SB203580, a specific inhibitor of p38-MAPK pathway, is a new reversal agent of P-glycoprotein-mediated multidrug resistance

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Abstract

P-glycoprotein (P-gp) is the plasma membrane transport pump responsible for efflux of chemotherapeutic agents from cells and is one of the systems that secures multidrug resistance (MDR) of neoplastic cells. In the present study, drug sensitive L1210 and multidrug resistant L1210/VCR (characterized by overexpression of P-gp) mouse leukemic cell lines were used as an experimental model. We have found that SB203580, a specific inhibitor of p38-MAPK pathway, significantly reduced the degree of the vincristine resistance in L1210/VCR cells. This phenomenon was accompanied by a decrease in the LC50 value of vincristine from 3.20±0.52 to 0.55±0.08 μM. The LC50 value of sensitive cells for vincristine was about 0.011 μM. The effect of SB203580 on L1210/VCR cells was associated with significantly increased intracellular accumulation of [3H]-vincristine in the concentration dependent manner. Prolonged exposure of resistant cells to 30 μM SB203580 did neither significantly influence the gene expression of P-gp, nor change the protein levels of p38-MAPK. Western blot analysis revealed that the MDR phenotype in L1210/VCR cells was associated with increased level and activity of cytosolic p38-MAPK. In resistant cells, the enhanced phosphorylation of both, p38-MAPK and ATF-2 (endogenous substrate for p38-MAPK) was found as well. In conclusion we could remark that SB203580, an inhibitor of p38 kinase pathway, reversed the MDR resistance of L1210/VCR cells. MDR phenotype of these cells is connected with increased levels and activities of p38-MAPK. These findings point to the possible involvement of the p38-MAPK pathway in the modulation of P-gp mediated multidrug resistance in the L1210/VCR mouse leukemic cell line. However, the mechanisms of SB203580 action should be further investigated. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Multidrug resistance (MDR) of neoplastic cells describes the phenomenon when cells are resistant to structurally and functionally diverse groups of drugs (Endicott and Ling, 1989; Gottesman and Pastan, 1993). It was found that development of the MDR phenotype is often associated with overexpression and drug transport activity of plasma membrane P-glycoprotein (P-gp; Juliano and Ling, 1976; Gottesman and Pastan, 1993). P-gp is proposed to operate as an ATP-driven efflux pump, transporting the broad spectrum of structurally unrelated cytotoxic drugs (including the Vinca alkaloids, anthracyclines, epipodophyllotoxins, and taxanes) through the plasma membrane (Biedler and Riehm, 1970; Endicott and Ling, 1989; Ford, 1995).

The role of protein phosphorylation in modulation of the multidrug resistance was suggested in several studies (Wu et al., 1998; Ratnasinghe et al., 1998). P-gp contains sites with structural features mimicking phosphorylation sites for protein kinase C (PKC) and protein kinase A (PKA) (Chambers et al., 1994). This transport system is also phosphorylated in vivo and the major phosphorylation domain has been identified as the linker region (amino acids 629–686; Glavy et al., 1997). It is known that P-gp can serve as a substrate for several protein kinases (PKC, PKA, casein kinase II, P-gp specific kinase, Chambers et al., 1994; Chambers et al., 1995; Glavy et al., 1997) but functional significance of its phosphorylation is not clear yet. Several modulators of protein phosphorylation/de-
phosphorylation were found to modulate the P-gp mediated multidrug resistance (Gekeler et al., 1996; Ramachandran et al., 1998; Castro et al., 1999; Sampaio-Maia et al., 1999), but some results indicate that certain protein kinase blockers inhibit drug transport by a mechanism independent of P-gp phosphorylation (Gekeler et al., 1996; Castro et al., 1999).

In our study were used sensitive (L1210) and multidrug resistant (L1210/VCR) mouse leukemic cell lines. Phorbol myristate acetate (PMA, direct activator of PKC) may influence the vincristine resistance of L1210/VCR cells with MDR phenotype mediated by P-gp (Barančík et al., 1995). Moreover, significant changes in content of some mitogen-activated protein kinases were also found in L1210/VCR cells, namely p38-MAPK (Barančík et al., 1999). Based on their results, Wu et al. (1998) also indicated the role of mitogen-activated protein kinases (MAPKs) in acquired resistance of cancer cells to anticancer drugs. In detail, these authors found increased amounts of activated ERKs (extracellular-signal regulated kinases) and p38-MAPK in KTFU-4 resistant cells after treatment with 5-fluorouracil. Moreover, treatment of human KB-3 carcinoma cells with adriamycin resulted in a time- and dose-dependent activation of JNKs (c-Jun N-terminal protein kinases, Osborn and Chambers, 1996).

