Short communication

Effect of Phorbol Myristate Acetate (PMA) on P-glycoprotein Mediated Vincristine Resistance of L1210 Cells

M. BARANČÍK[1], Z. ŠTEFANKOVÁ[1], A. BREIER[2]

1 *Institute for Heart Research, Slovak Academy of Sciences, Dúbravská cesta 9, 842 33 Bratislava, Slovakia*
2 *Institute of Molecular Physiology and Genetics, Slovak Academy of Sciences, Vlárska 5, 833 34 Bratislava, Slovakia*

Abstract. Effect of phorbol myristate acetate (PMA) on P-glycoprotein (P-GP)-mediated vincristine resistance of the multidrug resistant mouse leukemic cell line L1210/VCR was studied by one hour lasting incubation of cells in the presence of PMA, and after three days of cultivation in the presence of the same substance. After the incubation with 100 $\mu g.l^{-1}$ PMA the accumulation of [^{3}H]-vincristine by the above cells was significantly depressed. Moreover, full reverse of verapamil-induced stimulation of [^{3}H]-vincristine accumulation was observed in the presence of PMA. In contrary, when cells were cultivated three days in the presence of PMA, only slight but non-significant increase of [^{3}H]-vincristine accumulation was observed. Slight increase of vincristine accumulation by cells cultivated in the presence of PMA was also supported by higher sensitivity of these cells to vincristine.

Key words: P-glycoprotein — Multidrug resistance — Phorbol myristate acetate — Protein kinase C — Vincristine — L1210 cells

Phosphorylation of P-glycoprotein (P-GP) probably plays a role in the regulation of its drug transport activity, i.e., P-GP phosphorylation may be important for the development of multidrug resistance (MDR) phenotype. Several protein kinases may be involved in the regulation of this transport activity. The precise mechanism of P-glycoprotein phosphorylation is not yet known, but Fine et al. (1986, 1988 a) described co-expression of protein kinase C (PKC) and P-GP in multidrug resistant P388/ADR cell line. Thus phosphorylation of P-GP by PKC may be involved in the regulation of P-GP drug transport activity (Chambers et al. 1990). PKC is a Ca-phospholipid dependent protein kinase that phosphorylates several membrane proteins (Nishizuka 1986). P-glycoprotein has been shown to contain several sites with structural features mimicking phosphorylation sites for PKC (Chambers et al. 1994). PKC activity may be elevated by phorbol esters such

as phorbol myristate acetate (PMA) which is generally known as a direct activator of this enzyme. Potentiation of multidrug resistance of human breast cancer cells has been observed under the influence of phorbol esters (Fine et al. 1988 b). All the above facts indicate that protein phosphorylation by PKC may play a role in the regulation of P-GP drug transport activity.

In previous papers (Poleková et. al. 1992; Breier et al. 1994) over-expression of P-GP (induced by long term adaptation of cells on vincristine) accompanied by a reduction of vincristine (VCR) accumulation in L1210/VCR mouse leukemic cell line was described. In the present study we investigated the effects of PMA on [^{3}H]-vincristine accumulation by the above cell line, and cytotoxicity of vincristine to the above cell line.

Cell cultivation was carried out in standard RPMI 1640 medium supplemented with 5% heat inactivated fetal bovine serum and gentamycin in a humidified atmosphere of 5% CO_2 at 37°C. The medium was exchanged every 3–4 days. Cytotoxicity of vincristine was measured by cultivating cells with vincristine (0.25 10 $mg.l^{-1}$) in the presence or absence of PMA 100 $\mu g.l^{-1}$. Viable cells were counted in haemocytometer after staining with methylene blue.

Accumulation of [^{3}H]-vincristine was checked by the following procedure: Cells ($2 \times 10^5/100$ μl) were incubated for 1 hour in full cell cultivation medium containing 0.2 $mg.l^{-1}$ [^{3}H]-vincristine (0.25 $mCi.ml^{-1}$) in humidified atmosphere (5% CO_2) in the presence or absence of both, verapamil (final concentration 20 $\mu mol.l^{-1}$) and PMA (final concentration 100 $\mu g.l^{-1}$) at 37°C. The cells were then spun down ($300 \times g$), washed twice with PBS and resuspended in bidistilled water. Aliquots of the suspension were added to the Bray scintillation solution and measured in a 1214 Rackbeta liquid scintillation counter (LKB, Sweden). Specific radioactivity was expressed in $DPM.mg^{-1}$ membrane protein. The protein content was estimated using the method of Lowry et al. (1951).

