EUROPEAN JOURNAL OF PHARMACEUTICAL SCIENCES 29 (2006) 426–434

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LY294,002, a specific inhibitor of PI3K/Akt kinase pathway, antagonizes P-glycoprotein-mediated multidrug resistance

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ARTICLE INFO

Article history:
Received 4 May 2006
Received in revised form
17 July 2006
Accepted 10 August 2006
Published on line 22 August 2006

Keywords:
Mouse leukaemia cells L1210
Multidrug resistance
P-glycoprotein
Vincristine
Akt kinase
Protein kinase inhibitors
LY 294,002
Protein phosphorylation

ABSTRACT

The transmembrane transport pump P-glycoprotein (P-gp) causes the efflux of chemotherapeutic agents from cells and is an important system that secures multidrug resistance (MDR) of neoplastic cells. In the present study drug sensitive L1210 and multidrug resistant L1210/VCR mouse leukemic cell lines were used as an experimental model. We found that LY 294,002, a specific inhibitor of PI3K/Akt kinase pathway, reduced the degree of vincristine resistance in L1210/VCR cells significantly and in a concentration-dependent manner. This was accompanied by decrease in IC_{50} value to vincristine from 3.195 ± 0.447 to 1.898 ± 0.676 μmol/l for 2 μmol/l, to 0.947 ± 0.419 μmol/l for 4 μmol/l, and to 0.478 ± 0.202 μmol/l for 8 μmol/l LY294,002. The IC_{50} value of sensitive cells for vincristine was about 0.010 μmol/l. FACS analysis of the proportion of cells in apoptosis or necrosis by annexin-V apoptosis kit showed the following: (i) vincristine-induced apoptosis in resistant cell to a much lower extent than in sensitive cells; (ii) LY294,002 alone did not induce apoptosis or necrosis in both sensitive and resistant cells; (iii) LY294,002 applied together with vincristine significantly increased the number of apoptotic cells. Transport activity of P-gp in resistant cells was monitored using calcein/AM as substrate and was depressed by LY294,002 in a concentration dependent manner. Significant differences in calcein retention were not observed when cells were preincubated with LY294,002 at different times from 0.5 to 24 h. Sensitive and resistant cells contain similar amounts of uncleaved (i.e., unactivated) caspase-3 but in latter cells the activation of caspase-3 by proteolytic cleavage was decreased. The reversal of vincristine resistance by LY294,002 was associated with marked activation of caspase-3. Western blot analysis revealed that the development of MDR phenotype in L1210/VCR cells was also associated with increased level of Bcl-2 protein.

All the above findings point to the possible involvement of PI3K/Akt kinase pathway in modulation of P-gp mediated multidrug resistance in L1210/VCR mouse leukemic cell line. MDR reversal effect of LY294,002 is accompanied with this compound's influence on vincristine-induced apoptosis.

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doi:10.1016/j.ejps.2006.08.006

1. Introduction

A major problem in cancer chemotherapy is that tumors often develop a multidrug resistance (MDR) phenotype. Many mechanisms may contribute to the drug resistance, including drug inactivation, decreasing the amount of drugs in the intracellular space, influencing (blocking) apoptotic pathway induced by toxins (drugs), and other mechanisms. Drug transport activity of P-glycoprotein (P-gp, a product of the *mdr* gene, ABCB1 member of ABC transporter family) that protects intracellular space of neoplastic cells against elevated drug concentrations is generally known as a predominant cause of MDR (Gottesman and Pastan, 1993; Breier et al., 2005). P-gp exerts broad specificity for transported substances such as several anticancer drugs (vinca alkaloids, anthracyclines, antibiotics), linear and cyclic peptides (like valinomycin and gramicidin) (Wiese and Pajeva, 2001; Kvačkajová-Kišucká et al., 2001), inhibitors of HIV proteases, and several other substances including ethidium bromide, rhodamine 123 (Weaver et al., 1991), calcein/AM (Eneroth et al., 2001), or Fluo-3/AM (Orlický et al., 2004). The drug efflux activity of P-gp represents the mechanism that tumor cells use to escape from death induced by different chemotherapeutics agents. We found previously that modulators of some protein kinases pathways, especially MAPK (ERK, p38-MAPK), can influence drug transport activity of P-gp in multidrug resistant L1210/VCR cell line (Barančík et al., 2001; Kišucká et al., 2001). Also, other studies demonstrated the ability of modulators of protein phosphorylation/dephosphorylation to control the P-gp mediated multidrug resistance (Ding et al., 2001; Ganeshaguru et al., 2002). It is known that P-gp can serve as a substrate for several protein kinases (PKC, PKA, casein kinase II, P-gp specific kinase) but the functional significance of its phosphorylation is not yet clear and some reports indicate that certain protein kinases blockers inhibit drug transport by mechanism independent of P-gp phosphorylation (Castro et al., 1999; Newman et al., 2000). However, in several cancer cells a parallel expression of P-gp and activation and/or expression of mitogen-activated protein kinases (MAPKs), protein kinase C (PKC), Akt kinase was found. Moreover, these protein kinase pathways have been reported to modulate the expression or activity of P-gp in some multidrug resistant cell lines (Barančík et al., 2001; Kuo et al., 2002; Chen et al., 2004). It was also found that blocking these pathways using specific inhibitors (Go697, UO126, SP600125, LY294002) reduced basal levels of P-gp in the rat endothelial cells (Nwaozuzu et al., 2003). These facts point to some positive input of kinase pathways in the expression and function of P-gp.