In the present study we investigated the effect of SB203580, pyridinyl imidazole compound that is known as a specific inhibitor of p38-MAPK signaling pathway (Young et al., 1997), on vincristine resistance of L1210/VCR cell line. Furthermore, the effect of prolonged exposure of resistant cells to SB203580 on expression of p38-MAPK and P-gp was tested. We determined also the levels, phosphorylation state and activities of p38-MAPK in sensitive L1210 and resistant L1210/VCR cells.

2. Materials and methods

2.1. Cell culture

Parental sensitive mouse leukemic cell line L1210 and multidrug resistant cell line L1210/VCR obtained by long-term adaptation of sensitive cells to vincristine (Poleková et al., 1992) were used as an experimental model. Additional details concerning cross-resistance of L1210/VCR cells to other drugs, reversal of this resistance by different chemosensitizers, as well as role of P-gp as a dominant system responsible for MDR phenotype of L1210/VCR cells were described elsewhere (Barančík et al., 1994; Breier et al., 2000; Boháčová et al., 2000). Both, sensitive and resistant cells were grown in RPMI medium supplemented with 4% fetal bovine serum.

2.2. Effect of SB203580 on sensitive and resistant cells and vincristine cytotoxicities

SB203580 (Sigma) is a pyridinyl imidazole compound (structure is shown in Fig. 1) and is known as a specific inhibitor p38-MAPK signaling pathway. The influence of SB203580 on the survival of sensitive L1210 and resistant L1210/VCR cells was tested by culture of cells in the presence or absence of SB203580 in concentration range 0–150 μM. The effect of SB203580 on the sensitivity of L1210/VCR cells to vincristine was measured by a culture of cells in the vincristine containing medium (0–5.4 μM), in the presence or absence of 30 μM SB203580. Stock solution of SB203580 (60 mM) was prepared in dimethylsulfoxide (DMSO) and the culture of cells in the presence 0.05% DMSO served as a negative control. After a culture period of 3 days, cells were stained with trypan blue and counted in a haemocytometer. LC50 values represent medians of the lethal concentrations and were used for evaluation of effects of vincristine and SB203580.

2.3. Measurement of intracellular accumulation of radiolabeled vincristine

Accumulation of [3H]-vincristine in sensitive and resistant cells was determined by the following procedure: cells were cultivated for 24 h with 0.22 μM [3H]-vincristine (0.25 μCi/ml) and in the presence (15, 30 and 60 μM), or absence of SB203580. After the culture period, amounts of viable cells were determined and cells were pelleted by centrifugation (at 1200×g, 3 min), washed with PBS and resuspended in bidistilled water. Aliquots of suspensions were added to the Bray scintillation solution. The radioactivity was measured in a 1214 Rackbeta liquid scintillation counter and specific radioactivity was expressed in DPM/106 cells.

2.4. Preparation of cytosolic and particulate fractions

Cells were homogenised in ice-cold buffer A containing (in mM): Tris–HCl 20.0, EDTA 1.0, EGTA 1.0, DTT 1.0, sodium orthovanadate 0.1, PMSF 0.5 (pH 7.4). After homogenization with a pestle Teflon-glass homogenizer, the homogenate was spun down at 10,000×g for 30 min at 4°C. The supernatant containing the cytosolic fraction was collected and the pellet containing nuclei was resuspended
in buffer A (with 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.

2.5. Immunoblot analysis

Proteins were separated on 10% SDS–polyacrylamide gels and transferred onto nitrocellulose membranes. Anti-p38–MAPK (Santa Cruz), specific anti-phospho-p38–MAPK (reacts only with dual phosphorylated p38–MAPK on both, Thr180 and Tyr182, New England Biolabs) and anti-phospho-ATF-2 (activating transcription factor-2, endogenous substrate of p38–MAPK; New England Biolabs) antibodies were used as primary antibodies. The peroxidase labeled anti-rabbit immunoglobulin (Amersham) was used as the secondary antibody. Peroxidase reactions were detected by the ECL Western blot detection method (Amersham).

