Short communication

Pentoxifylline Influences Drug Transport Activity of P-Glycoprotein and Decreases mdr1 Gene Expression in Multidrug Resistant Mouse Leukemic L1210/VCR Cells

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Abstract. The effects of pentoxifylline (PTX) on intracellular accumulation of doxorobicin (DOX), DOX cytotoxicity and expression of Pgp in multidrug resistant L1210/VCR cell line were investigated. PTX (100 mg/l) was able to enhance the DOX accumulation in resistant cells. The maximum intracellular levels of DOX were reached after treatment with PTX for 24 hours (total duration of PTX-treatment was 72 hours). The levels of mdr1 mRNA (measured by RT-PCR method) were decreased 2-fold in the presence of 100 mg/l PTX (minimum reached within 48 hours) in comparison to control cells.

Key words: Pentoxifylline — Doxorubicin — mdr1 mRNA — P-glycoprotein — Multidrug resistance — Vincristine — L1210 cells

P-glycoprotein (Pgp) is a membrane drug transporting system that causes multidrug resistance of neoplastic cells against a wide range of cytotoxic agents (for review see Kvačkajová-Kišucká et al. 2001). Overcoming of multidrug resistance (MDR) by chemosensitizers belongs to the methods that can be considered from the point of successful clinical pharmacology (chemotherapy). Chemosensitizers are diverse groups of drugs with ability to depress efflux activity of P-glycoprotein (Pgp) in multidrug resistant cells and to increase the intracellular accumulation of cytostatics to the cytotoxic concentration for these cells (Chlebowski et al. 1982; Jusa and Tsuruo 1989; Yang et al. 1989; Chambers et al. 1992; Barančík et al. 1994; Barančík et al. 1995).

Effects of several xanthine derivatives on the resistance of L1210/VCR cells have been tested in previous reports (Breier et al. 1994a; Štefanková et al. 1996). It was found that only pentoxifylline (PTX) of the tested xanthine derivatives was able to reverse the vincristine resistance of the L1210/VCR cells (Štefanková et al.

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1996). It is worth noticing that the ability of PTX to interfere in MDR is not common for other tested xanthine derivatives and could not be explained on the basis of known biological activities of xanthines (such as inhibition of phosphodiesterase activity, inhibition of TNF-α synthesis, activation of calcium induced calcium release channels). Our present study is focused on further investigation of the features involved in PTX-mediated reversal of Pgp-mediated multidrug resistance.

Cell lines were maintained in standard RPMI 1640 medium supplemented with 4% fetal bovine serum and cultivated in atmosphere of 5% CO_2 at 37℃. Cytotoxicity of the doxorubicin (0–1 mg/l) was estimated after 3 days lasting cultivation of the L1210/VCR cells in the presence or absence of PTX (100 mg/l). The number of viable cells was determined using MTT cytotoxicity test (Mosmann 1983; Carmichael et al. 1987). The effects of PTX (100 mg/l) on the accumulation of doxorubicin in L1210/VCR cells was performed using modified method used in the study of Leonce et al. (1992). Cells were incubated in phenol red-free medium containing 100 mg/l PTX. The cells were collected, washed twice and resuspended in phenol red-free medium at several time points (0–72 hours). Aliquots of the suspension were incubated for 3 hours with 50 μmol/l DOX at 37℃. The mean DOX fluorescence of 1×10^4 cells was measured at 4℃ by FACScan flow cytometry (Becton Dickinson, Mountain View, CA, USA) and calculated using LYSIS software.

For the determination of the mdr1 mRNA expression, RT-PCR method was used. Total cellular RNA was extracted from the cells incubated for 48 hours with 100 mg/l PTX and from control cells (untreated with PTX) using LiCl/urea method (Walther and Stein 1994). RT-PCR was performed with the Gene Amp RNA PCR kit (Perkin Elmer via Rocher Molecular Systems Inc., Branchburg, NJ, USA) as described previously (Stein et al. 1996). PCR primers used for amplification were: for mdr1-specific sequences, the primer pair 5'-CCC ATC ATT GCA ATA GCA GG-3' (sense strand) and 5'-GTT CAA ACT TCT GCT CCT GA-3' (antisense strand) which yield a 167 bp product (Noonan et al. 1990); for β-actin-specific sequences, the primer pair 5'-ATC ATG TTT GAG ACC TTC AA-3' (sense strand) and 5'-CAT CTC TTG CTC GAA GTC CA-3' (antisense strand) (Wu et al. 1992) producing a 316 bp product. PCR products were separated on 1.5% agarose gel (Gibco). Gels were examined on UV transilluminator and were semiquantitated as relative mdr1 expression (mdr1 expression/β-actin expression) from video images by densitometry using the NIH-Image 1.44611 Public Domain software.

