Short Communication

Reversal Effect of Specific Inhibitors of Extracellular-Signal Regulated Protein Kinase Pathway on P-glycoprotein Mediated Vincristine Resistance of L1210 Cells

J Kišucká[1], M Barančík[1], V Boháčova[2] and A Breier[2]

1 *Institute for Heart Research, Slovak Academy of Sciences, 842 33 Bratislava, Slovakia*
2 *Institute of Molecular Physiology and Genetics, Slovak Academy of Sciences, Bratislava, Slovakia*

Abstract. Effect of specific inhibitors of extracellular-signal regulated protein kinase (ERK) pathway, PD98059 and UO126, on P-glycoprotein (Pgp)-mediated vincristine resistance of L1210/VCR cells was investigated Both test inhibitors significantly reduced the survival of L1210/VCR cells in the presence of vincristine and this was associated with a decrease of LC_{50} values to vincristine from 2 65 ± 0 43 to 0 67 ± 0 28 μmol/l and to 0 69 ± 0 09 μmol/l after treatment with 50 μmol/l PD98059 and 25 μmol/l UO126, respectively Moreover, the effects of PD98059 are connected also with an increased intracellular accumulation of radiolabeled vincristine in resistant L1210/VCR cells in concentration dependent manner The results of this study demonstrate that inhibitors of ERK signaling pathway are reversal agents of vincristine resistance in L1210/VCR cells The precise mechanism of PD98059 and UO126 action in modulation of MDR is not resolved yet, but the role of ERK-mediated phosphorylation cascade could be considered

Key words: P-glycoprotein — Multidrug resistance — Extracellular-signal regulated protein kinases — Inhibitors — Vincristine — L1210 cells

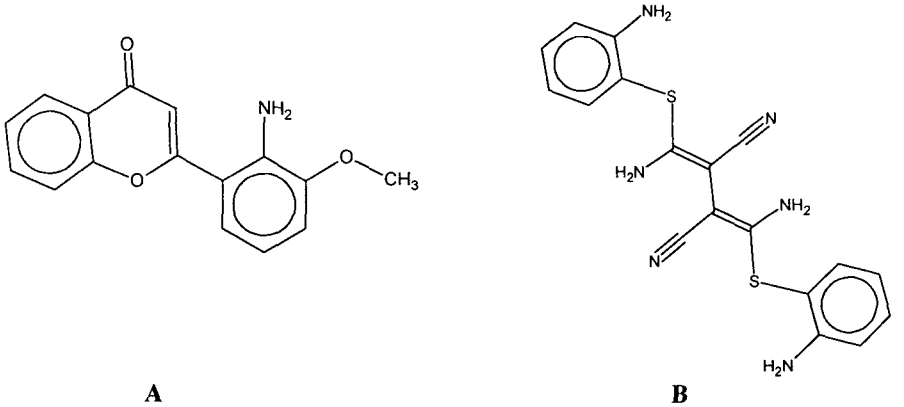
P-glycoprotein (P-gp) is a membrane drug transporting system that causes resistance of neoplastic cells against a wide range of cytotoxic agents (for review, see Litman et al 2001, Kvačkajova-Kišucka et al 2001) The mechanism of this resistance, known as multidrug resistance (MDR), involves reduction of intracellular accumulation of these drugs by P-gp-mediated transport It has been found that P-gp contains sites with structural features mimicking phosphorylation sites for some protein kinases, such as PKC and PKA (Chambers et al 1994) Results of

Correspondence to Dr Albert Breier, Institute of Molecular Physiology and Genetics, Slovak Academy of Sciences, Vlarska 5, 833 34 Bratislava, Slovakia
E-mail usrdtylo@savba sk

other studies also suggest that phosphorylation of P-gp can accelerate the P-gp-mediated drug transport (Sato et al 1990, Ratnasinghe et al 1998) As we have found previously, the multidrug resistance in L1210/VCR cells (cells adapted originally to vincristine) can be influenced by the action of protein kinases C activator as well as inhibitor of p38 MAPK signaling pathway (Barančik et al 1995, 2001) Another kinase pathway that can be activated by PKC and acts paralell to p38-MAPK is extracellular-signal regulated protein kinases (ERKs) cascade The ERKs are Ser/Thr protein kinases that belong to the family of mitogen-activated protein kinases (MAPKs) and which are activated mainly by trophic and mitogenic factors (Robinson and Cobb 1997, Bogoyevitch et al 1994) The ERKs play an important role in regulation of several cellular processes, such as proliferation and differen tiation Several studies suggest also the involvement of MAPKs (ERKs) in the acquired resistance of cancer cells to cytostatic drugs (Osborn and Chambers 1996, Wu et al 1998, Yang et al 2001)

In the present study we tested the effect of two specific inhibitors of ERK signaling pathway, PD98059 and UO126 (Fig 1), on P-gp-mediated multidrug resistance Sensitive (L1210) and multidrug resistant (L1210/VCR) mouse leukemic cell lines were used as an experimental model Multidrug resistant cells were prepared by adaptative cultivation of sensitive cells with vincristine, overexpresion of P-glycoprotein is the main characteristic feature of these resistant cells (Polekova et al 1992) Other specific features concerning crossresistance towards cytostatic agents other than vincristine and, if any, only minor role of glutathione detoxifi cation system for multidrug resistance of this cell line were described previously (Bohačova et al 2000, Breier et al 2000)

