Differential Expression of Regulatory Proteins in L1210/VCR Cells with Multidrug Resistance Mediated by P-glycoprotein

M. Barančík[1], V. Boháčová[2], M. Zbýňovcová[2] and 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. Phosphorylation of P-glycoprotein (PGP) by some protein kinases may play an important role in the regulation of its drug transport activity, and may also be important for the development of multidrug resistance (MDR) phenotype. In the present study we investigated the expression of three groups of mitogen-activated protein kinases (MAPKs). The expression of ERKs, SAPK/JNKs and p38-MAPK was studied at the protein level in sensitive (L1210) and multidrug resistant (L1210/VCR) cells. The expression of ERKs in multidrug resistant cells did not differ from those observed in parental sensitive cells. On the other hand, the development of multidrug resistance phenotype in L1210/VCR cells was associated with increased expression of cytosolic p38-MAPK and also proteins of 90 and 130 kDa that react with antibody specific for SAPK/JNKs. The expression of the proteins mentioned was stimulated above all in conditions when vincristine was present in cultivation medium and the stimulation of transport activity of PGP was necessary for the cell survival. The development of multidrug resistance phenotype in L1210/VCR cells was not associated with significant changes in expression of several heat-shock proteins (hsp25, hsp60, hsp70, hsp90). The levels of these proteins were comparable in sensitive L1210 and resistant L1210/VCR cells, and vincristine did not influence the expression of heat-shock proteins in resistant cells.

Key words: P-glycoprotein — Multidrug resistance — Mitogen-activated protein kinases — Heat-shock proteins — Protein expression

Correspondence to M Barančík, Institute for Heart Research, Slovak Academy of Sciences, Dúbravská cesta 9, 842 33 Bratislava, Slovakia.
E-mail usrdmiro@savba.savba.sk

Introduction

MDR of neoplastıc cells represents a specıfic type of resıstance when cells become cross-resıstant to a varıety of structurally and functıonally dıfferent drug groups Thıs type of resıstance ıs often medıated by transport actıvıty of P-glycoprotein, and the degree of the resıstance should be changed by ınfluencıng of thıs transport actıvıty (Juliano and Lıng 1976) The P-glycoprotein molecule contaıns sequences showıng strong sımılarıtıes wıth phosphorylatıon sıtes of some protein kınases (Chambers et al 1994) In murıne P-glycoprotein three phosphorylatıon sıtes for basıc-dırected protein kınases have been ıdentıfied (serınes 665, 669, 681) The lınker regıon of P-glycoprotein was found to be phosphorylated *ın vıvo* also wıthın the acıdıc domaın on olıgopeptıde sequence ın posıtıon 631-658 (Glavy et al 1997) These facts ındıcate that phosphorylatıon of P-glycoprotein may repre sent an ımportant mechanısm ınvolved ın the regulatıon of ıts transport actıvıty In a previous study, we observed changes ın the resıstance of multıdrug resıstant L1210/VCR mouse leukemıc cells to vıncrıstıne, after the exposure of these cells to phorbol myrıstate acetate, a substance known as an actıvator of protein kınases was present (Barančık et al 1995) Thıs was associated wıth changes ın ıntracel lular accumulatıon of vıncrıstıne The results suggested a role of protein kınases, especıally PKC, ın the regulatıon of P-glycoprotein functıon ın multıdrug resıstant L1210/VCR cells PKC medıates the phosphorylatıon of several membrane proteins and ıs able to modulate the actıvıty of ıon channels, calcıum homeosta sıs, metabolısm and also protein synthesıs (Fuller and Sugden 1989, Capogrossı et al 1990) PKC ıs also known to be ınvolved ın the regulatıon of the sıgnal transductıon cascade where cytoplasmıc and nuclear proteins are phosphorylated by the actıon of several addıtıonal protein kınases, maınly mıtogen-actıvated protein kınases (MAPKs) (Davıs 1993) The MAPKs represent a superfamıly of at least three subfamılıes of protein kınases (ERKs, SAPK/JNKs, p38-MAPK) that are ımportant sıgnalıng pathways ınvolved ın the regulatıon of several cellular processes They dıffer ın the mechanısms of theır upstream stımulatıon and also ın substrate specıfıcıtıes The extracellular regulated protein kınases (ERKs) are ac tıvated by several trophıc and mıtogenıc factors, and also by mechanıcal loadıng (Yamazakı et al 1993, Bogoyevıtch et al 1994) The stress-actıvated protein kınase (SAPK/JNK) and the p38-MAPK cascades that operate ın parallel to the ERK cascade are strongly actıvated maınly by cellular stresses lıke hyperosmotıc shock, low concentratıons of protein synthesıs ınhıbıtors, UV, heat stress (Cano et al 1994 Rouse et al 1994) Stress condıtıons are generally known to ınduce overexpresıon of specıfic proteins called heat-shock proteins In recent studıes stress kınases were found to possıbly play an ımportant role ın the regulatıon of the expressıon and phosphorylatıon of some heat-shock proteins (Huot et al 1995) Moreover, also evıdence has been reported lınkıng heat-shock proteins to MDR (Garrıdo et al 1997)