In our experiments the mouse leukemic cell line L1210/VCR with resistance against vincristine and several other cytostatics has been used (Boháčová et al. 2000; Breier et al. 2000). This cell line was selected from the parental L1210 cell line by cultivation in the medium with stepwise increasing concentrations of VCR. L1210/VCR cells exhibit the MDR phenotype that is associated with increased mdr1/Pgp expression (Poleková et al. 1992). Several specific features of this cell line were described elsewhere (Barančík et al. 1999, 2001; Kišucká et al. 2001). Previous studies showed that PTX was able to overcome the Pgp-mediated vincristine

resistance of L1210/VCR cells (Breier et al. 1994a; Štefanková et al. 1996). The [^{3}H]-vincristine accumulation studies revealed the time-dependent accumulation of [^{3}H]-vincristine and differences in response of the L1210/VCR cells to short-term and long-term exposure to PTX (Breier et al. 1994a,b). To estimate Pgp transport activity after PTX treatment, we performed cellular drug accumulation studies using doxorubicin (DOX) in both PTX-pretreated and untreated L1210/VCR cells. We found that pretreatment with PTX (100 mg/l) was able to influence the DOX accumulation in resistant cells. The maximum intracellular levels of DOX were reached at 24 hours of PTX treatment. As shown in Fig. 1, in the L1210/VCR cells pretreated with PTX (100 mg/l) for 24 hours a significant enhancement (2-fold) of DOX accumulation was observed when compared with the PTX-untreated cells (the mean fluorescence *per* cell 870.35 *versus* 429.44). The results indicate that the effect of PTX is associated with its ability to decrease efflux activity of Pgp in L1210/VCR cells. In order to resolve the precise mechanism by which PTX influences Pgp, further investigations are required. Reed and DeGowin (1992) showed that biotransformation of PTX give rise to seven metabolites. From this point of view the time-dependent effects of PTX may involve the effect of one or several

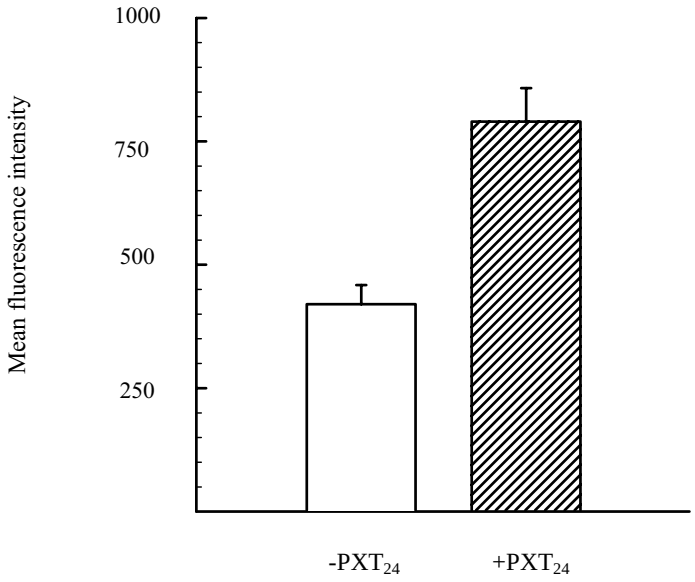

Figure 1. Effect of pentoxifylline (PTX) on doxorubicin (DOX) accumulation in L1210/VCR cells. Resistant cells were incubated in the presence or absence of 100 mg/l PTX. The graph shows the accumulation of DOX after pretreatment of cells with PTX for 24 hours. DOX fluorescence was measured with a FACScan flow cytometry and expressed as mean fluorescence of 1×10^4 cells. Results are expressed as the mean DOX fluorescence in PTX treated cells ($+PTX_{24}$) compared with the mean fluorescence of untreated cells ($-PTX_{24}$).

PTX metabolites, which are probably more potent in inhibition of Pgp transport activity compared to the parental molecule of PTX.

