doi: 10.4149/gpb_2014015

Vincristine-induced expression of P-glycoprotein in MOLM-13 and SKM-1 acute myeloid leukemia cell lines is associated with coexpression of nestin transcript

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Abstract. Nestin is a class 6 filament protein typically expressed in neural stem/progenitor cells. However, nestin expression has been observed in other tissues during mammalian embryogenesis. In human neural stem/progenitor cells, coexpression of nestin and P-glycoprotein (P-gp, ABCB1 member of the ABC transporter family) was detected. P-gp-mediated drug efflux is the most common molecular cause of multidrug resistance in neoplastic cells. Nestin expression has also been detected in various human solid tumours as well as in the corresponding established cell lines. Interestingly, expression of nestin in different leukemia cells has been recently reported. Here, we show that expression of P-gp is associated with the simultaneous expression of nestin in acute myeloid leukemia cell lines (MOLM-13 and SKM-1) under the selective pressure of vincristine, a substance that may induce P-gp expression in neoplastic cells.

Key words: P-glycoprotein — Nestin — Vincristine — SKM-1 AML cell line — MOLM-13 AML cell line

Introduction

Nestin (neural stem cell protein) is a class 6 filament protein that is a dimerization partner of vimentin and α-internexin (class 3 filament proteins) and is believed to be a marker of neural stem/progenitor cells (Gilyarov 2008). Although nestin is typically found in neural stem cells, it has also been detected in other cell types and tissues during mammalian embryogenesis, such as in skeletal muscles, umbilical cord blood, cardiac muscles, sterol and interstitial testicular cells, odontoblasts, hair follicle sheath cells, hepatic cells and renal progenitor cells (reviewed in Krupkova et al. 2010). Nestin expression has also been detected in various types of human solid tumours as well as in the corresponding established cell lines (Krupkova et al. 2010). Further, nestin expression is correlated with cell proliferation rates (Krupkova et al. 2010; Yang et al. 2010; Kruger et al. 2012). Nestin expression has also been detected in blood vessels within glioblastoma, prostate cancer, colorectal cancer, and pancreatic cancer, and its expression is more specific for newly formed blood vessels than other endothelial cell, which makes nestin an angiogenesis marker (Matsuda et al. 2013).

Interestingly, Loja et al. (2013) have shown nestin expression in several leukemia cell lines. In human neural stem/progenitor cells, correlation of nestin and the drug transporter P-glycoprotein (P-gp, ABCB1 member of ABC transporter family) have been described (Yamamoto et al. 2009). This efflux pump transports large groups of structurally unrelated chemicals across biological membranes and is involved in the formation of several barriers, such as the blood-brain barrier and barrier between fetal and maternal circulation (Breier et al. 2005; Molsa et al. 2005). In neoplastic cells, drug efflux activity of P-glycoprotein is the dominant molecular mechanism of cell resistance toward large groups of structurally unrelated anti-

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cancer agents that differ in pharmacological effect, i.e., it causes multidrug resistance (MDR) (Breier et al. 2005, 2013a).

The current study shows that the induction of P-gp expression by vincristine (VCR, a known P-gp inductor (Breier et al. 2013b)) in two acute myeloid leukemia (AML) cell lines (SKM-1 and MOLM-13) is associated with nestin coexpression.

Materials and Methods

Cell culture conditions

Two cell lines derived from patients with AML, which developed from myeloblastic syndrome (supplied by Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany), were used in this study: 1) SKM-1 (ACC 547) – derived from the peripheral blood of a 76-year-old patient (AML M5) and 2) MOLM-13 (ACC 554) – derived from the peripheral blood of a 20-year-old patient (AML FAB M5a). Cells (inoculums 5×10^5) were cultured in 5 ml of RPMI medium containing 12% fetal bovine serum (Biotech, SR), 100,000 units/l of penicillin and 50 mg/l of streptomycin (Sigma Aldrich, USA) for two days in humidified atmosphere containing 5% CO_2 at 37°C. Both cell lines were adapted to vincristine over a 6-month period with repeated passages in medium containing stepwise increases in drug concentrations beginning at 0.01 nmol/l VCR (Gedeon Richter Co., Hungary). This procedure yielded vincristine-resistant SKM-1/VCR and MOLM-13/VCR cell variants capable of growth in medium containing 2.50 nmol/l VCR.