The susceptibility of cancer cells to apoptosis induced by chemotherapeutic drugs depends on the balance between important pro-apoptotic and anti-apoptotic (survival) signals. ERK (extracellular-signal regulated kinases) and PI3K/Akt kinase cascades play key roles in regulation of apoptotic responses and are known as important survival cell signaling pathways. Inhibition of these systems may also be crucial from the point of effective cancer chemotherapy. It was found previously that specific inhibitors of ERK pathway (PD98059 and UO126) reversed the drug resistance in L1210/VCR cells (Kišucká et al., 2001). Recently it was found that the phosphatidylinositol-3-kinase (PI3K/Akt) pathway inhibitor LY294,002 blocks drug transport from HT29RDB colon carcinoma cells overexpressing MRP1 protein (Abdul-Ghani et al., 2006). The potentiation of doxorubicin-induced apoptosis was connected with increased drug accumulation, blocking of Akt kinase phosphorylation, and activation of caspases 9 and 3. On the other hand, LY294,002 induced a caspase-3-dependent cleavage of P-gp in human VBL100 T-lymphoblastoid CEM cells (Mantovani et al., 2006).

The aim of our present study was to investigate the effects of LY294,002 on P-glycoprotein-mediated vincristine resistance, transport activity of P-gp, and on vincristine-induced apoptosis in L1210/VCR cells.

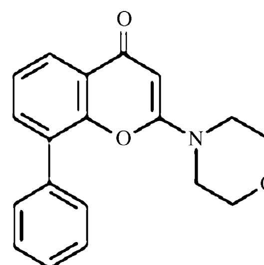
2. Materials and methods

2.1. Cell culture

Parental sensitive mouse leukemic cell line L1210 (S) and multidrug resistant cell line L1210/VCR (R) obtained by long-term adaptation of sensitive cells to vincristine (additional details characterizing this cell line were described elsewhere (Boháčová et al., 2000; Fiala et al., 2003; Sulová et al., 2005)) were used as the experimental model. Both sensitive and resistant cells were under baseline conditions grown in RPMI-1640 medium supplemented with 1 mg/ml L-glutamine, 4% fetal bovine serum and 1 μl/ml gentamycin (all from Life Technologies, Scotland, UK), in an atmosphere of 5% CO_2 at 37 °C.

2.2. Effect of LY294,002 on sensitive L1210 and resistant L1210/VCR cells

LY294,002 (structure see in Fig. 1) is known as a specific inhibitor of the PI3K/Akt kinase pathway. The influence of LY294,002 (Alexis Biochemicals) on the survival of sensitive L1210 and resistant L1210/VCR cells was tested by cultivation of these cells in the presence or absence of LY294,002 (concentration range 0–80 μM). Stock solution of LY294,002 (40 mM) was prepared in dimethylsulfoxide (DMSO); for this reason control cultivations have been done in the presence of DMSO (concentrations equal to the corresponding cultivation with LY294,002). After a cultivation period of 3 days, the viable cells were counted in a haemocytometer. Cytotoxicities of LY294,002 were evaluated as decrease of cell survival induced by its presence in cultivation medium (IC_{50} values for LY294,002).



2-(4-Morpholiny1)-8-pheny1-4*H*-1-benzopyran-4-one

Fig. 1 – Chemical structure of LY294,002.

2.3. Effect of LY294,002 on vincristine resistance of L1210/VCR cells

The effect of LY294,002 on sensitivity of L1210/VCR cells to vincristine was measured by cultivation of cells in the medium containing vincristine (0–5 μM) in the presence or absence of LY294,002 (concentrations 2, 4, 8 μM). Stock solution of LY294,002 (40 mM) was prepared in dimethylsulfoxide (DMSO); during cultivation DMSO was present with a concentration of 0.05%. For this reason, cultivation of cells in the presence DMSO alone in the same concentration served as a negative control. After a cultivation period of 3 days, the viable cells were counted in a haemocytometer. Multidrug resistance reversal effect was evaluated as decrease of IC_{50} for vincristine induced by the presence of LY294,002.

2.4. Detection of apoptotic cells by FACS analysis

Induction of apoptosis was detected by flow cytometry using Annexin V-FITC Apoptosis detection kit (Calbiochem). Sensitive or resistant cells were preincubated for 24 h in the absence or presence of vincristine (1 μg/ml), LY294,002 (2, 4 and 8 μmol/l) in a 37 °C, 5% CO_2 incubator. After this incubation, cells were washed two times with PBS and resuspended in 500 μl of binding buffer (10 mM HEPES/NaOH, pH 7.5 containing 140 mM NaCl and 2.5 mM $CaCl_2$) at concentration approximately 1×10^6 cells/ml. The cell suspension was added to plastic test tubes and to each cell suspension 1.25 μl of Annexin V-FITC (resulting concentration 0.5 μg/ml) and 10 μl of propidium iodide (resulting concentration 0.6 μg/ml) were added. The tubes were incubated at room temperature for 10 min, and thereafter the fluorescence of the cells was determined using a Coulter Epics Altra flow cytometer.