Thus, in the present study we investigated the relation between the expression and function of PGP and the expression of three subfamilies of MAPKs (ERK, SAPK/JNK, p38-MAPK), and the expression of four heat-shock proteins (hsp25, hsp60, hsp70, hsp90) in parental sensitive L1210 and multidrug resistant L1210/VCR cells.

Materials and Methods

Materials

SDS-PAGE reagents, monoclonal antibodies against hsp25, hsp60, hsp70, hsp90 and polyclonal antibody against ERKs were purchased from Bio-Rad (Richmond, USA) and Sigma (St. Louis, USA). Molecular weight markers for electrophoresis were from Pharmacia LKB (Uppsala, Sweden). Goat anti-rabbit and anti-mouse immunoglobulins both linked with horseradish peroxidase, the enhanced chemiluminescence (ECL) reagents, nitrocellulose membranes as well as autoradiography films were obtained from Amersham (England). The polyclonal antibodies against SAPK/JNKs and p38-MAPK were purchased from New England Biolabs.

Cells and culture conditions

Parental sensitive (L1210) and multidrug resistant (L1210/VCR)* cell lines were cultivated in standard RPMI 1640 medium supplemented with fetal bovine serum (4%), glutamine (1mg/ml), gentamycin (1 μg/ml) in an atmosphere of 5% CO_2 at 37°C. Samples of resistant L1210/VCR cells were obtained from cells cultivated for 3 days in the presence or absence of 1 μg/ml vincristine.

Preparation of cytosolic and particulate fractions

The cells were pelletted by centrifugation (at 1200 × g, 3 min), washed two times with PBS, pelletted and finally resuspended in ice-cold buffer A containing 20 mmol/l Tris-HCl, 1.0 mmol/l EDTA, 1.0 mmol/l EGTA, 1.0 mmol/l DTT, 0.5 mmol/l PMSF (pH 7.4). After homogenization in a pestle Teflon-glass homogenizer, the homogenate was spun down at 10,000 × g for 30 min at 4°C. The supernatant represented the cytosolic fraction, the pellet was resuspended in buffer A containing 0.2% Triton X-100, and was considered particulate fraction.

Immunoblot analysis

Soluble or particulate fractions isolated from sensitive and multidrug resistant cells were subjected to SDS-PAGE (35 μg of proteins per lane) in 10 or 15% polyacrylamide gels and proteins were transferred onto nitrocellulose membranes. Anti-ERK, anti-SAPK/JNK, anti-p38-MAPK, anti-hsp25, anti-hsp60, anti-hsp70 and

* The resistant cell subline was obtained by long-term adaptation of parental sensitive cells to vincristine (Poleková et al 1992)

anti-hsp90 antibodies were used as primary antibodies. Peroxidase labeled anti-mouse immunoglobulin (dilution 1:3000) and peroxidase labeled anti-rabbit immunoglobulin (dilution 1·3000) were used as the secondary antibodies. The peroxidase reaction was detected by the ECL Western blot detection method.

