Hypoxia Increases Cell Death in Multidrug-Resistant Leukemia Cells. Differences in Viability and Ultrastructure between Sensitive and Multidrug-Resistant L1210 Mouse Leukemic Cells under Hypoxia

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Abstract. The comparative study of sensitive and multidrug-resistant L1210 cells under 24 hours of hypoxia (2 % O_2 and 5 % CO_2 at 37 °C) was done to see if differences in energetic metabolism between both cell lines are paralleled by differences in cellular morphology. During the dye exclusion assay the viability of sensitive cells was about 70 to 90 %, whereas only 30 to 50 % of resistant cells were viable. Electron microscopic study of sensitive and resistant L1210 cells under hypoxia has shown cells of different ultrastructural appearance in both cell lines. Cells with necrotic changes (swollen mitochondria, lysed cells) prevailed in resistant cells. The highest incidence of cells with normal or slightly dense mitochondria was found among the sensitive L1210 cells. Additionally, cells with pyknotic nuclei, shrunken cytoplasm and dense mitochondria, reminiscent of apoptosis, could be found sporadically, especially in the sensitive L1210 cell line. These results are in agreement with flow cytometry measurements: in resistant cells the number of necrotic cells was on the average 2.3 times higher than in sensitive cells. Ultrastructural differences and differences in the numbers of necrotic cells as measured by flow cytometry between sensitive and resistant L1210 cells under hypoxia are consistent with differences in energetic metabolism between these cell lines, as described in earlier studies, and document an increased cell death in the resistant L1210 cell line.

Key words: L1210 cells — Multidrug resistance — Hypoxia — Necrosis — Apoptosis — Mitochondria

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Introduction

The development of multidrug resistance (MDR) in neoplastic cells presents a serious obstacle in the treatment of malignant diseases and elucidation of differences in metabolic pathways between drug-sensitive and drug-resistant cells may help to design more effective therapies for tumours and leukemias. P-glycoprotein (P-gp), a product of mdr1 gene, is in many cases a primary cause of MDR or, at least, participates in it (for a review, see Kvačkajová-Kišucká et al. 2001). P-gp is an integral glycoprotein (170 kDa) of plasma membrane in animal cells and is a member of the family of ABC (ATP binding cassette) transporters. Functionally, P-gp is an efflux ATPase with a low affinity to transported substances. Several phosphorylating sites were identified in the P-gp primary structure (Chambers et al. 1994) indicating that P-gp may exist as a phosphoglycoprotein and its phosphorylation may represent an important regulatory mechanism of its activity.

Mouse leukemic cell line L1210, first described by Law et al. (1949), can be turned into MDR cell line by stepwise adaptation to vincristine (VCR) (Poleková et al. 1992). The resistance of L1210/VCR cells may be depressed by chemosensitizers, the drugs able to reverse MDR (Barančík et al. 1994; Kišucká et al. 2001; Drobná et al. 2002; Kupsáková et al. 2002). The basic ultrastructural features of L1210 cells (Brandes et al. 1966), some staining properties (El-Saggan and Uhrík 2002) and structural differences between sensitive and MDR L1210 cells (Breier et al. 1994a,b; Uhrík et al. 1994) have already been described.

An indication of differences in energetic metabolism between L1210 and L1210/VCR cells resulted from the comparative study of the level of oxygen consumption and glucose incorporation in the presence of VCR (Poleková et al. 1992): Resistant cells were distinguished by increased velocity of O_2 consumption and a moderate decrease in glucose incorporation whereas in sensitive cells a decreased O_2 consumption with a strong reduction of glucose incorporation could be observed. These observations were tentatively explained by an assumption of decreasing rate of glycolysis with increasing level of mitochondrial oxidative phosphorylation under Pasteur effect in resistant cells.

In the present study the viability and ultrastructural features of L1210 and L1210/VCR cells under 24 hours hypoxia were compared to see if differences in energetic metabolism between both cell lines are paralleled by differences in cellular morphology.

Materials and Methods

Cell lines

Sensitive (L1210) and resistant (L1210/VCR) mouse leukemic cell lines were used. L1210/VCR cells with P-gp overexpression were prepared by adaptive cultivation of the parental cell line in RPMI medium with increasing concentrations of VCR

(Poleková et al. 1992). Several characteristics of L1210/VCR cells were described previously (Boháčová et al. 2000; Breier et al. 2000).