Cell cultivation was carried out in standard RPMI 1640 medium supplemented with 4% heat inactivated fetal bovine serum and gentamycin in the atmosphere of 5% CO_2 at 37°C Cytotoxicity of vincristine was measured by cultivating cells with vincristine (0–5 4 μmol/l) in the presence or absence of either PD98059 (5, 10, 25 and 50 μmol/l) or UO126 (3 125, 6 25, 12 5 and 25 μmol/l) Viable cells



A B

Figure 1. Chemical structure of PD98059 (A) and UO126 (B)

were counted in haemocytometer after staining with methylene blue. The effect of PD98056 on growth of L1210/VCR cells was also investigated The resistant cells were cultivated in the presence or absence of 10 μmol/l and 50 μmol/l PD98059 and in the presence of 0.1% DMSO (control). In several time periods (3, 6, 9, 27, 30, 33, 51, 54, 57 hours) the amounts of viable cells were determined and data were expressed in % of control (0 h). Accumulation of [^{3}H]-vincristine after PD98059 treatment was controlled by the following procedure· cells (2×10^5/100 μl) were incubated for 24 hours in medium containing 0.22 μmol/l [^{3}H]-vincristine (0 25 mCi/ml) in the absence or presence of PD98059. Concentrations of ERK cascade inhibitor used were 12.5, 25 and 50 μmol/l. After 24 hours the numbers of viable cells were determined and the cells were then spun down by centrifugation (1200 $\times$ g, 3 min) After washing with cold PBS the cellular pellets were resuspended

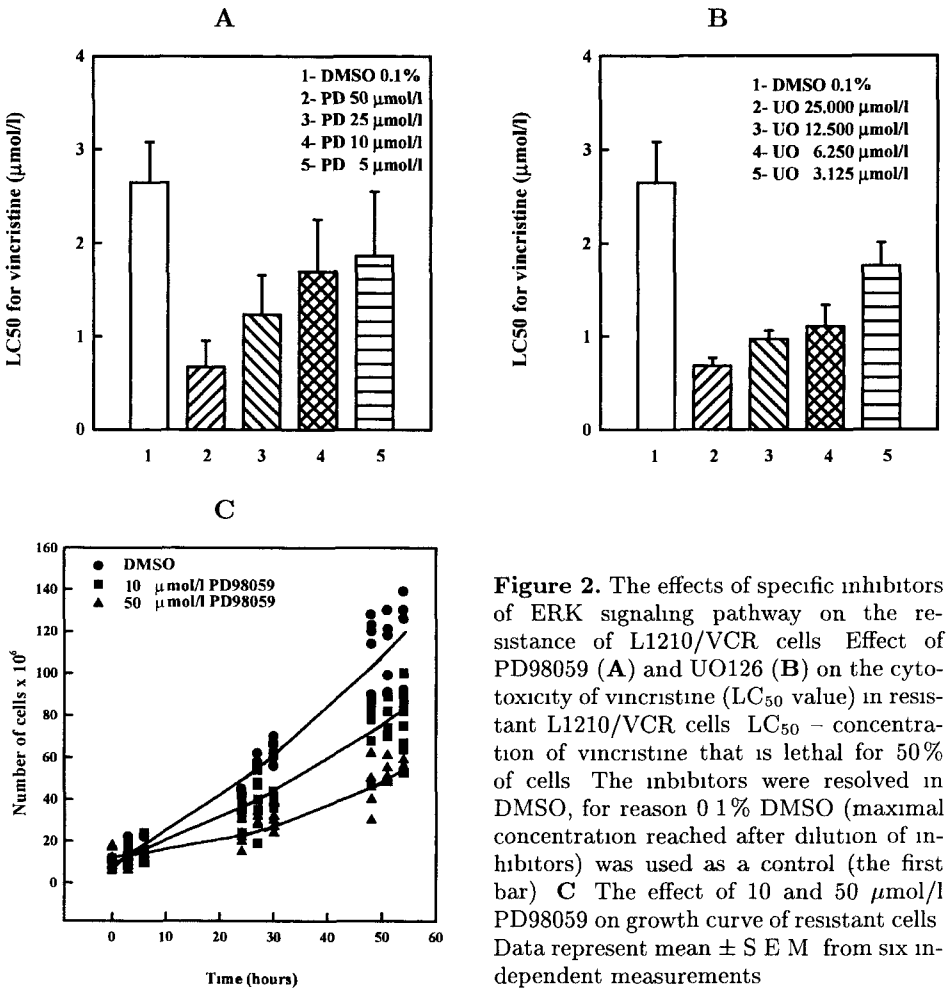


Figure 2. The effects of specific inhibitors of ERK signaling pathway on the resistance of L1210/VCR cells Effect of PD98059 (**A**) and UO126 (**B**) on the cytotoxicity of vincristine (LC_{50} value) in resistant L1210/VCR cells LC_{50} – concentration of vincristine that is lethal for 50% of cells The inhibitors were resolved in DMSO, for reason 0 1% DMSO (maximal concentration reached after dilution of inhibitors) was used as a control (the first bar) **C** The effect of 10 and 50 μmol/l PD98059 on growth curve of resistant cells Data represent mean ± S E M from six independent measurements