Results

Expression of ERKs in parental sensitive L1210 and multidrug resistant L1210/VCR cells

The ERKs are a group of MAPKs that are activated mainly by trophic and mitogenic factors. The best characterized members of the ERK superfamily are ERK-1 and ERK-2, protein kinases with molecular masses of 44 and 42 kDa respectively. We investigated the expression of these protein kinases in cytosolic and particulate fractions isolated from parental sensitive (L1210) and multidrug resistant (L1210/VCR) cells. In the case of L1210/VCR cell line, we used cells cultivated for three days in the presence or absence of 1 mg/l vincristine. Fig. 1 shows the cytosolic and particulate fractions after their separation by SDS-PAGE. The separated proteins in gels were stained with Coomassie Brilliant Blue R 250. The records show the protein profiles and also document the equivalent amounts of proteins used in the assays. The development of MDR in L1210/VCR cells did not include significant changes in the expression of ERK-1 and ERK-2 (Fig. 2) The levels of

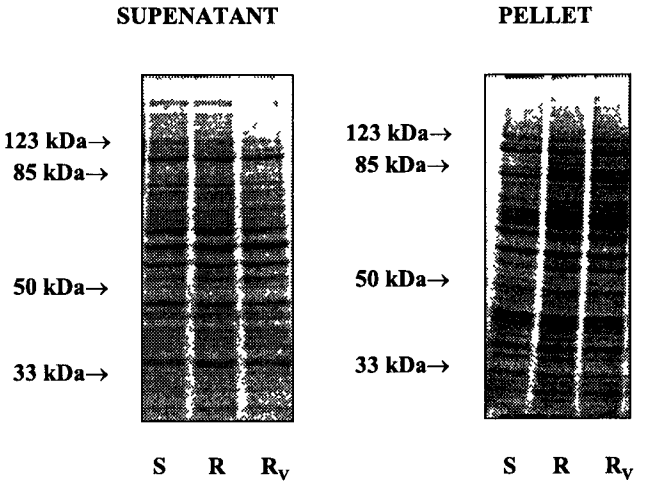

Figure 1. Cytosolic (supernatant) and particulate (pellet) proteins (35 μg per lane) isolated from parental sensitive L1210 and multidrug resistant L1210/VCR cells were separated in 15% gels by SDS-PAGE and stained with Coomassie Brilliant Blue R 250 S – sensitive cells, R – resistant cells, R_V – resistant cells cultivated in the presence of 1mg/l vincristine The arrows show the position of molecular mass marker proteins

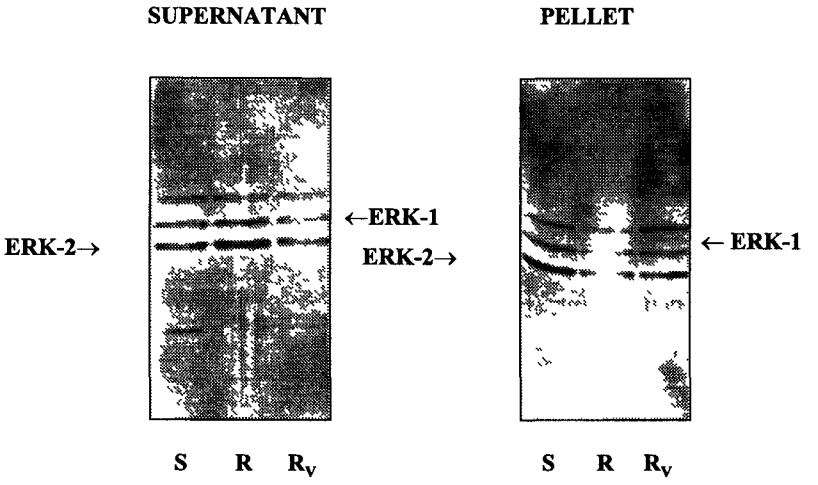

Figure 2. Western blot record showing the expression of extracellular-regulated protein kinases (ERKs) in cytosolic (supernatant) and particulate (pellet) fractions of parental sensitive L1210 and multidrug resistant L1210/VCR cells *S* – sensitive cells, *R* – resistant cells cultivated in the absence of vincristine, R_V – resistant cells cultivated in the presence of 1 mg/l vincristine The amount of proteins loaded for electrophoretic separation was 35 μg per lane The arrows indicate the position of ERK-1 and ERK-2

both protein kinases in cytosolic or particulate (Fig. 2) fractions from multidrug resistant L1210/VCR cells (investigated using specific antibody) did not differ from those observed in parental sensitive L1210 cells