Cytotoxicity experiments were performed to determine whether the simultaneous application of PTX and DOX results in enhanced drug cytotoxicity. Cells were incubated in the presence of different concentrations of DOX (0–1 mg/l) and in the presence or absence of PTX (100 mg/l) for 72 hours. No significant effects of PTX on DOX cytotoxicity were observed (data not shown). Interesting is the fact that PTX (100 mg/l) is highly effective in improvement of cytotoxic effect of such cytostatics as vincristine, actinomycin D and mitomycin C, and the presence of PTX reversed the resistance of L1210/VCR cells against these cytostatics (results not shown). The results obtained with DOX may be partially explained on the basis of spectrophotometry studies that showed the ability of PTX to interact directly with the planar aromatic molecule of the DOX (Traganos et al. 1991). Formation of methylxanthine-drug complex in solution effectively lowers the concentration of the free drug and thereby reduces its pharmacological activity. Similar principle, i.e., direct interaction, was assumed for explanation why verapamil is not able to reverse the resistance of L1210/VCR cells to DOX (Breier et al. 1998).

To investigate the level of mdr1 mRNA gene expression the RT-PCR method was used. The experiments were performed in the same treatment conditions as for

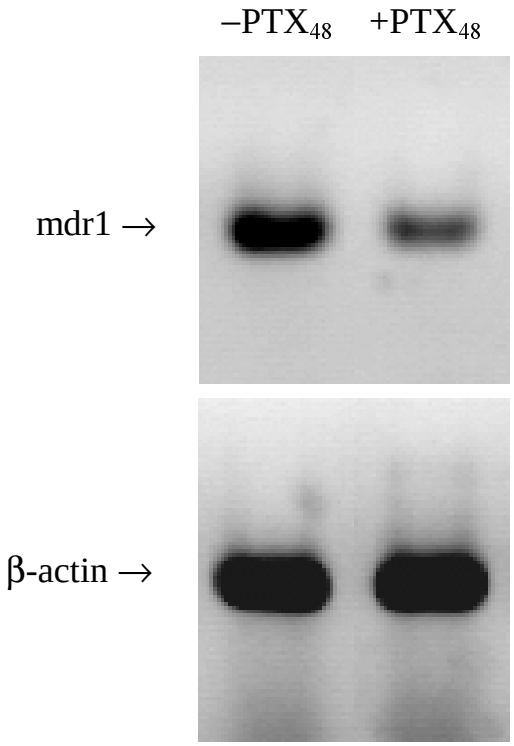

Figure 2. Effect of pentoxifylline (PTX) on mdr1 mRNA expression in L1210/VCR cells. Exponentially growing cells were cultured in the presence ($+PTX_{48}$) or in the absence ($-PTX_{48}$) of PTX at concentration 100 mg/l. The total RNA was extracted after 48 hours. Both, upper and lower panels demonstrate the results of one representative experiment (total of two separate experiments). The sizes for the specific RT-PCR products are 167 bp for mdr1 and 316 bp for β-actin.

the investigation of DOX accumulation. As shown in Fig. 2, approximately a 2-fold decrease in mRNA levels for mdr1 gene occurred within 48 hours of treatment of L1210/VCR cells with 100 mg/l PTX. The decreased mdr1 mRNA expression in PTX-treated L1210/VCR cells was associated with increased DOX accumulation. This indicates that PTX could act by a similar mechanism as described in the studies of Muller et al. (1994, 1995) for verapamil. In this study verapamil was able to down-regulate the mdr1/Pgp expression in multidrug resistant human leukemic cell lines and the effect of verapamil was associated with decreased Pgp activity. However, relatively high doses of verapamil were required to down-regulate Pgp expression. From this point of view, PTX with its lower cytotoxicity should be considered as an alternative approach for the reversal of multidrug resistance. This drug can be used also by the study of a potentially novel mechanism of action of chemosensitizers.

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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 leukemic cell line. Drugs Exp. Clin. Res. **20,** 13—18

Barančík M., Štefanková Z., Breier A. (1995): Effect of phorbol myristate acetate (PMA) on P-glycoprotein mediated vincristine resistance of L1210 cells. Gen. Physiol. Biophys. **14,** 171—175

Barančík M., Boháčová V., Zbýňovcová M., Breier A. (1999): Differential expression of regulatory proteins in L1210/VCR cells with multidrug resistance mediated by P-glycoprotein. Gen. Physiol. Biophys. **18,** 45—56

Barančík M., Boháčová V., Kvačkajová J., Hudecová S., Križanová O., Breier A. (2001): SB203580, a specific inhibitor of p38-MAPK pathway, is a new reversal agent of P-glycoprotein-mediated multidrug resistance. Eur. J. Pharm. Sci. **14**, 29—36