2.6. Measurement of p38–MAPK activities by “in gel” phosphorylation

Equivalent amounts of proteins were separated on 10% SDS–polyacrylamide gels containing 0.5 mg/ml of GST-MAPKAP-K2 fusion protein. MAPKAP-K2 is a protein kinase phosphorylated directly by p38–MAPK and is known as a physiological substrate for p38–MAPK (Kumar et al., 1999). Proteins in gels were denatured by incubation with 50 mM Tris–HCl, pH 8.0, containing 6 M guanidine–HCl and renaturation was achieved by incubation with 50 mM Tris–HCl, pH 8.0, containing 0.1% (v/v) Nonidet P-40 and 5 mM 2-mercaptoethanol. The “in gel” phosphorylation of substrate was performed in 40 mM Hepes (pH 8.0), 0.5 mM EGTA, 10 mM magnesium chloride, 1.0 μM protein kinase A inhibitory peptide, 25 μM [γ-32P]-ATP (5 μCi/ml) for 4 h at room temperature. After extensive washing in 5% (w/v) trichloracetic acid containing 2% (w/v) sodium pyrophosphate, gels were dried and quantitative analysis was performed using a Phosphorimager SF (Molecular Dynamics). All additional details were published elsewhere (Barančík et al., 2000).

2.7. Effect of SB203580 on the expression of P-gp in L1210/VCR cells

L1210/VCR cells were exposed for 72 h to treatment with 30 μM SB203580 or 0.05% DMSO (control). RNA was isolated afterwards according to the procedure of Chomczynski and Sacchi (1987), using guanidine isothiocyanate (Fisher Scientific, USA) and phenol–chloroform extraction.

Reverse transcription was done from 3 μg of total RNA using First-Strand cDNA Synthesis Kit (Pharmacia Biotech, Sweden). We used primer pd(N)6 (Pharmacia, Sweden). Specific PCR was performed using MDR1 (5’-CCC ATC ATT GCA ATA GCA GG-3’) and MDR2 (5’-GTT CAA ACT TCT GCT CCT GA-3’) primers (Noonan et al., 1990). After the initial denaturation, 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and polymerization at 72°C for 1 min were performed. The estimated product size was 167 bp. PCR products were analyzed on 2% agarose gels. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) is commonly used as an internal control in many cell systems. Therefore, as a control for semi-quantitative evaluation of PCR, GAPDH primers (GAPDH1: 5’-AGA TCC ACA ACG GAT ACA TT-3’; GAPDH2: 5’-TCC CTC AAG ATT GTC AGC AA-3’) were used to amplify a 309 bp fragment from each first strand molecule. After the initial denaturation at 94°C for 5 min, 30 cycles of PCR at 94°C for 1 min, 60°C for 1 min and 72°C for 3 min were performed.

3. Results

3.1. Effect of SB203580 on the resistance of L1210/VCR cells

The exposure of L1210/VCR cells to 30 μM SB203580 resulted in the significant reduction of resistance of these cells against vincristine. This effect was accompanied by the lowering of the LC50 value of vincristine from 3.203±0.521 to 0.557±0.082 μM (Fig. 2A). For sensitive cells, the LC50 value of vincristine was 0.011 μM and so the resistance index (ratio of LC50 for resistant and sensitive cells) changed from 291 for L1210/VCR cells cultivated in the absence of SB203580 to 51 for cells in the presence of 30 μM SB203580. When SB203580 was applied in lower concentrations (10 μM) the value of resistance index was changed slightly to 217. The effects of SB203580 on L1210/VCR cells were associated with significantly increased intracellular accumulation of [3H]-vincristine (Fig. 2B). Moreover, the elevation of intracellular accumulation of [3H]-vincristine was induced proportionally with an increased concentration of SB203580.

The influence of SB203580 on the survival of sensitive L1210 and resistant L1210/VCR cells was tested by a culture of these cells in the presence or absence of SB203580 (concentration range 0–150 μM). We have found that the LC50 value of SB203580 for sensitive L1210 cells was 39.2±2.2 μM and for resistant cells 52.0±7.6 μM (Fig. 2C).

