Short Communication

Carbonyl Group of Aliphatic Side Chain of Pentoxifylline does not Play Role for P-Glycoprotein Antagonizing Effect of Pentoxifylline

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Abstract. Previously we have found that pentoxifylline (PTX), but not caffeine, theophylline, or 1-methyl-3-isobutylxanthine, affects sensitivity of L1210/VCR cells, a line with multidrug resistance mediated by P-glycoprotein (P-gp) to vincristine (VCR) and doxorubicine. Comparison of chemical structure of PTX with other above xanthines has revealed only one marked difference. PTX contains extended aliphatic chain containing reactive electrophilic carbonyl group in the position N1. The investigation of possibility that this group is crucial for PTX-induced MDR reversal represents the aim of the current paper. To prove this hypothesis, we used the new synthesized PTX derivative in which the carbonyl group is modified by a substance containing amino-group and the product of reaction is the respective Schiff base (SB). Successful reaction was observed when PTX reacted with 3,5-diaminobenzenesulfonyl acid (DABS). The product of reaction of DABS with carbonyl group of aliphatic part of PTX was proved using NMR and IR spectroscopy. We found that the resulting PTX derivative PTX-SB revealed higher cytotoxicity on both sensitive L1210 and multidrug resistant L1210/VCR cells than PTX. Moreover, PTX-SB exerts more pronounced MDR reversal effect on L1210/VCR cells than PTX. These results indicate that electrophilic carbonyl group on aliphatic chain located in position N1 of PTX is not essential for MDR reversal effects of PTX.

Key words: Pentoxifylline — *mdr1* gene product — P-glycoprotein — Multidrug resistance — Vincristine — L1210 cells

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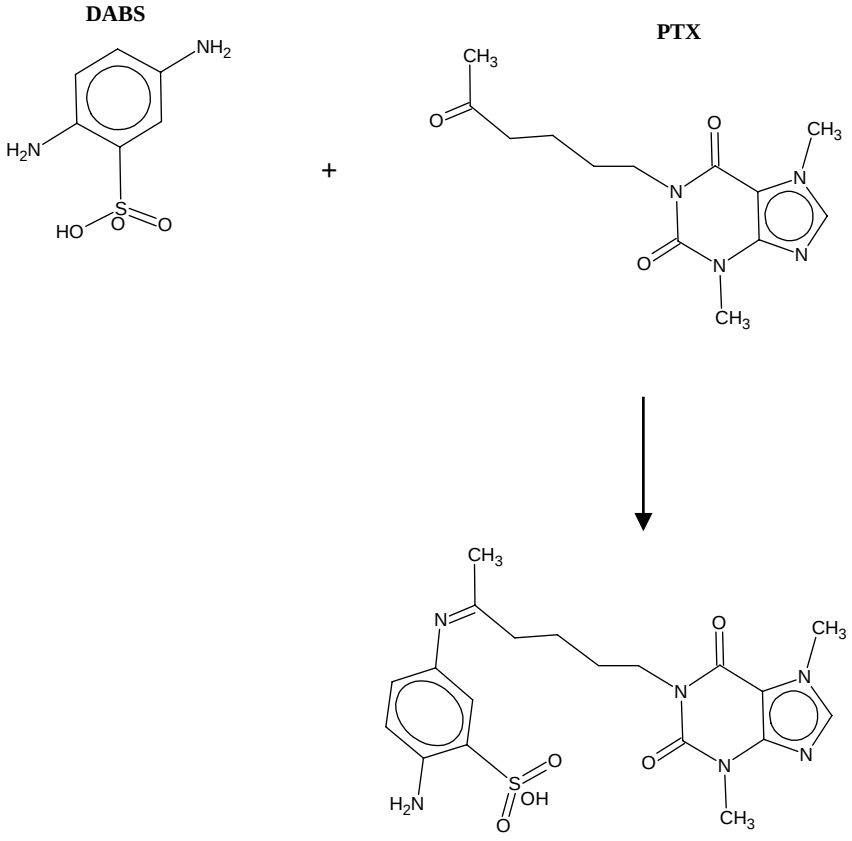
Multidrug resistance (MDR) is a phenomenon when neoplastic cells become resistant to a wide range of structurally and functionally dissimilar unrelated anticancer drugs (Ling 1997). The MDR phenotype is frequently observed in rodent and human cell lines selected for resistance to a single agent (Nielsen and Skovsgaard 1992; Litman et al. 2001). MDR is often caused by activity of drug-efflux ATP-dependent pump, called P-glycoprotein (P-gp). P-gp is 170 kDa integral membrane protein located in cell plasma membrane, which exports structurally diverse groups of substances out from cytoplasm (Kvačkajová-Kišucká et al. 2001). This glycoprotein is product of *mdr1* gene (Juliano and Ling 1976; Gottesman et al. 1995) and is a member of the ATP-binding cassette (ABC) proteins family that involves many ATP-dependent prokaryotic and eukaryotic membrane transporters (Higgins 1992). Several drugs called chemosensitizers (calcium channel blockers, calmodulin inhibitors, coronary vasodilators, indole alkaloids, quinolines, hormones, cyclosporines and others; Ford and Hait 1990, 1993; Avendano and Menendez 2002) are able to depress MDR through partial or full abolishment of P-gp transport activity by several mechanisms including direct competition with cytostatics, inhibition of ATP binding and hydrolysis by P-gp, influencing the processes connected with P-gp activation or regulation of P-gp expression (Kvačkajová-Kišucká et al. 2001).