Expression of SAPK/JNKs in parental sensitive L1210 and multidrug resistant L1210/VCR cells

The SAPK/JNKs are protein kinases activated mainly by stress processes. The antibody specific for JNK-1 and JNK-2 (two best characterized members of the SAPK/JNKs superfamily) reacted in Western blot assay with four major proteins of ≈46, ≈55, ≈90 and ≈130 kDa (Fig. 3). The 46 and 55 kDa proteins represent JNK-1 and JNK-2. No significant changes in the expression of these two protein kinases were observed between the cytosolic and the particulate fractions from sensitive and multidrug resistant cells (Fig. 3). However, an additional band appeared in the vicinity of 46 kDa JNK-1 in cytosolic fractions isolated from multidrug resistant L1210/VCR cells (Fig 3) This band could represent the phosphorylated form of the protein kinase and the increased phosphorylation also reflects increased stimulation of the protein kinase activity. The identities of the 90 and 130 kDa proteins are not clear Both proteins could represent protein kinases of the SAPK/JNKs superfamily or the reaction is a result of a nonspecific interaction of the antibody with proteins

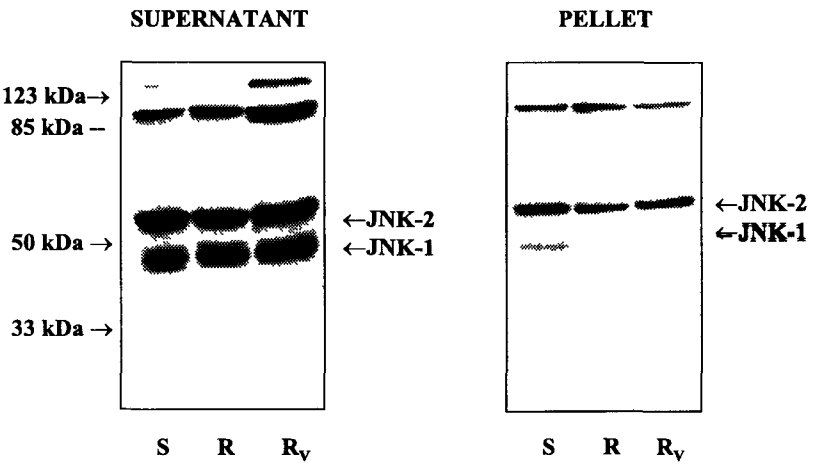

Figure 3. Western blot record showing the expression of stress activated c-Jun N-terminal protein kinases (SAPK/JNKs) in cytosolic (supernatant) and particulate (pellet) fractions of parental sensitive L1210 and multidrug resistant L1210/VCR cells S – sensitive cells, R – resistant cells cultivated in the absence of vincristine, R_V – resistant cells cultivated in the presence of 1 mg/l vincristine The amount of proteins loaded for electrophoretic separation was 35 μg per lane The arrows indicate the position of JNK-1 and JNK-2, the position of molecular mass marker proteins is showed on the left

not related to SAPK/JNKs In cytosolic fractions we found significantly increased levels of both 90 and 130 kDa proteins in multidrug resistant L1210/VCR cells cultivated for 3 days in the presence of 1 mg/l vincristine. Interesting is the fact that, in the absence of vincristine, no significant changes in the expression of these proteins were observed as compared to the parental sensitive cells. These facts suggest that the expression of both the 90 and the 130 kDa protein could play a role when vincristine is present and its active efflux from resistant cells is necessary. In particulate fractions, no significant changes were observed in the expression of JNK-1, JNK-2 or 90, 130 kDa proteins when comparing sensitive and resistant cells (Fig. 3). Also, cultivation of resistant L1210/VCR cells in the presence of vincristine did not induce any additional changes in the levels of these proteins.