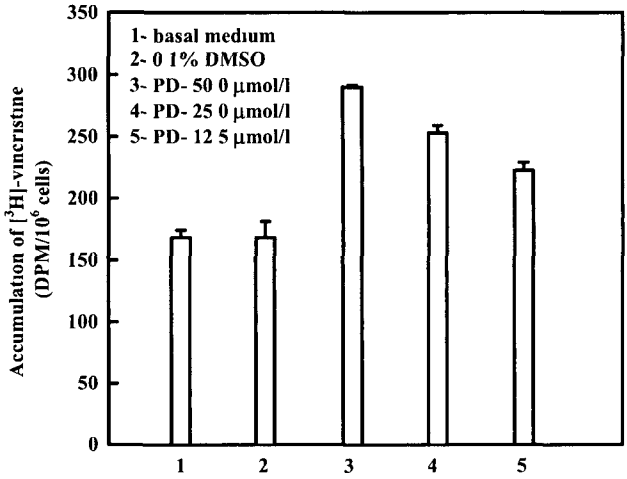


Figure 3. Influence of PD98059 on intracellular accumulation of [^{3}H]-vincristine in resistant L1210/VCR cells Accumulation of [^{3}H]-vincristine was determined after 24 hours cultivation in the presence or absence of 12 5, 25 and 50 μmol/l PD98059 or after treatment with DMSO (negative control) Specific radioactivity was expressed in DPM/10^6 cells Data represent mean ± S E M from six independent measurements

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/10^6 cells

We have found that the exposure of L1210/VCR cells to both PD98059 and UO126 resulted in reduction of their resistance to vincristine in concentration dependent manner (Fig 2A and B) The LC_{50} values for vincristine dropped down from 2 65 ± 0 43 to 0 67 ± 0 28 μmol/l and to 0 69 ± 0 09 μmol/l after treatment with 50 μmol/l PD98059 and 25 μmol/l UO126, respectively At lower concentra tions, effects of tested inhibitors on vincristine resistance were not so expressive In other experiments the reversal impact of PD98059 inhibitor on L1210/VCR cells growth was observed As shown in Figure 2C, after cultivation of resistant cells with this inhibitor, the lag phase of growth curve prolonged This prolongation was dependent on concentration of tested inhibitor Results concerning the effect of PD98059 on [^{3}H]-vincristine accumulation by multidrug resistant cell line are summarized in Fig 3 Maximal increase of [^{3}H]- vincristine accumulation was ob served during the incubation of cells in a medium containing 50 μmol/l PD98059 from 168 ± 6 dpm/10^6 cells (control) to 290 ± 2 dpm/10^6 cells

The effect of PD98059 and UO126 on vincristine resistance of L1210/VCR cells suggests that both substances are reversing agents of P-gp mediated MDR They represent specific inhibitors of ERK signaling pathway with action at the level of MEK1/2 and ERKs (Alessi et al 1995, Favata et al 1998) This implicates also the role of ERK pathway in MDR modulation However, the precise action mechanism

of both PD98059 and UO126 is not resolved yet. As we have found previously, in L1210/VCR cells no significant changes in levels of ERKs were occured when compared to sensitive cells (Barančík et al. 1999). Neverthless, results of our preliminary studies indicate that the presence of vincristine stimulates the increased phosphorylation of ERKs in resistant cells (not shown). The possible role of ERK signaling pathway in development of multidrug resistance phenotype has been suggested in a recent study (Yang et al. 2001), where heat stress and growth factors were found to induce increased expression of endogenous MDR1 mRNA in human renal carcinoma cells. The authors suggested involvement of phospholipase C activation and important role of Raf-MAPK (ERK) pathway in mediating of MDR1 expression. Moreover, as they reported, the inhibition of ERK signaling pathway by PD98059 and UO126 blocked the expression of MDR1. Also some protein kinase C blockers were found to influence P-gp mediated drug transport (Chambers et al. 1992; Gekeler et al. 1996; Hu and Robert 1997; Castro et al. 1999). However, the latter blockers act probably by a mechanism independent of P-gp phosphorylation (Gekeler et al. 1996; Castro et al. 1999). On the other hand, it seems that direct interaction of respective blockers with P-gp molecule may be important It is possible that also reversal effect of ERK cascade inhibitors is mediated through direct interaction of these drugs with P-gp. Neverthless, the ability of ERK pathway inhibition to change phosphorylation of some proteins that could influence the transport properties of P-glycoprotein cannot be excluded either The elucidation of precise mechanism of PD98059 and UO126 action needs further investigations, but the results of our present study clearly show that both inhibitors of ERK signaling pathway can be considered as new reversing agents of P-gp-mediated multidrug resistance

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