Multidrug resistant mouse leukemic cell line L1210/VCR used in the present paper was selected from the parental L1210 cell line by cultivation in the medium with increasing concentrations of VCR (Poleková et al. 1992).This cell line exhibits MDR phenotype that is associated with increased P-gp expression (Breier et al. 1998), but not with changes in activity of glutathion S-transferase (Boháčová et al. 2000) and exerts high degree of cross-resistance (30 to 500 times) to actinomycin D, dexamethasone, cyclophosphamide, vinblastine, mitomycine C and doxorubicin (Breier et al. 2000). Several specific features of this cell line were described elsewhere (Barančík et al. 2001; Kišucká et al. 2001). Xanthine pentoxifylline (PTX) was found to be able to overcome vincristine (VCR) and doxorubicine resistance of P388 multidrug resistant leukemia cells (Chitnis et al. 1990; Viladkar and Chitnis 1994) and also in mouse leukemic cell line L1210/VCR (Breier et al. 1994; Drobná et al. 2002). The presence of PTX in cultivation medium caused approximately twofold decrease in mdr1 mRNA level in L1210/VCR cells (Drobná et al. 2002). Reversal effect of PTX was not found to be common for other xanthines like caffeine, theophylline, and 1-methyl-3-isobutylxanthine (Štefanková et al. 1996). The only marked difference between PTX and other tested xanthines follows from their chemical structure. Only PTX contains aliphatic chain in position N1 with polar electrophilic carbonyl group. Modifications of this part of PTX molecule were found to alter the reversal efficacy of PTX (Ojima et al. 1998). In the present paper we were aiming to investigate whether the presence of carbonyl group was essential for ability of PTX to reverse MDR. For this purpose, we prepared new derivative PTX-SB in which the carbonyl group on the aliphatic chain was modified and we studied its effect on resistance of L1210/VCR cells to vincristine.

The PTX-SB derivative was prepared by reaction of amino-group of 2,4-

diaminobenzenesulfonic acid (DABS), purum 98.0 % (Sigma-Aldrich Chemie, GmbH, Taufkirchen, Germany) with carbonyl groups located on aliphatic chain of PTX (Slovakofarma Hlohovec, Slovakia). The resulting reaction product was the respective Schiff base according to Scheme 1. Reaction was carried out in 50 ml of bidistiled water containing 5 mmol of both PTX and DABS for two hours at 95 °C. The product was separated by chromatography on Silicagel 200 (Sigma-Aldrich Chemie, GmbH, Taufkirchen, Germany) using the system methanol : ethanol (10 : 1). The purity of product was ascertained by thin layer chromatography (TLC) on Silufol (Kavalier, Votice, Czech Republic) in methanol and product was detected in UV



Scheme 1. Reaction of pentoxifylline (PTX) with 2,4-diaminobenzenesulfonic acid (DABS) yields a new PTX derivative

lamp. Structure of new derivative was elucidated on the basis of ^{1}H NMR and IR spectroscopy.