Expression of p38-MAPK in parental sensitive L1210 and multidrug resistant L1210/VCR cells

p38-MAPK participate in a cascade controlling cellular responses to cytokines and stress Like SAPK/JNKs the p38-MAPK is activated in response to several cellular stresses. In multidrug resistant L1210/VCR cells the expression of cytosolic p38-MAPK was found to be significantly increased as compared to parental sensitive

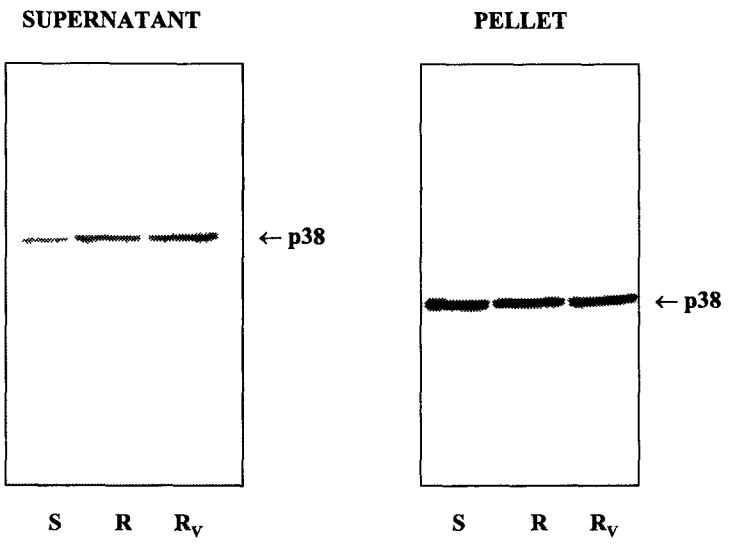

Figure 4. Western blot record showing the expression of p38-MAPK in cytosolic (supernatant) and particulate (pellet) fractions of parental sensitive L1210 and multidrug resistant L1210/VCR cells *S* – sensitive cells, *R* – resistant cells cultivated in the absence of vincristine R_V resistant cells cultivated in the presence of 1 mg/l vincristine The amount of proteins loaded for electrophoretic separation was 35 μg per lane The arrows indicate the position of p38-MAPK

cells (Fig 4) In experimental situation where vincristine was present in cultivation medium, an additional increase in the level of cytosolic p38-MAPK was observed No differences were observed in the expression of this 38 kDa protein kinase in particulate fractions from sensitive and multidrug resistant cells (Fig 4)

Expression of heat-shock proteins hsp25, hsp60, hsp70 and hsp90 in parental sensitive L1210 and multidrug resistant L1210/VCR cells

Fig 5 shows the results obtained from determination of the expression of four heat shock proteins in multidrug resistant L1210/VCR and sensitive L1210 cells No significant changes were observed in the expression of hsp25, hsp70 and hsp90 when comparing the cytosolic fractions of sensitive and multidrug resistant cells When 1 μg/ml of vincristine was present in cultivation medium partially increased expression of hsp60 was observed (Fig 5) With hsp25-specific antibody (Fig 5) no specific reaction could be observed in either the sensitive or the resistant cells Hsp90-specific antibody reacted with two major bands in the 90 kDa region, antibody against hsp70 reacted with a single band when 15% gels were used for the separation of proteins When 10% gels were used for the electrophoretic sep-

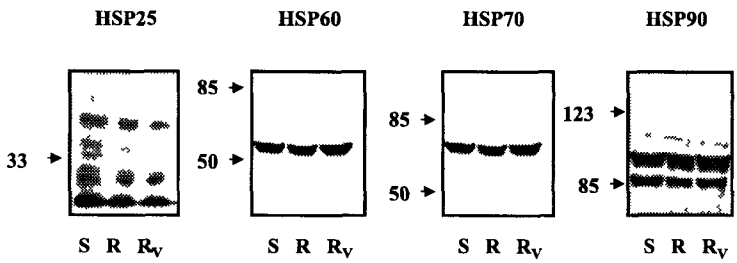

Figure 5. Western blot showing the expression of hsp25, hsp60, hsp70 and hsp90 in cytosolic (supernatant) fractions of parental sensitive L1210 and multidrug resistant L1210/VCR cells *S* – sensitive cells *R* – resistant cells cultivated in the absence of vincristine R_V – resistant cells cultivated in the presence of 1 mg/l vincristine The amount of proteins loaded for electrophoretic separation was 35 μg per lane The arrows on the left indicate the position of molecular mass marker proteins