Results concerning the effect of verapamil and PMA on [^{3}H]-vincristine accumulation by multidrug resistant cell line are summarized in Fig. 1. A significant increase of [^{3}H]- vincristine accumulation ($p < 0.05$) was observed during the incubation of cells in a medium containing 20 $\mu mol.l^{-1}$ verapamil (from 8.13 ± 1.05 $DPM.mg^{-1}$ cellular proteins for control to 17.03 ± 2.28 $DPM.mg^{-1}$ protein for cells incubated with verapamil). In contrast, PMA present in cultivation medium (100 $\mu g.l^{-1}$) induced a significant depression ($p < 0.05$) of [^{3}H]- vincristine accumulation (to 4.14 ± 0.70 $DPM.mg^{-1}$ protein). The effect of verapamil may be considered as a reversal action of this drug on MDR (for review see Vendrik et al. 1992). On the other hand, the effect of PMA on vincristine accumulation may be discussed from the point of PKC-induced phosphorylation of P-GP (Chambers et al. 1990). However, the possibility that PKC phosphorylated other protein(s) and these phosphorylated proteins were subsequently regulating the P-GP transport activity could not be excluded either. When the cells were incubated for 1 hour

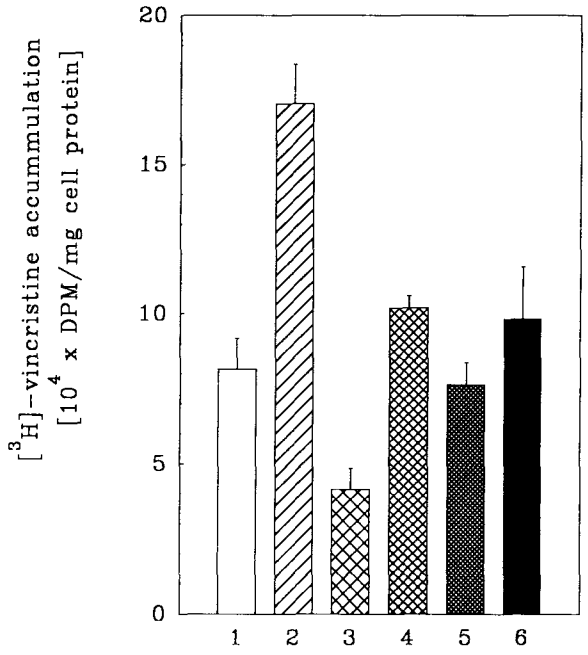

Figure 1. Effect of verapamil and PMA on [^{3}H]-vincristine accumulation by multidrug-resistant mouse leukemic cell line L1210/VCR. Prior to the experiment, the cells were pre-cultivated in the absence (1–4) or in the presence of PMA at concentration 100 $\mu g.l^{-1}$ (5, 6). After the pre-cultivation period the cells were incubated in full cell cultivation medium containing 0.2 $mg.l^{-1}$ [^{3}H]-vincristine (0.25 $mCi.ml^{-1}$) for 1 hour in a humidified atmosphere (5% CO_2) in the absence of verapamil and PMA (1, 5) or in the presence of verapamil in a final concentration of 0.02 $mmol.l^{-1}$ (2, 4) as well as PMA in a final concentration of 100 $\mu g.l^{-1}$ (3, 4, 6) at 37 °C. The result are mean ± S.E.M. from nine measurements.

with both, 20 $\mu mol.l^{-1}$ verapamil and 100 $\mu g.l^{-1}$ PMA the accumulated radioactivity was 10.02 ± 0.92 $DPM.mg^{-1}$ protein (Fig. 1.) indicating that the effects of these substances were mutually interfering. In other words, PMA is able to abolish the reversal effect of verapamil on MDR. On the other hand, verapamil like other calcium entry blockers may inhibit the PKC activity (Schatzman et al. 1981).

Differences in response of the cells to short-term and long-term exposure to PMA were also observed. Prior to the experiment, the cells were precultivated for 96 hours in the presence of 100 $\mu g.l^{-1}$ PMA. After the precultivation, the cells accumulated [^{3}H]- vincristine during subsequent incubation with the labelled drug similarly as did controls (Fig. 1 bars 1 and 5). When PMA was present during the incubation of the cells with [^{3}H]- vincristine, an only nonsignificant increase of

