig. 4. *Activity of glutathione S-transferase in sensitive L1210 cells (S) and resistant L1210/VCR cells (R) in the absence and presence of ATD, DXB, MMC, VCR and VNB. Data represent mean values ± S.D. from 6 independent values.*

Volm *et al.* (1992) recently described a significant effect of LBSO on the reversal of MDR in sarcoma tumor (Sa 180) cells in which the MDR phenotype was found to be accompanied by an overexpression of both PGP and glutathione S-transferase (isoenzyme π). The lack of the LBSO effect on MDR reversal in L1210/VCR cells (Fig. 2) only points to a minor (if any) role of the glutathione detoxification system (GDS) in the MDR phenotype of these cells. Interestingly, the resistant cells were considerably less sensitive to LBSO than the sensitive cells (Fig. 2). The possibility that LBSO may be considered as the substrate for PGP and be eliminated by this transport system from the intracellular space of resistant cells is improbable. In the case that LBSO represented the substrate of PGP, and was thus transported by PGP, it should compete with cytostatics for the binding site on the PGP molecule. In this case, LBSO should induce a MDR reversal effect similar to that described for verapamil, nicardipine, cyclosporine A and progesterone (Naito *et al.* 1988, 1989, Naito and Tsuruo 1989). However, no appreciable reversal effect induced by LBSO on the resistance of L1210/VCR cells to vincristine has been observed (Fig. 2). On the other hand, it could be speculated that, when GDS is not functional due to depletion of glutathione (induced by LBSO), there still exists another effective system (e.g. PGP) in L1210/VCR cells for elimination of toxic products of cell metabolism. Thus, resistant cells could also survive in the situation when activity of GDS is significantly reduced. The study of GDS involvement in the MDR character of L1210/VCR cells using depletion of glutathione by LBSO had the following reasons. The MDR phenotype based on the acceleration of GDS depends on the activity of several enzymes that ensure glutathione regeneration, formation of glutathione-drug conjugates and additional processing of these conjugates. Thus, this type of MDR phenotype may be present in the cells even when intracellular levels

of GST are not changed. For example, the resistance accompanied by a significant elevation of glutathione reductase and glutathione peroxidase, but not of GST activity, was observed after treatment of cancer cells with mitomycin C (Perry *et al.* 1993). It is therefore not so simple to obtain information about the effectivity of GDS by detecting the expression of one enzyme only, as could be done for PGP-mediated MDR. On the other hand, the depletion of glutathione by LBSO should reduce the efficacy of GDS significantly and it thus represents a better approach for characterizing the involvement of GDS in MDR phenotype of L1210/VCR cells. The possibility that GDS may be involved in MDR character of L1210/VCR cells was also contradicted by the fact that GST activities in resistant cells were not changed in the presence of vincristine, vinblastine, mitomycin C, doxorubicin and actinomycin D. Moreover, the activities of these enzymes in sensitive and resistant cells were not different. The possibility that the MDR character of L1210/VCR cells could be mediated by alterations of topoisomerase I and II activities contradicts the fact that this type of MDR is often accompanied by unaltered sensitivities of resistant cells to actinomycin D and vincristine, as was demonstrated in lung fibrosarcoma cells (Lelievre *et al.* 1995). All these facts indicate that the MDR phenotype of our cells is predominantly mediated by PGP.

Acknowledgements

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Abbreviations and symbols

PGP, P-glycoprotein; MDR, multidrug resistance; VCR, vincristine; ATD, actinomycin D; DXB, doxorubicin; MMC, mitomycin C; VBL, vinblastine, LBSO, L-buthionine sulfoximine; GDS, glutathione detoxification system; IC_{50}, median inhibitory concentration; GST, glutathione S-transferase.

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Reprint requests

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