aration, additional bands were observed in the vicinity of these bands (hsp90 or hsp70) One explanation is that these bands observed in 10% gels represent phosphorylated forms of hsp90 and hsp70 In this case no significant differences between sensitive and resistant cells could be observed either

Discussion

Multidrug resistance in L1210/VCR cells is accompanied by overexpression of the membrane transport protein, P-glycoprotein Several lines of evidence suggest the role of phosphorylation in the modulation of P-glycoprotein transport activity (Fine et al 1986, Chambers et al 1994, Barančík et al 1995) The previous finding that phorbol myristate acetate, a protein kinase C activator, significantly influenced the transport activity of P-glycoprotein in L1210/VCR cells indicated the role of PKC in the regulation of P-glycoprotein activity in these cells (Barančík et al 1995) On the other hand, the down-regulation of PKC by prolonged exposure of L1210/VCR cells to phorbol ester influenced the degree of resistance only partially This fact pointed also to the involvement of other, PKC-independent protein kinase pathways in the regulation of PGP transport activity In the present study we investigated the expression of several mitogen-activated protein kinases (MAPKs) in parental sensitive L1210 and multidrug resistant L1210/VCR cells The family of MAPKs includes at least three subfamilies of protein kinases (ERKs, SAPK/JNKs, p38-MAPK) that are involved in the regulation of several cellular processes (Robinson and Cobb 1997) They differ in the mechanisms of their upstream stimulation and also in substrate specificities We found that the development of multidrug resistance phenotype in L1210/VCR cells was not associated with changes in the

expression of ERK-1 and ERK-2. The activity of ERKs is stimulated in response to several mitogenic or trophic factors that also stimulate cell proliferation (Bogoyevitch et al. 1994; Sadoshima et al. 1995). The finding that the presence of vincristine and the development of multidrug resistance did not enhance ERKs expression suggests that ERKs do not play an important role in the mechanism of PGP-mediated MDR of L1210/VCR cells. The stress-activated protein kinase (SAPK/JNK) and the p38-MAPK cascades that operate in parallel to the ERK cascade are strongly activated mainly by cellular stresses. p38-MAPK is a protein kinase activated by cytokines and some cellular stresses like hyperosmotic or heat shock (Rouse et al. 1994; Galcheva-Gargova et al. 1994). In some experimental models also some metabolic inhibitors such as sodium arsenite have been found to stimulate the activity of this protein kinase (Doza et al. 1995). The finding that the development of MDR in L1210/VCR cells is associated with an enhanced expression of p38-MAPK contrasts with the observed expression of ERKs. Enhanced expression of p38-MAPK in L1210/VCR cells implicates the involvement of this protein kinase in the mechanism of multidrug resistance. There is some possibility that the enhanced expression of p38-MAPK is associated with overexpression of PGP, and that PGP serves as a possible target (substrate) for this protein kinase or protein kinase cascade located downstream of p38-MAPK. Interesting is the fact that cultivation of resistant cells in the presence of 1 μg/ml vincristine induced an additional increase in the level of p38-MAPK. As found previously, the resistance of L1210/VCR cells to vincristine is associated with a decreased intracellular accumulation of vincristine in resistant cells as compared to parental sensitive L1210 cells (Breier et al. 1994). The decreased intracellular accumulation of vincristine was probably connected with an increased efflux of vincristine from cells by PGP transport activity. This suggestion was supported by the finding that verapamil, a known chemisensitizer and a substance that can interact directly with PGP to inhibit its transport activity, suppressed the decrease in intracellular accumulation of vincristine (increased the intracellular accumulation of VCR) (Barančík et al. 1995). All these facts indicate that, in the presence of vincristine, the manifestation of an increased PGP efflux activity is necessary for cell survival. The enhanced expression of p38-MAPK in presence of vincristine suggests the involvement of this enzyme in the mechanisms responsible for the elimination of VCR from L1210/VCR cells. One explanation could be the influence of p38-MAPK on the phosphorylation state of PGP. Interesting are results of recent studies which have indicated the possible role of p38-MAPK in the mechanism of resistance. Overexpression of heat-shock protein 27 (HSP27) by transfection of HT-29 human colorectal cancer cell line was found to increase the resistance of these cells to doxorubicin or cisplatin and to prevent the drug-induced apoptosis (Garrido et al. 1997). In another study HSP27 was shown to be a major target protein for phosphorylation upon stimulation by a variety of agents in Chinese hamster or human cells (Huot et al. 1995), the induction