2.5. Calcein/AM assay

S or R cells (5×10^5) were washed two times in PBS containing 0.2% bovine serum albumin, filled up to 200 μl with the same buffer and were preincubated in the absence or presence of vincristine (1 μg/ml), LY294,002 (2, 4 and 8 μmol/l) for 30 min and 24 h in a 37 °C, 5% CO_2 incubator. Calcein/AM (Sigma–Aldrich, resulting concentration 200 nmol/l) and propidium iodide (resulting concentration 0.6 μg/ml) were added directly to the incubation medium after the preincubation period and then the samples were incubated for 20 min at 37 °C. After incubation, cells were washed two times in ice-cold PBS containing 0.2% BSA. Fluorescence measurements were made in the Coulter Epics Altra flow cytometer.

2.6. Preparation of cytosolic and particulate protein fractions

The cells were homogenized in ice-cold buffer A containing (in mM): Tris–HCl 20.0, EGTA 1.0, DTT 1.0, sodium orthovanadate 0.1, PMSF 0.5 (pH 7.4). After homogenisation with a pestle Teflon homogeniser, the homogenate was spun down at 14,000 × *g* for 30 min at 4 °C. The supernatant represented the cytosolic fraction, the pellet containing also nuclei was resuspended in buffer A containing 0.2% Triton X-100 and was designed as a particulate fraction. Protein concentrations were determined according to the method of Bradford (1976) using bovine serum albumin as a standard.

2.7. Immunoblot analysis

Proteins (50 μg/lane) were separated on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Anti-Akt kinase, anti-Bcl-2 (from Santa Cruz Biotechnology), specific anti-phospho-Akt kinase (reacts with phosphorylated Akt kinase (Ser473)), anti-caspase-3, anti-cleaved caspase-3 (all from Cell Signaling Technology), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Chemicon International) antibodies were used as the primary antibodies. The peroxidase labelled anti-rabbit or anti-mouse immunoglobulins (Amersham) were used as the secondary antibodies. Peroxidase reactions were detected by the ECL Western blot detection method.

3. Results

3.1. Effect of LY294,002 on vincristine resistance of L1210/VCR cells

The exposure of L1210/VCR cells to LY294,002 resulted in significant reduction of resistance of these cells against vincristine in a concentration dependent manner. This reduced resistance was accompanied by lowering of IC_{50} value to vincristine from 3.195 ± 0.447 to 1.898 ± 0.676 μmol/l for 2 μmol/l, to 0.947 ± 0.419 μmol/l for 4 μmol/l, and to 0.478 ± 0.202 for 8 μmol/l LY294,002 (Fig. 2A). For sensitive L1210 cells represented IC_{50} to vincristine 0.0102 ± 0.0004 μmol/l and the presence of LY294,002 did not infuence the sensitivity of these cells to VCR since 0.0112 ± 0.0005, 0.0114 ± 0.0006 and 0.0098 ± 0.0005 μmol/l were obtained as IC_{50} to vincristine in the presence of 2, 4 and 8 μmol/l LY294,002, respectively. The resistance index (ratio of IC_{50} for resistant and sensitive cells) changed from 313 for L1210/VCR cells cultivated in the absence of LY294,002 to 190, 95, and 48 for cells in the presence of 2, 4, and 8 μmol/l LY294,002.

3.2. Cytotoxicity of LY294,002 on sensitive and resistant cells

The influence of LY294,002 on survival of sensitive L1210 and resistant L1210/VCR cells was tested by cultivating these cells in the presence or absence of this substance (concentration range 0–80 μmol/l). We did not observe significant differences in the effects of LY294,002 on the viability of sensitive and resistant cells. This is documented by IC_{50} values to LY294,002 which represented 7.6 ± 0.2 μmol/l for sensitive L1210 and 8.9 ± 0.3 μmol/l for resistant L1210/VCR cells (Fig. 2B).

3.3. Effect of LY294,002 on induction of apoptosis in sensitive and resistant cells

Induction of apoptosis was detected by flow cytometry using Annexin V-FITC Apoptosis detection kit. Necrotic changes were determined using propidium iodide. Results demonstrate that the presence of vincristine significantly increased the percentage of early apoptotic and late apoptotic or necrotic