Cell cultivation under hypoxia

L1210 and L1210/VCR cells were incubated in DMEM (PAA) supplemented with 5% FCS, 2 mmol/l glutamine, 4.5 g/l glucose and penicillin-streptomycin (both at 100μg/ml) mixture in the atmosphere of 5% CO_2 and 2% O_2 at 37℃ for 24 h.

Dye exclusion test

After 24 hours of hypoxic exposure sensitive and resistant L1210 cells were stained with erythrosin or trypan blue solution and ratios of viable/death cells were counted in hemocytometer.

Flow cytometry

Both cell lines after 24 hours incubation in hypoxic conditions were 2 times washed with PBS and then double stained with Annexin V-FITC Apoptosis Detection Kit (Sigma). Measurements were performed on flow cytometer Coulter Epics Altra (Beckman Coulter). Both fluorescent dyes propidium iodide (PI) and Annexin V-FITC were excited at 488 nm. Apoptotic cells were stained with Annexin V-FITC and fluorescent signal was detected on second channel PMT2 – 525 nm. Necrotic cells were stained by PI with emission on third channel PMT3 – 585 nm. Experiments were performed 3 times. Cells were sorted according to fluorescent intensity and displayed in four quadrants as survival, necrotic or apoptotic fraction.

Electron microscopy

After cultivation cells were centrifuged at $1000 \times g$ for 3 min, washed in phosphate buffer and fixed for 60 min with 2% glutaraldehyde in 0.1 mol/l Na-cacodylate buffer (CB) at pH 7.2. During fixation the cells were centrifuged at $1000 \times g$ for 3 min to form a sediment. The specimen was then washed 3 times for 7 min with CB and postfixed with 1% osmium tetroxide in CB for 60 min. The samples were rinsed in H_2O, stained overnight with 2% uranyl acetate, dehydrated in increasing concentrations of ethanol, cleared in propylene oxide and embedded in Durcupan. Ultrathin sections were cut with a microtome Porter-Blum MT2, stained with lead citrate and examined in a JEOL JEM-1200 EX electron microscope at 80 kV.

Results

Dye exclusion test

During the dye exclusion assay the viability of sensitive cells incubated under hypoxic conditions was about 70 to 90%, whereas only 30 to 50% of resistant cells were viable. The viability of both cell lines under normoxia was above 95%.

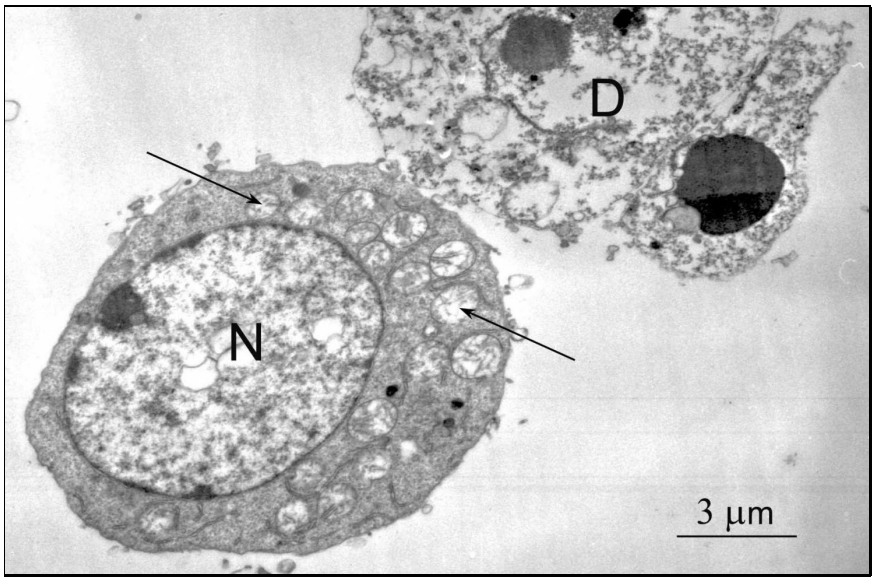

Figure 1. A resistant L1210 cell under hypoxia with swollen mitochondria (arrows). D, a cell debris as a result of necrotic cell lysis; N, cell nucleus.