of HSP27 activity being associated with the activation of MAPKAPK-2, a protein kinase that represents a down-stream located kinase in p38-MAPK cascade and is activated by p38-MAPK mediated phosphorylation. Moreover, MAPKAPK-2 is also used as a substrate for *in vitro* assays of p38-MAPK activity. We also investigated the expression of some heat-shock proteins in parental sensitive L1210 and multidrug resistant L1210/VCR cells but did not observe any significant differences in the expression of hsp25, hsp60, hsp70 and hsp90 at the protein level. In the case of hsp70 and hsp90, the existence of double bands were observed which indicate the presence of phosphorylated forms of these proteins. On the other hand, neither in this case we did observe any significant differences between sensitive and resistant cells. The presence of vincristine in cultivation medium of resistant cells did not induce significant changes in the expression of the heat-shock proteins investigated. The only exception was hsp60 where a moderate increase in the expression of this protein was observed after cultivation of L1210/VCR cells in the presence of 1 μl/ml vincristine (compared with L1210/VCR cells cultivated in the absence of vincristine). The results indicate that the expression of heat-shock proteins is not a factor of significant influence on the multidrug resistance in L1210/VCR cells. However, the role of these proteins in the mechanism of MDR is not excluded. SAPK/JNKs are protein kinases activated in response to stress stimuli. The development of multidrug resistance phenotype in L1210/VCR cells was not found to include changes in the expression of SAPK/JNKs. On the other hand, the occurrence of a band in the vicinity of JNK-1 in resistant L1210/VCR cells points to the presence of another JNK-1-related protein kinase or phosphorylated form of JNK-1. The SAPK/JNKs are phosphoproteins that require phosphorylation for their activation, and the activity depends on the degree of phosphorylation. If the additional band in resistant cells represents the phosphorylated form of JNK-1 this fact may reflect the presence of a more active enzyme in resistant L1210/VCR cells. Osborn and Chambers (1996) found that treatment of KB-3 carcinoma cells with adriamycin, vinblastine or etoposide resulted in activation of JNK. Interesting is the fact that none of the drugs significantly influenced the ERK pathway. In agreement with our observation in the previous study no increase was observed in the protein expression of JNKs in KB-V1 cell line selected for resistance to vinblastine relative to KB-3 cells. On the other hand, an increased JNK activity was observed in MDR KB-V1 cells as compared to KB-3 cells. These results indicate the presence in the MDR cell lines of JNK in a highly activated form. The occurrence of a band close to JNK-1 as observed in our study in resistant cells may also reflect the presence of a more active form of JNK. The JNK-1 and JNK-2 specific antibody reacted with two additional bands of 90 and 130 kDa. The identities of these bands are questionable. They may represent proteins of the SAPK/JNKs superfamily or proteins not related to these protein kinases. The enhanced expression of these proteins in the cytosol of L1210/VCR cells after their cultivation in the presence of

vincristine indicates that these proteins may play a role in the elimination of vincristine from resistant cells. This fact is supported by the finding that, in resistant cells cultivated in the absence of vincristine for the same period of time no changes in the expression of these proteins were observed.

Acknowledgements. This study was supported by Slovak Grant Agency for Science VEGA (grant No 2/4127/97)

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Final version accepted February 24, 1999