Investigation of SB203580 effects on the gene expression of P-gp, under conditions when the resistance was reversed, showed that the SB203580 treatment did not significantly change the expression of P-gp (Fig. 3B). Moreover, SB203580 treatment also did not significantly influence protein levels of p38–MAPK (Fig. 3A).
Fig. 2. The effects of specific p38-MAPK inhibitor, SB203580, on the resistance of L1210/VCR cells. A – Effect of SB203580 on the cytotoxicity of vincristine (LC50 value) in resistant L1210/VCR cells. R – Cytotoxicity of vincristine on L1210/VCR cells; SB – cytotoxicity of vincristine on L1210/VCR cells in the presence of 30 μM SB203580; S – cytotoxicity of vincristine on parental sensitive L1210 cells. LC50 – concentration of vincristine that is lethal for 50% of cells. B – Effect of SB203580 on intracellular accumulation of [H]-vincristine in L1210/VCR cells. The resistant cells were treated with 15, 30 and 60 μM SB203580 or 0.1% DMSO (negative control) and the accumulation of vincristine was determined as described in Materials and methods. R – resistant L1210/VCR cells cultivated in the basal medium; RD – L1210/VCR cells cultivated in the presence of 0.1% DMSO (negative control); SB1 – resistant cells treated with 60 μM SB203580; SB2 – resistant cells treated with 30 μM SB203580; SB3 – resistant cells treated with 15 μM SB203580; S – intracellular accumulation of [H]-vincristine in sensitive L1210 cells. Results represent means ± S from three independent measurements. C – Cytotoxicity of SB203580 on sensitive L1210 (S) and resistant L1210/VCR (R) cells. LC50 – Concentration of SB203580 that is lethal for 50% of cells.

3.2. p38-MAPK levels and activities in sensitive L1210 and multidrug resistant L1210/VCR cells

Levels of p38-MAPK in a cytosolic fraction isolated from the multidrug resistant L1210/VCR cells were considerably increased in comparison to corresponding data for sensitive L1210 cells (Fig. 4A). In contrast, any significant changes in the levels of this protein kinase in particulate fractions were not observed when the resistant and sensitive cells were compared (not shown). The p38-MAPK activity is stimulated via its phosphorylation mediated by upstream-located protein kinases. For this reason we investigated the content of phosphorylated (activated) form of p38-MAPK in sensitive and resistant cells, using a specific antibody that reacts with dual phosphorylated p38-MAPK (Thr180/Tyr182; New England Biolabs). A significantly higher content of phosphorylated form of cytosolic p38-MAPK was found in resistant cells when compared to sensitive cells (Fig. 4B). Activities of p38-MAPK in sensitive and resistant cells were determined by means of phosphorylation of specific substrates (GST-MAPKAP-K246_400) directly in gel after SDS–PAGE. In L1210/VCR cells we found increased activities of cytosolic p38-MAPK when compared to L1210 cells (Table 1). Changes in the p38-MAPK phosphorylation and activities in L1210/VCR cells were associated also with an increased phosphorylation of ATF-2 in particulate fractions (Table 1). ATF-2 is transcription factor located downstream of p38-MAPK and can serve also as an endogenous substrate for this protein kinase.