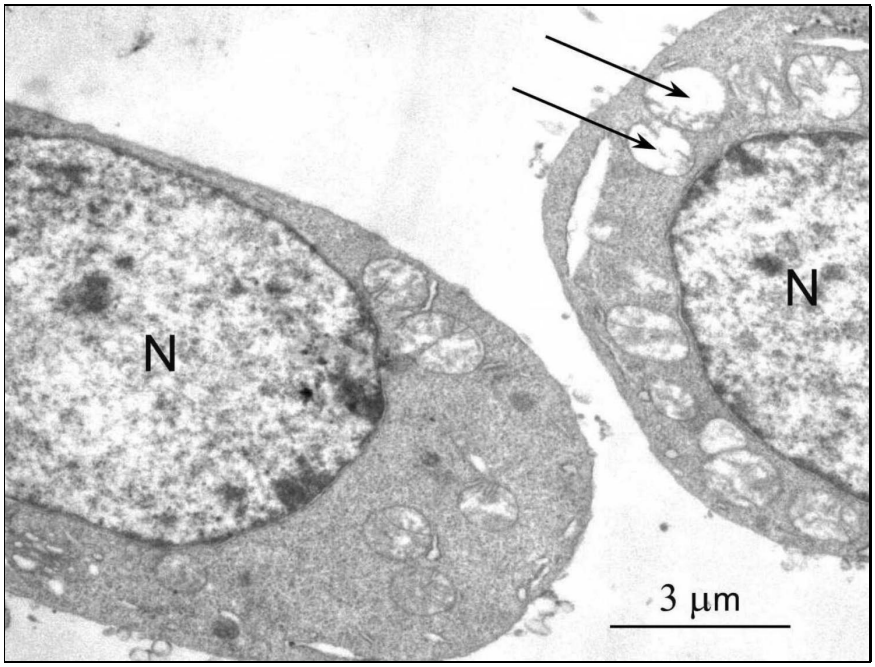

Figure 2. Resistant L1210 cells under hypoxia. In the cell on the right cristae lysis in some mitochondria is prominent (arrows). N, cell nucleus.

Electron microscopy

Electron microscopy of cells exposed to hypoxia has shown cells of different ultrastructural appearance in both cell lines: a) Cells with swollen mitochondria displaying the image of necrosis (Figs. 1, 2). In many mitochodria the lysis of cristae was prominent (Fig. 2). The incidence of these cells was highest in the resistant L1210/VCR cell line. b) Disintegrated cell remnants as a result of cell lysis (Fig. 1, D). The patterns of lysed cells prevailed again in the resistant L1210/VCR cell line. c) Cells with normal or slightly dense mitochondria (Figs. 3a,b). These cells were characteristic for the sensitive L1210 cell line. d) Cells with pyknotic nuclei, shrunken cytoplasm and dense mitochondria (Fig. 4). The overall appearance of these cells was reminiscent of apoptosis and they could be found sporadically, especially in the sensitive L1210 cell line.

Flow cytometry

Increased number of damaged L1210/VCR cells observed under hypoxic conditions using dye exclusion assay prompted us to use double-colour (PI-Annexin V-FITC) flow cytometry to resolve if such cells undergo also apoptosis. We have found that hypoxia increased necrosis in L1210/VCR cells 2.3 times in comparison with sensitive L1210 cells. The majority of cells were found in the left quadrants (viable and necrotic) and no significant differences in numbers of apoptotic cells between both cell lines could be observed (Fig. 5).

Discussion

The main form of the cell death under 24 h hypoxia in both L1210 and L1210/VCR cell populations, as measured by flow cytometry, has been necrosis. The striking difference in the numbers of necrotic cells between L1210 and L1210/VCR cells is in agreement with differences in ultrastructural appearance: The characteristic signs of necrotic changes, swelling of cytoplasm and mitochondria and the cell lysis, have been most pronounced in the L1210/VCR cell line.

The development of MDR and overexpression of P-gp in the mouse leukemic L1210 cells resulted in increased sensitivity to hypoxia. Ultrastructural differences between sensitive and resistant L1210 cells under hypoxia and the results of dye exclusion viability assay and flow cytometry are consistent with differences in energetic metabolism between these cell lines, indicating an increased level of mitochondrial oxidative phosphorylation in resistant cells (Poleková et al. 1992).