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. (Prague) **49**, 447—453

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., Barančík M. (1998): Direct interaction between verapamil and doxorubicin caused the lack of reversal effect of verapamil on P-glycoprotein mediated resistance to doxorubicin *in vitro* using L1210/VCR cells. Neoplasma **45,** 248—253

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

Carmichael J., DeGraff W. G., Gazdar A. F., Minna J. D., Mitchell J. B. (1987): Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. Cancer Res. **47,** 936—942

Chambers T. C., Zheng B., Kuo J. F. (1992): Regulation by phorbol ester and protein kinase C inhibitors, and by a protein phosphatase inhibitor (okadaic acid), of P-glycoprotein phosphorylation and relationship to drug accumulation in multidrug-resistant human KB cells. Mol. Pharmacol. **41,** 1008—1015

Chlebowski R. T., Block J. B., Cundiff D., Dietrich M. F. (1982): Doxorubicin cytotoxicity enhanced by local anesthetics in a human melanoma cell line. Cancer Treat. Rep. **66,** 121—125

Jusa K., Tsuruo T. (1989): Reversal mechanism of multidrug resistance by verapamil: direct binding of verapamil to P-glycoprotein on specific sites, and transport of verapamil outward across the plasma membrane of K562/ADM cells. Cancer Res. **49,** 5002—5006

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

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

Leonce S., Pierre A., Anstett M., Perez V., Genton A., Bizzari J. P., Atassi G. (1992): Effects of a new triazinoaminopiperidine derivative on adriamycin accumulation and retention in cells displaying P-glycoprotein-mediated multidrug resistance. Biochem. Pharmacol. **44,** 1707—1715

Mosmann T. (1983): Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods **65,** 55—63

Muller C., Bailly J. D., Goubin J., Laredo J., Jaffrezou J. P., Bordier C., Laurent G. (1994): Verapamil decreases P-glycoprotein expression in multidrug resistant human leukemic cell lines. Int. J. Cancer **56,** 1—6

Muller C., Goubin F., Ferrandis E., Cornil-Schwartz I., Bailly J. D., Bordier C., Benard J., Sikic B. I., Laurent G. (1995): Evidence for transcriptional control of human mdr1 gene expression by verapamil in multidrug-resistant leukemic cells. Mol. Pharmacol. **47,** 51—56

Noonan K. F., Beck C., Holymayer T. A., Chin J. E., Wunder J. S., Andrulis I. L., Gazdar A. F., Willman C. L., Griffith B., Vonhoff D. D., Roninson I. B. (1990): Quantitative analysis of MDR1 (multidrug resistance) gene expression in human tumors by polymerase chain reaction. Proc. Natl. Acad. Sci. U.S.A. **87,** 7160—7164

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

Reed W. R., DeGowin L. (1992): Suppressive effects of pentoxifylline on natural killer cell activity. J. Lab. Clin. Med. **119,** 763—771

Stein U., Walther W., Shoemaker R. H. (1996): Modulation of mdr1 expression by cytokines in human colon carcinoma cells: an approach for reversal of multidrug resistance. British J. Cancer **74,** 1384—1391

Štefanková Z., Barančík M., Breier A. (1996): Overcoming of P-glycoprotein mediated vincristine resistance of L1210/VCR mouse leukemic cells could be induced by pentoxifylline but not by theophylline and caffeine. Neoplasma **43,** 11—15

Traganos F., Kapuscinski J., Darzynkiewicz Z. (1991): Caffeine modulates the effects of DNA-intercalating drugs *in vitro*: A flow cytometric and spectrophotometric analysis of caffeine interaction with novantrone, doxorubicin, ellipticine, and the doxorubicin analogue AD198. Cancer Res. **51,** 3682—3689

Walther W., Stein U. (1994): Influence of cytokines on mdr1 expression in human colon carcinoma cell lines: increased cytotoxicity of MDR relevant drugs. J. Cancer Res. Clin. Oncol. **120,** 471—478

Wu L., Smythe A. M., Stinson S. F., Mullendore L. A., Monks A., Scudiero D. A., Paull K. D., Rubinstein L. V., Boyd M. R., Shomeaker R. H. (1992). Multidrug resistant phenotype of disease-oriented panels of human tumor cell lines used for anticancer drug screening. Cancer Res. **52,** 3029—3034

Yang C. P., DePinho S. G., Greenberger L. M., Arceci R. J., Horwitz S. B. (1989): Progesterone interacts with P-glycoprotein in multidrug resistant cells and in the endometrium of gravid uterus. J. Biol. Chem. **264,** 782—788

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