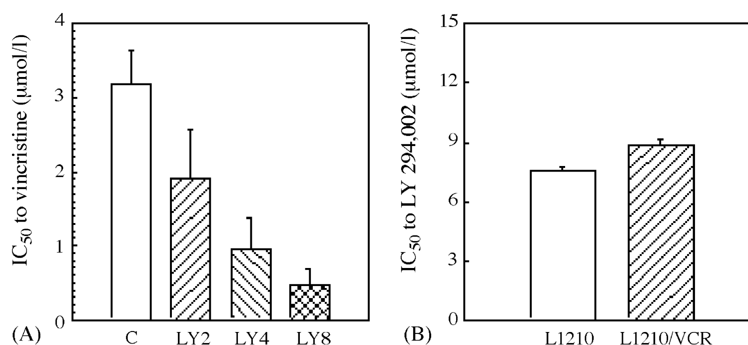


Fig. 2 – The effects of specific PI3K/Akt inhibitor, LY294,002, on resistance of L1210/VCR cells. (A) Effect of LY294,002 on cytotoxicity of vincristine (IC_{50} value) in resistant L1210/VCR cells. C: cytotoxicity of vincristine on L1210/VCR cells; LY2: cytotoxicity of vincristine on L1210/VCR cells in the presence of 2 µM LY294,002; LY4: cytotoxicity of vincristine on L1210/VCR cells in the presence of 4 µM LY294,002; LY8: cytotoxicity of vincristine on L1210/VCR cells in the presence of 8 µM LY294,002. IC_{50}: concentration of vincristine that is lethal for 50% of cells. (B) Cytotoxicity of LY294,002 on sensitive L1210 and resistant L1210/VCR cells. IC_{50}: concentration of LY294,002 that is lethal for 50% of cells.

cells in sensitive L1210 (Fig. 3B) but not in resistant L1210/VCR (Fig. 3F) cells. LY294,002 alone did not induce changes in the number of sensitive or resistant cells in early or late apoptosis and/or necrosis. Fig. 3C and G show the effect of 4 µmol/l LY294,002, and similar effects were also found for 2 and 8 µmol/l LY294,002. However, the presence of both vincristine and LY294,002 induced an increase in number of early apoptotic and late apoptotic and/or necrotic cells in both sensitive and resistant cells. Fig. 3D and H document the effect of 4 µmol/l LY294,002. Similar effects were found for 2 and 8 µmol/l LY294,002.

3.4. *Effect of LY294,002 on transport activity of P-glycoprotein*

Calcein/AM was described as a substrate of P-gp and calcein formed via the intracellular deesterification of calcein/AM is a substrate of MRPs (Litman et al., 2000). Cells in our experiments were preincubated with VCR (1 µg/ml), LY294,002 (2, 4, and 8 µmol/l) and loaded with calcein/AM. Samples were evaluated by flow cytometry. We found that sensitive cells L1210 were extensively loaded by calcein (Figs. 4 and 5A). The presence of vincristine and LY294,002 (documented are results

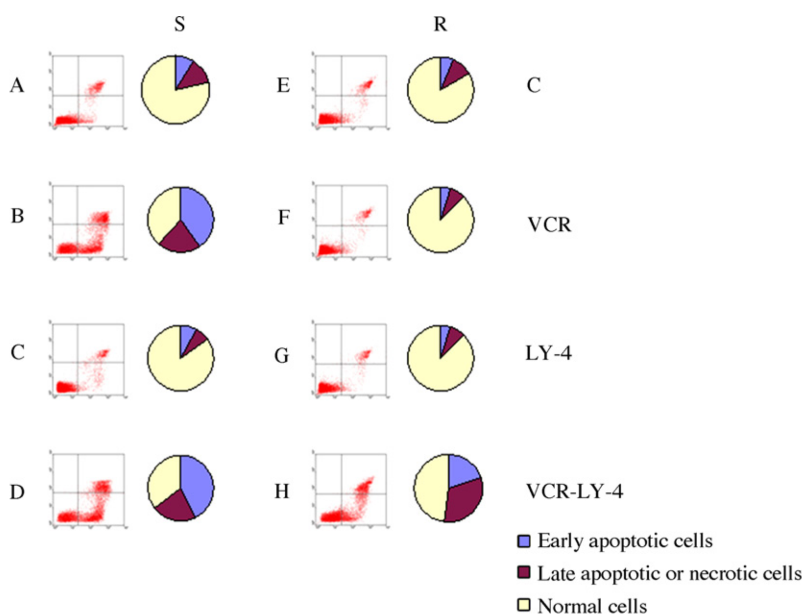


Fig. 3 – The effects of vincristine (VCR) and LY294,002 (LY) on apoptosis induction in sensitive (S) and multidrug resistant (R) cells. FACS histograms show the effects on cells exposed to 1 µg/ml VCR and/or 4 µmol/l LY294,002 for 24 h. Induction of apoptosis was detected by flow cytometry using Annexin V-FITC Apoptosis detection kit (Calbiochem). Fluorescence of the cells was determined using a Coulter Epics Altra flow cytometer. Lower right quadrant in FACS histograms shows early apoptotic cells, upper right quadrant shows late apoptotic and/or necrotic cells. The proportion of early apoptotic, late apoptotic/necrotic, and normal cells are summarized on pie diagrams for the respective experimental groups. C: control cells; VCR: cells treated with 1 µg/ml vincristine; LY-4: cells treated with 4 µmol/l LY294,002; VCR-LY-4: cells treated with 1 µg/ml vincristine and 4 µmol/l LY294,002.