Generally, the resistant cells are more dependent on the respiratory function of mitochondria. Under the hypoxic conditions the cells suffer from ATP-shortage and the permeability barrier of the cellular membrane will be damaged with consequences for ionic homeostasis resulting in swelling of cytoplasm and of all organelles, including mitochondria. The final outcome is necrosis and cell lysis.

More favourable energetic conditions and a better supply with ATP allow to switch to another way of cell death: apoptosis. The process of apoptotic shrinkage may last until depletion of energetic reserves (for a review, see McConkey 1998).

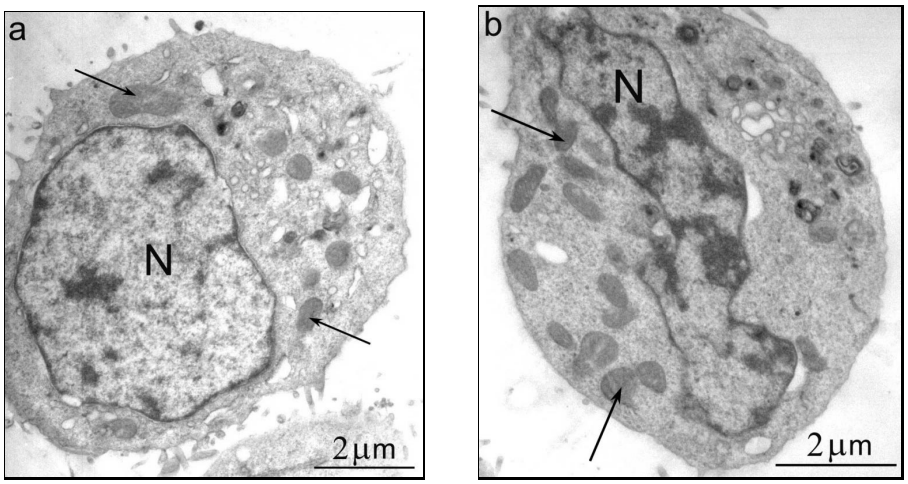

Figure 3. Sensitive L1210 cells under hypoxia. (a) a cell with mitochondria of normal density and (b) with slightly dense mitochondria and cytoplasm. The nucleus in (b) is shrunken. N, cell nucleus; arrows, mitochondria.

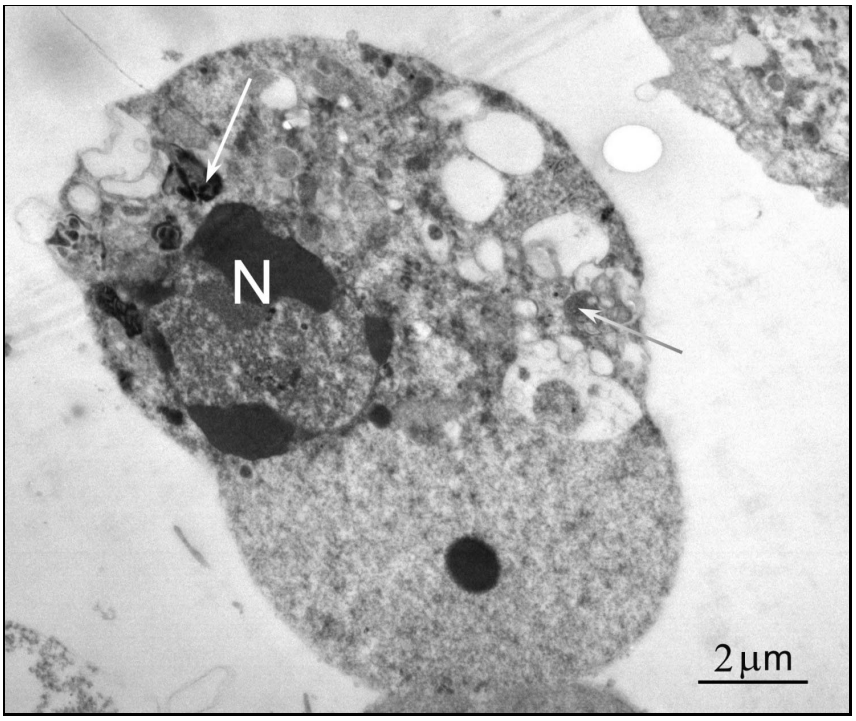

Figure 4. A sensitive L1210 cell under hypoxia with a pyknotic nucleus, shrunken cytoplasm and dense mitochondria. N, cell nucleus; arrows, mitochondria.