4. Discussion

We consider our observation that pyridinyl imidazole compound SB203580, specific inhibitor of p38-MAPK signaling pathway, may reverse the resistance of L1210/VCR cells to vincristine to be the most important finding. As we have found previously, the resistance of L1210/VCR cells to vincristine is mainly P-gp dependent and is associated with decreased intracellular accumulation of vincristine in resistant cells as compared to parental sensitive L1210 cells (Breier et al., 1994; Boháčová et al., 2000). These facts indicate that SB203580 is a new reversing agent of P-gp-mediated multidrug resistance. SB203580 is a pyridinyl imidazole derivative and is
known to be a specific inhibitor of p38-MAPK (Young et al., 1997). We found that the exposure of multidrug resistant L1210/VCR cells to SB203580 was associated with a reversal of resistance of these cells to vincristine and with significantly increased intracellular accumulation of [\(^{3}H\)]-vincristine. Moreover, the elevation of intracellular accumulation of [\(^{3}H\)]-vincristine was induced proportionally with increased concentration of SB203580. The investigation of SB203580 effects on the gene expression of P-gp, in conditions when the resistance was reversed, showed that SB203580 treatment did not change significantly the gene expression of mdr1 gene for P-gp. Moreover, SB203580 treatment did not significantly influence protein levels of p38-MAPK. These facts indicate that the observed reduction of resistance in L1210/VCR cells (connected with increased intracellular accumulation of vincristine) under the influence of SB203580 does not involve transcription changes and is most probably associated with the modulation of P-gp transport activity. However, the precise mechanism of SB203580 action is not resolved yet. In the recent study of Newman et al. (2000) an inhibitory effect of OC144-093 (substituted diarylimidazole i.e., substance with a structure partially related to SB203580) on multidrug resistance in several cell line expressing P-gp was described. The action of OC144-093 was connected with the inhibition of [\(^{3}H\)]-azidopine binding to P-gp and P-gp ATPase activities. Moreover, some PKC blockers were found to inhibit the
P-gp-mediated drug transport by mechanisms independent of P-gp phosphorylation (Gekeler et al., 1996; Castro et al., 1999). These results suggest that structures of com-

Table 1

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<th>Substrate</th>
<th>S</th>
<th>R</th>
<th>R&lt;sub&gt;VCR&lt;/sub&gt;</th>
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<tr>
<td>ATP-2</td>
<td>14.1±7.0</td>
<td>100.0±6.7</td>
<td>53.3±8.7</td>
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<tr>
<td>MAPKAP-K2</td>
<td>50.2±9.5</td>
<td>100±7.6</td>
<td>120±9.6</td>
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Activities of p38 MAPK were determined: (i) By Western blot analysis using a phospho-specific ATF-2 antibody where the phospho-ATF-2 levels were determined. Differences in content of the phosphorylated form of this transcription factor in particeulate fraction isolated from sensitive and resistant cells were compared. The results are expressed as a relative intensity of band after scanning and quantification of records after ECL detection. Intensity of signals from resistant cells in the absence of vincristine was arbitrarily taken as 100%. (ii) By in gel phosphorylation of MAPKAP-K2 as a substrate. The relative phosphate radioactivity was obtained after quantitative analysis of respective gel using Phosphorimager SF. Intensity of signals from resistant cells in the absence of vincristine was arbitrarily taken as 100%. The results are means±S<sub>E</sub> from five independent measurements.

The fact that L1210 cells were only slightly more sensitive to SB203580 as L1210/VCR cells (resistance index 1.3) is interesting. In the case, when SB203580 is a substrate for P-gp, a much higher resistance of L1210/VCR cells to this compound compared to sensitive cells could be expected. Based on results obtained with substituted diarylimidazol OC144-093 (Newman et al., 2000), it is possible to suggest that SB203580, an imidazol derivative, may act on MDR similarly, through direct interaction with P-gp.
However, its inhibitory effect on the p38-MAPK signaling pathway enables us to expect the involvement of this kinase cascade in direct or indirect regulation of P-gp activity.

In the present study, not only the effects of SB203580 on resistance, but also changes of p38-MAPK in sensitive and resistant cells were tested. We observed changes in levels, phosphorylation and activity of cytosolic p38-MAPK in MDR L1210/VCR cells when compared to sensitive L1210 cells. The p38-MAPK is a protein kinase that belongs to the mitogen-activated protein kinase (MAPK) superfamily and the p38-MAPK signaling pathway is activated mainly by cytokines, some kind of stress (UV radiation, metabolic stress, hyperosmotic, heat shock; Robinson and Cobb, 1997; Doza et al., 1995). Changes in content and activities of MAPKs were observed also in other experimental models with developed multidrug resistance phenomenon. In KTFU-4 resistant cells increased amounts of activated ERKs and p38-MAPK after treatment with 5-fluorouracil were observed (Wu et al., 1998) and in human KB-3 carcinoma cells it was found that treatment with Adriamycin resulted in a time- and dose-dependent activation of JNKs (Osborn and Chambers, 1996). These results show that in several cell lines the development of MDR with selecting agent (vincristine, adriamycin, etc.) led also to significant changes in such regulatory systems chemically stressed KB cells. FEBS Lett. 364, 223–228.

References

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References