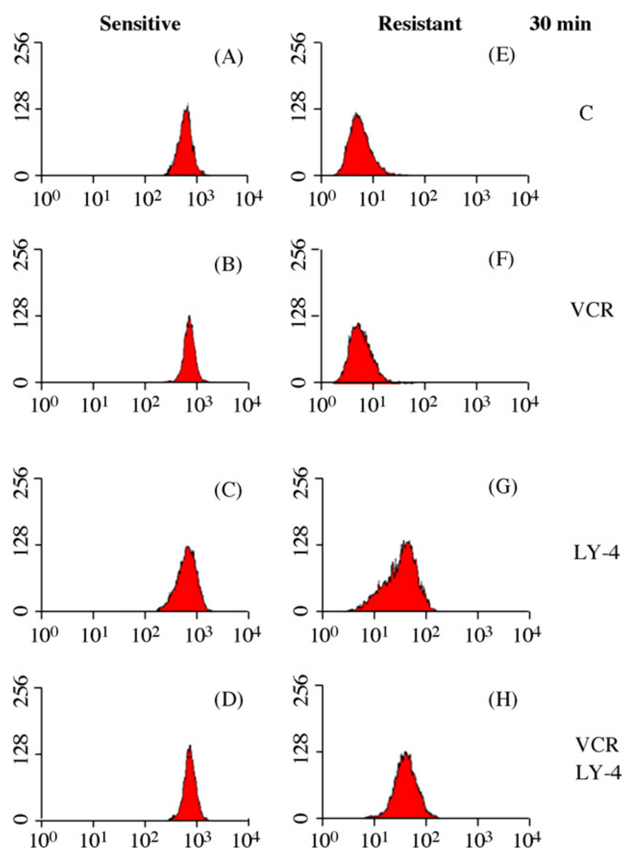


Fig. 4 – Representative FACS histograms showing the effects of 1 μg/ml vincristine (VCR) and 4 μmol/l LY294,002 (LY) on calcein retention in sensitive L1210 (S) and resistant L1210/VCR (R) cells. Cells were exposed to VCR and/or LY294,002 for 30 min. Intensity of calcein fluorescence was measured using FACS analysis. C: control cells; VCR: cells treated with 1 μg/ml vincristine; LY-4: cells treated with 4 μmol/l LY294,002; VCR-LY-4: cells treated with 1 μg/ml vincristine and 4 μmol/l LY294,002.

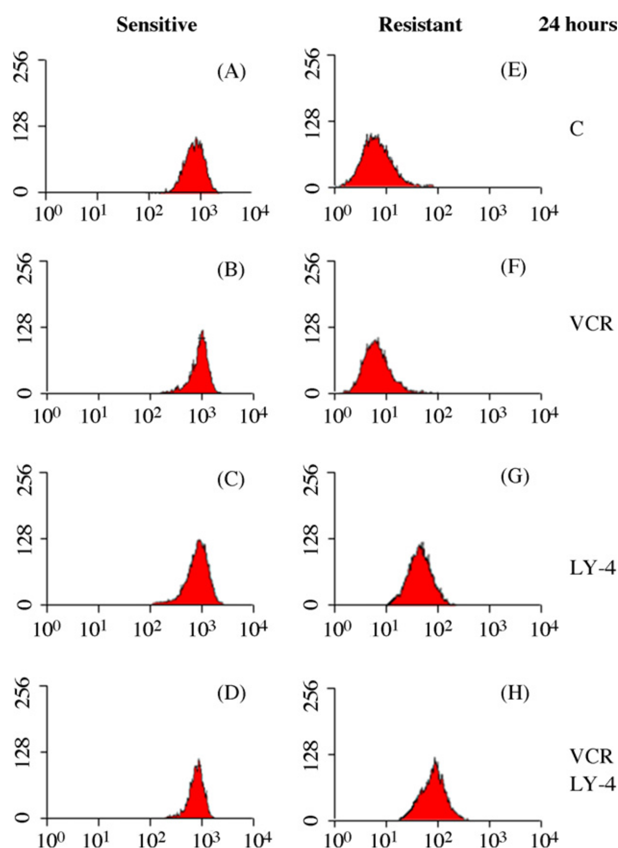


Fig. 5 – Representative FACS histograms showing the effects of 1 μg/ml vincristine (VCR) and 4 μmol/l LY294,002 (LY) on calcein retention in sensitive L1210 (S) and resistant L1210/VCR (R) cells. Cells were exposed to VCR and/or LY294,002 for 24 h. Intensity of calcein fluorescence was measured using FACS analysis. C: control cells; VCR: cells treated with 1 μg/ml vincristine; LY-4: cells treated with 4 μmol/l LY294,002; VCR-LY-4: cells treated with 1 μg/ml vincristine and 4 μmol/l LY294,002.

obtained with 4 μmol/l LY294,002) did not influence the loading of sensitive cells with calcein (Figs. 4 and 5B–D). In contrast to sensitive cells, P-gp in resistant L1210/VCR cells prevented the cells from retaining calcein (Figs. 4 and 5E). This effect was in resistant cells not altered by presence of vincristine (Figs. 4 and 5F). However, preincubation of L1210/VCR cells with LY294,002 for both 30 min (Fig. 4G) and 24 h (Fig. 5G), significantly influenced the loading with calcein. Moreover, the effects of LY294,002 were concentration – but not time-dependent (Fig. 6). The presence of both vincristine and LY294,002 did not induce additional changes in comparison to effects of LY294,002 alone (Figs. 4 and 5H).