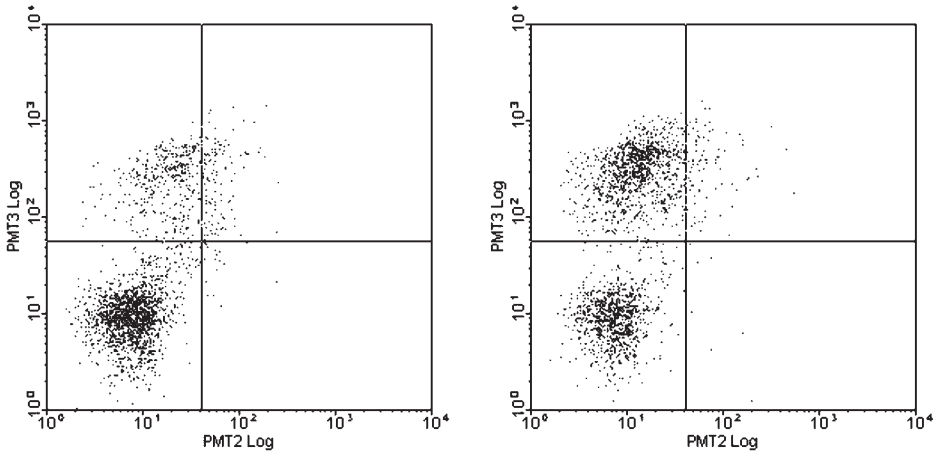

Figure 5. Induction of apoptosis *vs.* necrosis of L1210 sensitive (left panel) and L1210 resistant (right panel) murine leukemic cells. Flow cytometric analysis after 24 h hypoxia. *Abscissa*, PMT2 Log-fluorescence of Annexin V-FITC. *Ordinate*, PMT3 Log-fluorescence of propidium iodide. The necrotic cells may be seen in the upper left quadrants, the viable cells in the lower left quadrants, the early apoptotic cells in the lower right quadrants and the late apoptotic/necrotic cells in the upper right quadrants.

A modified glucose metabolism in cells with a resistant phenotype has been described also in other malignant cell lines. In comparison with adriamycin (ADM)-sensitive colon carcinoma cell line, the ADM-resistant cells showed a significant increase in the oxidative pathway of glucose metabolism as well as in acetyl-CoA production and the amount of glucose carbon atoms metabolized through the pentose phosphate pathway was significantly higher (Fanciulli et al. 1993). Similarly modified energy metabolism in malignant cells associated with the development of a resistant phenotype has been found in Ehrlich ascites tumor cells (Miccadei et al. 1996).

The higher incidence of cells with ultrastructural characteristics of apoptosis (Fig. 4) in sensitive L1210 cells as compared to resistant L1210/VCR cell line is rather difficult to interpret as yet and additional work is necessary to elucidate mechanisms, other than ATP shortage, determining the switch between the different ways of cell death under hypoxic conditions. A possible hint to explain these observations may be provided by findings of inhibitory effect of P-gp on apoptotic cell death (Robinson et al. 1997; Smyth et al. 1998; Johnstone et al. 1999).

The discrepancy between a very low incidence of apoptotic cells identified with the method of flow cytometry and their occasional findings (especially in sensitive cells) according to structural criteria will require a more thorough study with exposition of cells to more differentiated culture conditions including hypoxias of different duration.

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References

Barančík M., Poleková L., Mrázová T., Breier A., Stankovičová T., Slezák J. (1994): Reversal effect of several Ca^{2+}-entry blockers, neuroleptics and local anaesthetics on P-glycoprotein-mediated vincristine resistance of L1210/VCR mouse leukaemic cell line. Drugs Exp. Clin. Res. **20**, 13—18

Boháčová V., Kvačkajová J., Barančík M., Drobná Z., Breier A. (2000): Glutathione S-transferase does not play a role in multidrug resistance of L1210/VCR cell line. Physiol. Res. **49**, 447—453

Brandes D., Shofield B., Slusser R., Anton E. (1966): Studies of L1210 leukemia. I. Ultrastructure of solid and ascites cells. J. Natl. Cancer Inst. **37**, 467-485

Breier A., Barančík M., Štefanková Z., Uhrík B., Tribulová N. (1994a): Effect of pentoxifylline on P-glycoprotein mediated vincristine resistance of L1210 mouse leukemic cell line. Neoplasma **41**, 297—303