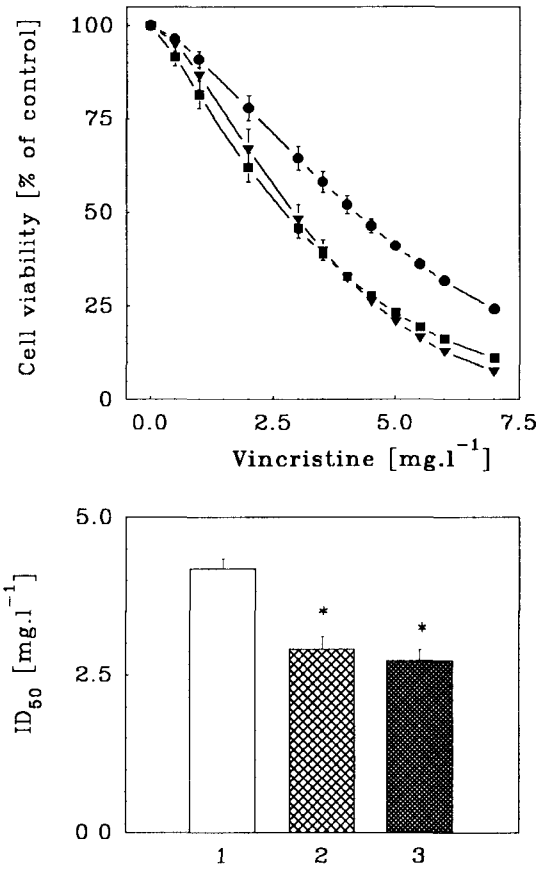

Figure 2. Effect of PMA on vincristine cytotoxicity of multidrug-resistant mouse leukemic cell line. Prior to the experiment, the cells were pre-cultivated in the absence of PMA (circles, and in the upper panel, 1 in the lower panel) or in the presence of 100 μg.l^{-1} PMA (squares and triangles in the upper panel, 2 and 3 in the lower panel). After the pre-cultivation, the cells were cultivated in the presence of vincristine only (circles and triangles in the upper panel, 1 and 2 in the lower panel) as well as in the presence of both vincristine and 100 μg.l^{-1} PMA (squares in the upper panel, 3 in the lower panel). The cells were counted using a haemocytometer. The results are mean $\pm$ S.E.M. from 6 independent measurements. $*$ – significance $p < 0.05$

[^{3}H]-vincristine accumulation from 7.62$\pm$0.74 DPM.mg^{-1} (in the absence of PMA) to 9.83$\pm$1.72 DPM.mg^{-1} (in the presence of PMA) was observed, in contrast to a significant depression of the drug accumulation induced by the same concentration of PMA in non preincubated cells (Fig. 1). This suggested that long-term cultivation of the cells in the presence of PMA caused a defect in the PMA stimulatory action on the drug transport activity of P-GP. Downregulation of PKC under long-term influence of phorbol esters described by Shoji et al. (1987) might be discussed from this point of view. Thus, when cells were cultivated in the presence of PMA, a depression of PKC activity could be expected. Lower activity of PKC may be accompanied by a lower drug transport activity of P-GP in these cells. This notion compares well with the data in Fig. 2, that the presence of PMA in the cultivation medium shifts down the ID_{50} value for vincristine (from 4.17 $\pm$ 0.17 mg.l^{-1} for control to 2.90 $\pm$ 0.21 mg.l^{-1} for cells cultivated in the presence of PMA and to 2.71 $\pm$ 0.23 mg.l^{-1} for cells twice cultivated in the presence of PMA). Neverthe-

less, resistant cells (L1210/VCR) remained resistant after cultivation with PMA and were more than 200 times less sensitive to vincristine than were nonmultidrug resistant cells (parental L1210) showing ID_{50} value of about 0.01 mg.l^{-1} (Poleková et al. 1992; Breier et al. 1994).

Acknowledgements. This work was supported in part by Slovak Grant Agency for Science (grant No. 2/571/93). The authors are grateful to Slovakofarma (Hlohovec, Slovakia) for partial support of this work.

References

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

Chambers T. C., Mc Avoy E. M., Jacobs J. W., Eilon G. (1990): Protein kinase C phosphorylates P-glycoprotein in multidrug resistant human KB carcinoma cells. J. Biol. Chem. **265,** 7679—7686

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

Fine R. L., Patel J., Hamilton T. C., Cowan K., Curt G. A., Friedman M. A., Chabner B. A. (1986): Activation of protein kinase C (PKC) increases vincristine (VC) efflux and resistance in drug sensitive MCF-7 cells. Proc. Amer. Assoc.Cancer Res. **27,** 271

Fine R. L., Patel J., Carmichael J., Cowan K. H., Chabner B. A. (1988a): Phosphoprotein and protein kinase C changes in human multidrug-resistant cancer cells. In: Mechanisms of Drug Resistance in Neoplastic Cells. (Eds. P. V. Woolley, K. D. Tew), p. 87, Academic Press, San Diego CA

Fine R. L., Patel J., Chabner B. A. (1988b): Phorbol esters induce multidrug resistance in human breast cancer cells. Proc. Nat. Acad. Sci. USA **85,** 582—586

Lowry O. H., Rosenbrought N. J., Farr A. L., Randall R. J. (1951): Protein measurement with Folin-phenol reagent. J. Biol. Chem. **193,** 265—275

Nishizuka Y. (1986): Studies and perspectives of protein kinase C. Science **233,** 305—312

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

Schatzman R. C., Wise B. C., Kuo J. F. (1981): Phospholipid-sensitive calcium-dependent protein kinase: inhibition by anti-psychotic drugs. Biochem. Biophys. Res. Commun. **98,** 669—676

Shoji M., Girard P. R., Charp P. A., Koeffler H. P., Vogler W. R., Kuo J. F. (1987): Effect of phorbol ester on translocation and down-regulation of protein kinase C and phosphorylation of endogenous proteins in human acute myeloid leukemia cell line KG-1 and its phorbol ester-resistant subline KG-1a. Cancer Res. **47,** 6363—6368

Vendrik C. P. J., Bergers J. J., De Jong W. H., Steerenberg P. A. (1992): Resistance to cytostatic drugs at the cellular level. Cancer Chemother. Pharmacol. **29,** 413—429

Final version accepted April 3, 1995