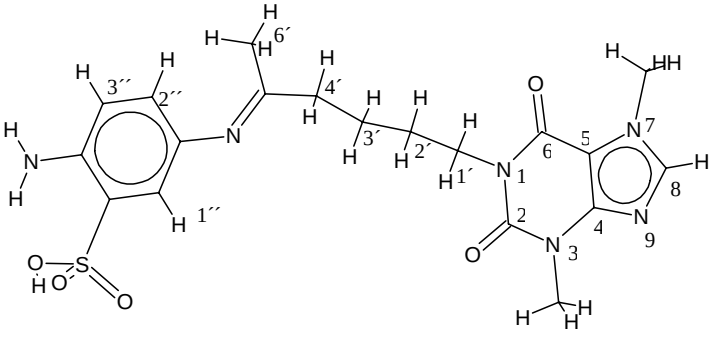
The procedure of PTX derivative synthesis described above supplied 6 mg of product (yield 0.267 %). Formation of product during reaction was detected using UV spectrometry, and TLC. Product was separated chromatographically from reaction mixture on Silicagel 200 and applied on TLC or, for estimation of its effect, on L1210/VCR cells immediately after separation. TLC chromatography revealed that product obtained after separation on Silicagel 200 gave only one spot (R_f = 0.84) different from spots of DABS and PTX characterized by R_f values amounting to 0.62 and 0.48, respectively. Structure of product was elucidated with the aid of IR and ^{1}H NMR spectrometry and confirms the structure of PTX-SB:

1. IR spectroscopy confirmed the presence of S═O binding (1150 and 1300 cm^{-1}) and NH_2 group (3025 cm^{-1}) both located on aromatic ring of aminobenzenesulfonic part of PTX-SB. Moreover, C═O groups on pyridine moiety remains unchanged according to signal at extremely low region of spectrum (1560 cm^{-1}).

2. ^{1}H NMR spectra confirmed the proposed structure of PTX-SB on the basis

Table 1. ^{1}H NMR signals of the new PTX-SB derivative

Position of proton	Signal	ppm
3	singlet	3.50
7	singlet	3.95
8	singlet	8.11
1′	triplet	3.80
2′, 3′	triplet	1.55
4′	triplet	2.65
6′	singlet	2.25
1″	singlet	7.65
2″	doublet	7.25
3″	doublet	7.45



of 10 characteristic proton signals originated from specific structural parts of the molecule (Table 1).

In the study mouse leukemic cell line L1210/VCR was used. The cells were cultured in standard RPMI 1640 medium supplemented with L-glutamine (1 mg/ml), fetal bovine serum (4%) and gentamycin (1 μl/ml; all from Life Technologies, Scotland, UK), in an atmosphere of 5% CO_2 at 37°C. MDR reversal effect of xanthines was determined by cultivation of L1210/VCR cells on 96 well plates (10^4 cells/200 μl) in medium containing vincristine (concentration range 0–6 mg/l) in the absence or presence of PTX or PTX-SB (10 and 50 mg/l). The effects of xanthines on viability of L1210/VCR cells were investigated by their cultivation in the presence or absence of PTX or PTX-SB (concentration range 0–500 mg/l). After 3 day's cultivation period the cells were stained with Trypan blue (Sigma-Aldrich Chemie, GmbH, Taufkirchen, Germany) and counted in heamocytometer (Bürker's chamber). Cytotoxicities of both xanthines were evaluated as decrease of cell survival induced by their presence in cultivation medium. MDR reversal effect was evaluated as decrease of LC_{50} for vincristine induced by the presence of the respective xanthine.

Cytotoxic effects of PTX and PTX-SB on L1210/VCR were estimated after three days lasting cultivation in the presence or absence of the respective xanthine.