Breier A., Štefanková Z., Barančík M., Tribulová N. (1994b): Time dependence of [^{3}H]-vincristine accumulation by L1210 mouse leukemic cells. Effect of P-glycoprotein overexpression. Gen. Physiol. Biophys. **13**, 287—298

Breier A., Drobná Z., Dočolomanský P., Barančík M. (2000): Cytotoxic activity of several unrelated drugs on L1210 mouse leukemic cell sublines with P-glycoprotein (PGP) mediated multidrug resistance (MDR) phenotype. A QSAR study. Neoplasma **47**, 100—106

Chambers T. C., Pohl J., Glass D. B., Kuo J. F. (1994): Phosphorylation by protein kinase C and cyclic AMP-dependent protein kinase of synthetic peptides derived from the linker region of human P-glycoprotein. Biochem. J. **299**, 309—315

Drobná Z., Stein U., Walther W., Barančík M., Breier A. (2002): Pentoxifylline influences drug transport activity of P-glycoprotein and decreases mdr1 gene expression in multidrug resistant mouse leukemic L1210/VCR cells. Gen. Physiol. Biophys. **21**, 103—109

El-Saggan A. H., Uhrík B. (2002): Improved staining of negative binding sites with ruthenium red on cryosections of frozen cells. Gen. Physiol. Biophys. **21**, 457—461

Fanciulli M., Bruno T., Castiglione S., Del Carlo C., Paggi M. G., Floridi A. (1993): Glucose metabolism in Adriamycin-sensitive and -resistant LoVo human colon carcinoma cells. Oncol. Res. **5**, 357—362

Johnstone R. W., Cretney E., Smyth M. J. (1999): P-glycoprotein protects leukemia cells against caspase-dependent, but not caspase-independent, cell death. Blood **93**, 1075—1085

Kišucká J., Barančík M., Boháčová V., Breier A. (2001): Reversal effect of specific inhibitors of extracellular-signal regulated protein kinase pathway on P-glycoprotein mediated vincristine resistance of L1210 cells. Gen. Physiol. Biophys. **20**, 439—444

Kupsáková I., Dočolomanský P., Rybár A., Barančík M., Breier A. (2002): Carbonyl group of aliphatic side chain of pentoxifylline does not play role for P-glycoprotein antagonizing effect of pentoxifylline. Gen. Physiol. Biophys. **21**, 471—478

Kvačkajová-Kišucká J., Barančík M., Breier A. (2001): Drug transporters and their role in multidrug resistance of neoplastic cells. Gen. Physiol. Biophys. **20**, 215—237

Law L. W., Dunn T. B., Boyle P. J., Miller J. H. (1949): Observations on the effect of a folic-acid antagonist on transplantable lymphoid leukemias in mice. J. Natl. Cancer Inst. **10**, 179—192

McConkey D. J. (1998): Biochemical determinants of apoptosis and necrosis. Toxicol. Lett. **99**, 157—168

Miccadei S., Fanciulli M., Bruno T., Paggi M. G., Floridi A. (1996): Energy metabolism of adriamycin-sensitive and -resistant Ehrlich ascites tumor cells. Oncol. Res. **8**, 27—35

Poleková L., Barančík M., Mrázová T., Pirker R., Wallner J., Sulová Z., Breier A. (1992): Adaptation of mouse leukemia cells L1210 to vincristine. Evidence for expression of P-glycoprotein. Neoplasma **39,** 73—77

Robinson L. J., Roberts W. K., Ling T. T., Lamming D., Sternberg S. S., Roepe P. D. (1997): Human MDR1 protein overexpression delays the apoptotic cascade in Chinese hamster ovary fibroblasts. Biochemistry **36**, 11169—11178

Smyth M. J., Krasovskis E., Sutton V. R., Johnstone R.W. (1998): The drug efflux protein, P-glycoprotein, additionally protects drug-resistant tumor cells from multiple forms of caspase-dependent apoptosis. Proc. Natl. Acad. Sci. USA **95**, 7024—7029

Uhrík B., Tribulová N., Klobušická M., Barančík M., Breier A. (1994): Characterization of morphological and histochemical changes induced by overexpression of P-glycoprotein in mouse leukemic cell line L1210. Neoplasma **41**, 83—88

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