3.5. *Effect of LY294,002 on protein levels and activation of Akt kinase*

Levels of Akt kinase in cytosolic and particulate fractions isolated from the multidrug resistant L1210/VCR cells were comparable with the levels of this kinase found in sensitive L1210 cells (Fig. 7). The incubation of cells for 24 h in the presence of vincristine and LY294,002 did not influence the protein levels of Akt kinase. The Akt kinase activity is stimulated via its phosphorylation mediated by upstream-located protein kinases. For this reason, we investigated the content of phosphorylated (activated) form of Akt kinase using antibody that reacts specifically with Ser473 phosphorylated Akt kinase. When sensitive and resistant cells were compared, we did not observe significant differences in Ser473 phosphorylation of Akt kinase. Also, the incubation of resistant L1210/VCR cells in the presence of VCR did not significantly influence the phosphorylation of Akt kinase. However, after exposure of resistant cells for 24 h to 4 μmol/l LY294,002 we found significantly decreased content of phosphorylated Akt kinase in particulate fraction (Fig. 7B). In cytosolic fraction, the amount of activated Akt kinase decreased only slightly (Fig. 7A).

3.6. *Effect of LY294,002 on protein levels and activation of caspase-3*

Caspase-3 is a key executioner of apoptosis. We did not find significant differences in the levels of its inactive zymogen between sensitive L1210 and resistant L1210/VCR cells.

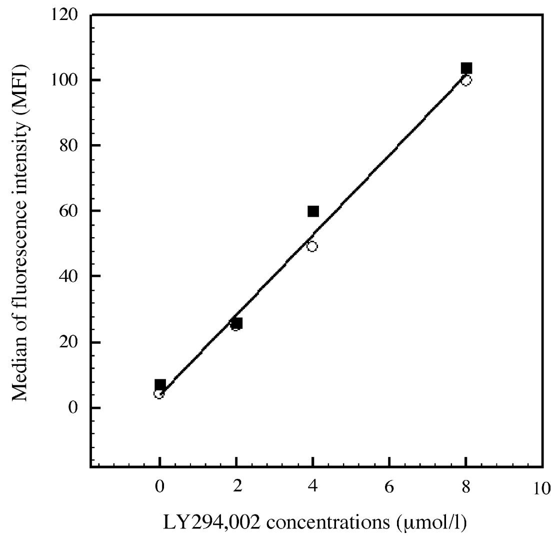


Fig. 6 – Graph showing the concentration dependent effects of LY294,002 on the intensity of calcein fluorescence in L1210/VCR cells. (○) Cells were exposed to LY294,002 for 30 min; (■) cells were exposed to LY294,002 for 24 h.

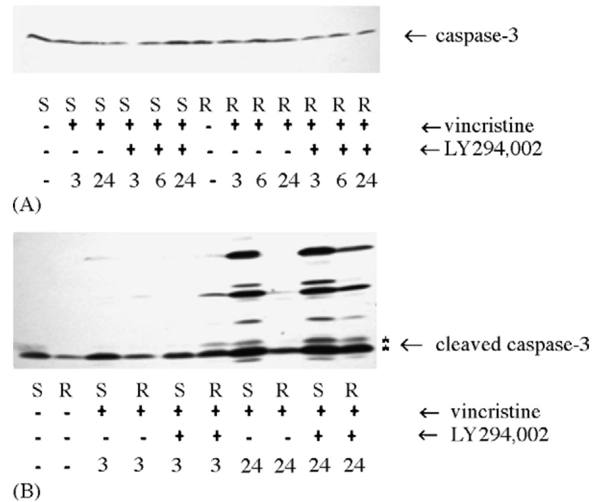


Fig. 8 – (A) Effect of vincristine and LY294,002 treatment on levels of caspase-3. The sensitive L1210 and resistant L1210/VCR cells were treated at several time points (3, 6, and 24 h) with 4 μmol/l LY294,002, 1 μg/ml vincristine; the caspase-3 kinase levels were determined by Western blot analysis using specific antibody. (B) Effect of vincristine and LY294,002 treatment on activation of caspase-3. The sensitive L1210 and resistant L1210/VCR cells were treated at several time points (3, 6, and 24 h) with 4 μmol/l LY294,002, 1 mg/l vincristine; the levels of fragments (17/19 kDa) of activated caspase-3 were determined using specific antibody.

Also, the presence of 1 μg/ml vincristine and/or 4 μmol/l LY294,002 did not influence the levels of inactive caspase-3 (Fig. 8A). Activation of caspase-3 requires proteolytic processing of its inactive zymogen into activated p17 and p12 fragments. Large fragment (17/19 kDa) levels of activated caspase-3 resulting from cleavage adjacent to Asp175 we determined using specific antibody. By comparison of sensitive and resistant cells, we found increased cleaved caspase-3 in sensitive cells. The presence of vincristine induced in sensitive cells the increase in the level of the 17/19 kDa fragment. Moreover, the presence of vincristine-induced increased immunoreactivity of the antibody with other proteins which were approximately 30 and 55 kDa. Similar effects of vincristine were not observable in resistant cells. Presence of LY294,002 did not have an additional effects on these immunoreactivities in sensitive cells. However, in resistant cells the presence of both LY294,002 and vincristine increased cleavage of caspase-3 (17/19 kDa fragment) and also