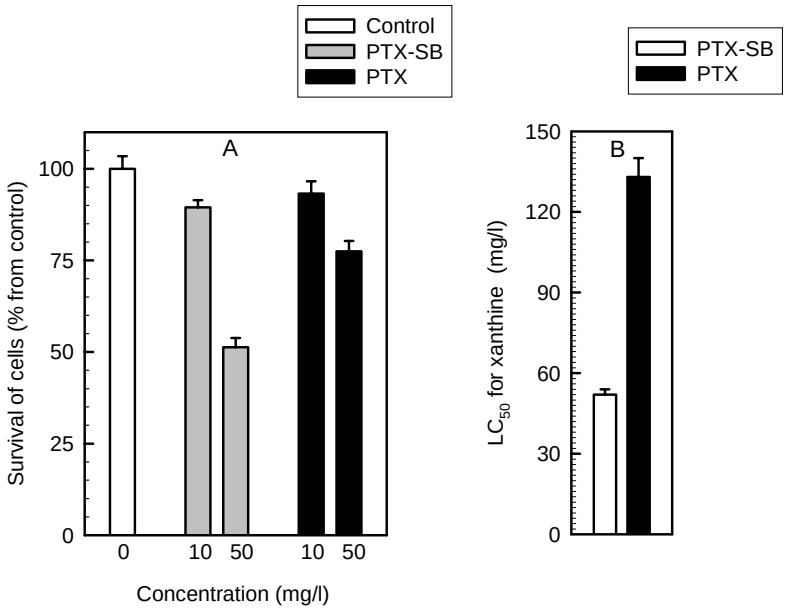


Figure 1. The effect of pentoxifylline (PTX) and new synthesized derivative PTX-SB on viability of resistant L1210/VCR cells. Panel A: survival of L1210/VCR cells in the presence of either PTX or PTX-SB at given concentrations. Panel B: the LC_{50} values obtained for PTX or PTX-SB. Data represent means ± S.E.M. from 3 independent measurements.

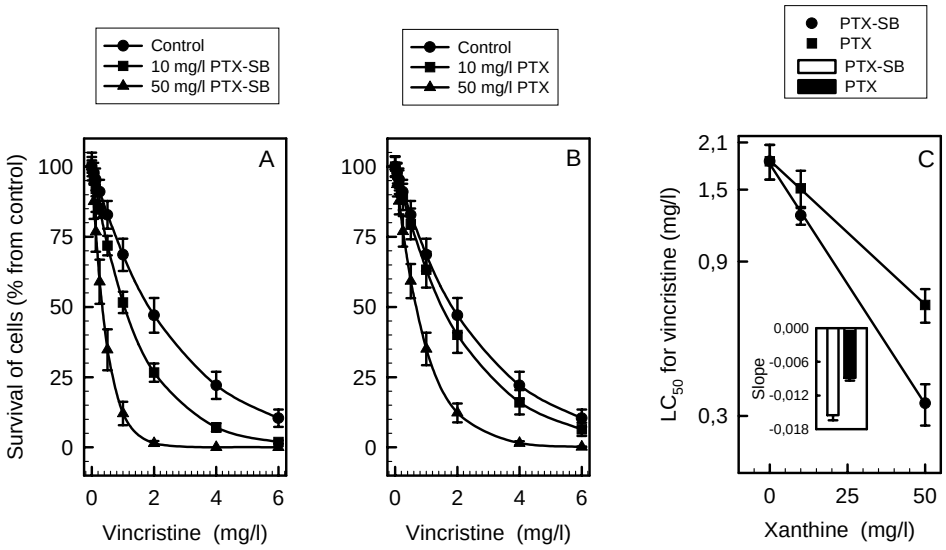


Figure 2. The effect of pentoxifylline (PTX) and new synthesized derivative PTX-SB on survival of L1210/VCR cells in medium containing different concentrations of vincristine. On the panel **A** and **B** are effects of PTX-SB and PTX, respectively. On the panel **C** are PTX-SB and PTX induced decreases of LC_{50} values for vincristine linear in semilogarithmic plot. Insert in panel C represents the slopes of the respective straight lines. Data represent means ± S.E.M. from 3 independent measurements.

The data obtained showed higher toxicity of PTX-SB on L1210/VCR cells when compared with PTX (Fig. 1A). LC_{50} for PTX-SB (52 ± 2 mg/ml) was found to be about 2.5 times lower as LC_{50} obtained for PTX (133 ± 8 mg/ml) (Fig. 1B). L1210/VCR cells exert high degree of resistance to vincristine that may be documented by two order higher LC_{50} for vincristine (1.8 mg/l) as compared with parental L1210 cells (0.01 mg/l). Presence of both PTX-SB (Fig. 2A) and PTX (Fig. 2B) increased vincristine cytotoxicity by concentration dependent manner. LC_{50} value for vincristine was considerably decreased from value 1.837 ± 26 mg/l to 1.248 ± 0.078 or 1.516 ± 0.200 mg/l in the presence of 10 mg/l PTX-SB or PTX and to 0.328 ± 0.048 and 0.661 ± 0.079 mg/l in the presence of 50 mg/l of PTX-SB or PTX. Thus efficacy of PTX-SB in reversal of MDR of L1210/VCR cell is greater than of PTX. Value of LC_{50} for vincristine as a function of PTX or PTX-SB concentration gave straight line in semilogaritmic plot (Fig. 2C). A steeper slope of this dependence was observed for PTX-SB as for PTX that again pointed to more pronounced MDR reversal effect of the new derivative. These findings and the fact that the new derivative is without carbonyl group on the aliphatic chain indicate that this carbonyl group is not essential for the ability of PTX to reverse the vincristine resistance.

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References

Avendano C., Menendez J. C. (2002): Inhibitors of multidrug resistance to antitumor agents (MDR). Curr. Med. Chem. **9**, 159—193

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. (1994): Effect of pentoxifylline on P-glycoprotein mediated vincristine resistance of L1210 mouse leukemic cell line. Neoplasma **41**, 297—303

Breier A., Drobná Z., Boháčová V., Barančík M. (1998): Resistance of L1210 mouse leukemic cell line characterized by overexpression of ATP dependent drug transporter to several drugs. Chem. Papers **52**, (Sp. Issue) 418—419

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

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

Ford J. M., Hait W. N. (1990): Pharmacology of drugs that alter multidrug resistance in cancer. Pharmacol. Rev. **42**, 155—199

Ford J. M., Hait W. N. (1993): Pharmacologic circumvention of multidrug resistance. Cytotechnology **12**, 171—212

Gottesman M. M., Hrycyna C. A., Schoenlein P. V., Germann U. A. Pastan I. (1995): Genetic analysis of the multidrug transporter. Annu. Rev. Genet. **29**, 607—649

Higgins C. F. (1992): ABC transporters: from microorganisms to man. Annu. Rev. Cell. Biol. **8,** 67—113

Chitnis M. P., Viladkar A. B., Juvekar A. S. (1990): Inhibition of DNA biosynthesis by vincristine and pentoxifylline in murine P388 leukemia cells resistant to doxorubicin. Neoplasma **37**, 619—626

Juliano R. L., Ling V. (1976): A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. Biochim. Biophys. Acta. **455**, 152—162

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—44

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

Ling V. (1997): Multidrug resistance: molecular mechanisms and clinical relevance. Cancer Chemother. Pharmacol. **40**, (Suppl.) S3—8

Litman T., Druley T. E., Stein W. D., Bates S. E. (2001): From MDR to MXR: new understanding of multidrug resistance systems, their properties and clinical significance. Cell. Mol. Life Sci. **58**, 931—959

Nielsen D., Skovsgaard T. (1992): P-glycoprotein as multidrug transporter: a critical review of current multidrug resistant cell lines. Biochim. Biophys. Acta **1139,** 169—183

Ojima I., Bounaud P. Y., Oderda C. F. (1998): Recent strategies for the treatment of multi-drug resistance in cancer cells. Expert Opin. Ther. Pat. **8**, 1587—1598

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

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

Viladkar A., Chitnis M. (1994): In vitro effects of pentoxifylline and doxorubicin on cell survival and DNA damage in sensitive and MDR-P388 leukemia cells. Cancer. Biother. **9**, 143—151

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