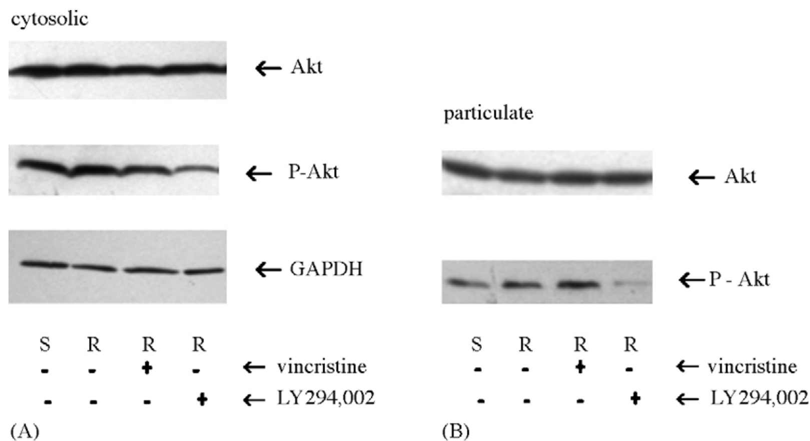


Fig. 7 – (A) Effect of LY294,002 treatment on levels and specific activation of Akt kinase in cytosolic fraction. The sensitive L1210 and resistant L1210/VCR cells were treated for 24 h with 4 μmol/l LY294,002, 1 μg/ml vincristine and the Akt kinase levels were determined by Western blot analysis using specific Akt kinase antibody (upper blot). Western blot record in the middle shows the specific (Ser473) phosphorylation of Akt kinase in sensitive and resistant cells after exposure to vincristine and LY294,002. Blot in lower part shows the levels of GAPDH. (B) Effect of LY294,002 treatment on levels and specific activation of Akt kinase in particulate fraction. Upper blot shows content of total Akt kinase, lower Western blot record shows the specific (Ser473) phosphorylation of Akt kinase.

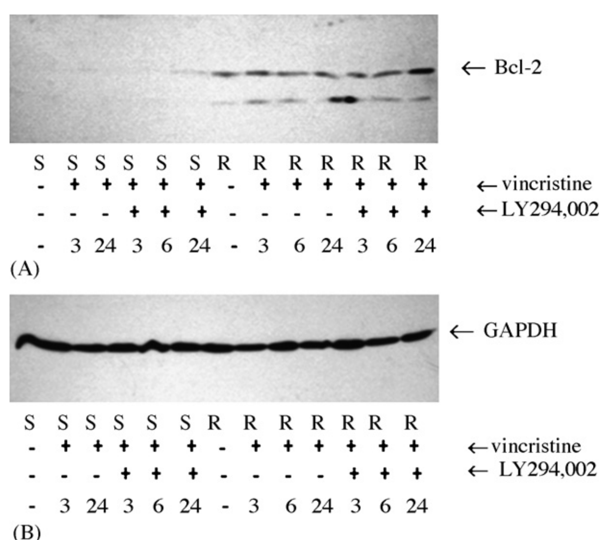


Fig. 9 – (A) Effect of vincristine and LY294,002 treatment on protein levels Bcl-2 protein. The sensitive L1210 and resistant L1210/VCR cells were treated at several time points (3, 6, and 24 h) with 4 μmol/l LY294,002, 1 μg/ml vincristine; Bcl-2 levels were determined by Western blot analysis using specific antibody. (B) Effect of vincristine and LY294,002 treatment on protein levels of GAPDH.

increased immunoreactivity of antibody at 30 and 55 kDa (Fig. 8B).

3.7. *Effect of LY294,002 on levels of Bcl-2 protein*

Bcl-2 has been demonstrated to exert a survival function in response to a wide range of apoptotic stimuli through inhibition of mitochondrial cytochrome c release. We found that resistant L1210/VCR cells had considerably increased levels of Bcl-2 protein when compared to sensitive L1210 cells. The presence of vincristine and/or LY294,002 did not significantly influence the levels of Bcl-2 protein in comparison to control conditions (Fig. 9A). Fig. 9B shows that we did not observe changes in the content of GAPDH as house-keeping protein in individual samples.

4. Discussion

Our most important finding is our observation that the compound LY294,002, a specific inhibitor of PI3K/Akt kinase signaling pathway, may reverse the P-glycoprotein-mediated multidrug resistance of L1210/VCR cells. We found that the exposure of these cells to LY294,002 was associated with reversal of resistance of these cells to vincristine and that was proportional to increased concentrations of LY294,002. On the other hand, the presence of LY294,002 did not modulate the sensitivity (IC_{50} values) of sensitive cells to VCR.

The investigation of LY294002 effects on the content and specific phosphorylation (activation) of Akt kinase, in conditions when the resistance in L1210/VCR cells was reversed, showed that LY294,002 treatment did not significantly change the protein levels of Akt kinase but inhibited the activation of this kinase. These facts indicate that the observed reduction of resistance in L1210/VCR cells under the influence of LY294,002 could involve changes in modulation of anti-apoptotic Akt pathway. Also, regulation of P-gp phosphorylation or indirect regulation of P-gp activities by Akt kinase cannot be excluded. In human hepatoma and embryonic fibroblast 293 cells, 2-acetylaminofluorene (2-AAF) induced activation of *mdr1* gene and this was connected with an activation of PI3 kinase pathway. Using transfection assay, it was also demonstrated that constitutively activated PI3K and Rac1 (downstream effector of PI3K) enhanced the activation of the *mdr1* promoter by 2-AAF (Kuo et al., 2002). These results suggested that the up-regulation of *mdr1* expression is mediated by effectors of PI3K/Akt pathway.

Another possible action of LY294,002 is interaction with P-gp. For some protein kinase blockers, inhibition of P-gp-mediated drug transport by mechanisms independent of P-gp phosphorylation was found (Castro et al., 1999; Newman et al., 2000). The studies suggested that structures of test compounds and their possible direct interaction with P-gp can play an important role in modulation of MDR, independent of or in addition to their other physiological actions. However, sensitive and resistant cells exert similar values of IC_{50} for LY294,002, therefore no differences between effects of LY294,002 alone on L1210 and L1210/VCR cells could be awaited. On the other hand, flow cytometry revealed that LY294,002 may influence the loading with calcein in resistant cells. These findings suggest that LY294,002 can interact with P-gp and so modulate its transport activity. But the fact that the presence of both vincristine and LY294,002 did not induce additional changes in comparison to the effects of LY294,002 alone indicates that the mechanism of LY294,002 action does not involve direct interaction of this compound with vincristine drug-binding site of P-gp. Moreover, LY294,002 treatment significantly influenced the activation of caspase-3 (Fig. 8) and stimulated the vincristine-induced apoptosis (Fig. 3) in multidrug resistant L1210/VCR cells. Acceleration of caspase-3 and caspase-9 activation was induced by LY294,002 in HT29RDB colon carcinoma cells overexpressing MRP (Abdul-Ghani et al., 2006). The PI3K/Akt kinase inhibitor blocked also the Akt phosphorylation and sensitized these drug-resistant cells to doxorubicin-induced apoptosis. Alteration in resistance and drug induced apoptosis may also be a consequence of changes in the expression of genes involved in the Bcl-2 pathway. We found that development of MDR in L1210/VCR cells is associated with up-regulation of Bcl-2 protein levels and decreased content of activated caspase-3. In MCF-7/Bcl-2 cells Raf-1 activation led to increased expression of P-gp; cells overexpressing constitutively active ΔRaf-1 showed higher levels of P-gp mRNA and protein and resistance to doxorubicin (Davis et al., 2003). Also in these cells, correlation between resistance to doxorubicin and Bcl-2 content at the mRNA and protein level were found. Ectopic Raf-1 expression increased both Bcl-2 expression and resistance. This suggests that the P-gp-mediated drug resistance could be in L1210/VCR cells directly related to the amount of Bcl-2 expressed in these cells. We observed decreased content of activated caspase-3 in L1210/VCR cells. Evidence suggests that cell lines expressing high levels of P-gp are less sensitive to caspase-mediated apoptosis induced by wide range of death

stimuli. Moreover, Mantovani et al. (2006) showed that P-gp is cleaved in a caspase-3 dependent manner during apoptosis of human T-lymphoblastoid CEM cells. The role of P-gp in processes of apoptosis was documented also in rat brain endothelial cells. It was found that P-gp levels increase after their exposure to H_2O_2 and LY294,002 inhibited these changes (Nwaozuzu et al., 2003). This suggests that increased P-gp levels can also provide protection of cells against ROS-induced apoptosis.

That LY294,002 was able to depress P-glycoprotein transport activity, was proved by calcein retention assay (Figs. 4 and 5). The fact that time of incubation (0.5 and 24 h) of resistant cells with LY294,002 did not change the quantity of the resulting effect indicates that this substance did not influence the P-gp expression. From this point it differs from the effects of pentoxifylline derivatives that were described to depress the P-gp expression in L1210/VCR cells (Kupsáková et al., 2004). Stimulatory effect of LY294,002 on vincristine-induced apoptosis in L1210/VCR cells was proved using Annexin V-FITC apoptosis detection kit. This effect of Akt kinase inhibitor could not be assumed as specific for P-gp mediated MDR of cells and for vincristine as selection agent because LY294,002 induced similar stimulatory effect also on HT29RDB colon carcinoma cells overexpressing MRP prepared by selection pressure of doxorubicin (Abdul-Ghani et al., 2006). Thus, the idea that suppression of the apoptotic pathway is an independent mechanism that may be additive to P-gp- or MRP1-mediated drug resistance mechanism could not be excluded. However, transport activity of both P-gp and MRP1 may have a common regulatory mechanism that involves PI3K/Akt kinase pathway and therefore its inhibitor similarly influences both P-gp and MRP1 based resistance.

Acknowledgements

This study was supported by Slovak Grant Agency for Science VEGA (grant No. 2/6080/26, 2/4155/26, 2/4154/26) and by APVT (grant No. 51-027-404).

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