Cell death effect of vincristine on sensitive and resistant variants of SKM-1 and MOLM-13 cells

Cells (5×10^4 cells/well) were cultured either with or without VCR (concentration range 0.1–1000.0 nmol/l) in 96-well culture plates. Vincristine was added directly to 200 µl of culture media as described in previous chapter. After 48 hours, cell viability was assayed using the MTT test (Gerlier and Thomasset 1986), which was performed by adding MTT ([3-(4,5-dimethyldiazol-2-yl)-2,5 diphenyltetrazolium bromide]) to a final concentration of 0.25 mg/ml *per* well. The cells were incubated with MTT for 2 hours. Next, the plates were centrifuged for 15 minutes (2500 rpm), and the cell sediment was extracted using dimethyl sulfoxide. The absorbance at 540 nm was measured using a Universal Microplate Spectrophotometer mQuant (BioTek Instruments, Inc. USA). Dose-response curves were fitted according to an exponential decay equation (Eq. 1) by non-linear regression as previously described (Kupsakova et al. 2004; Sulova et al. 2005):

$$N = 100\% \times \exp\left[\ln(0.5) \times (c/LC_{50})\right] \quad (1)$$

where N represents the percentage (from a control in the absence of vincristine) of cell viability after culture in the presence of VCR at a concentration c. The LC_{50} is the concentration of substance when N = 50%.

Determination of P-gp protein levels in sensitive and vincristine-resistant variants of SKM-1 and MOLM-13 cells

Cells were harvested and crude membrane fractions were prepared using a ProteomeExtract Subcellular Proteome Extraction Kit (Calbiochem, USA) according to the manufacturer's protocol. The proteins from the samples were separated by sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) using 8% polyacrylamide gels (Laemmli, 1970). The proteins were transferred by electroblotting to a nitrocellulose membrane (Towbin et al. 1979). P-gp was detected using the c219 anti-P-gp monoclonal antibody (Calbiochem, USA). A secondary anti-mouse antibody conjugated to horseradish peroxidase (GE Healthcare, USA) was used for detection. To provide an internal control for protein loading, a rabbit polyclonal antibody against GAPDH (Santa Cruz Biotechnology, USA) was used as a primary antibody, and goat anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology, USA) served as a secondary antibody. The protein bands were visualized using an ECL detection kit (GE Healthcare, USA) and a CF 440 imaging system (Kodak, USA).

Examination of P-gp function by the calcein/AM assay in sensitive and vincristine-resistant variants of SKM-1 and MOLM-13 cells

P-gp transport activity was measured using a previously described calcein retention assay (Orlicky et al. 2004). To remove VCR after cultivation, the cells were centrifuged ($500 \times g$) and washed three times with phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin. The washing procedure included three 10-min incubations in buffer to eliminate any VCR retained in the cells. The cells were resuspended in 500 µl of the same buffer. Calcein/AM (final concentration of 0.1 µmol/l; Sigma-Aldrich) and propidium iodide (final concentration of 0.9 µmol/l; Sigma-Aldrich) were added directly to the buffer, and the samples were incubated for 20 min at 37°C in a CO_2 incubator. Verapamil (10 µmol/l), cyclosporine A (0.83 µmol/l) and ketoconazole (10 µmol/l) were used as P-gp inhibitors and were added with Calcein/AM and propidium iodide. After incubation, the cells were washed twice with ice cold PBS. Fluorescence was measured using an Accuri C6 flow cytometer (BD Bioscience, USA). Only viable, non-propidium iodide-stained cells (more than 92% in each case) were counted.

Determination of the mRNA levels of P-gp and nestin in sensitive and vincristine-resistant variants of SKM-1 and MOLM-13 cells

Total RNA was isolated from cells using TRIzol reagent (Life Technology, Slovakia) according to the manufacturer's instructions. Reverse transcription (RT) was performed with 2 μg of DNAse I (Thermo Scientific, Germany)-treated RNA and the RevertAidTM H MinusFirst Strand cDNA Synthesis Kit (Thermo Scientific, Germany) according to the manufacturer's protocol. PCR was performed in a total volume of 25 μl consisting of 4 μl RT mixture, 1 × PCR buffer, 1.5 mmol/l $MgCl_2$, 0.2 mmol/l dNTP, 1 pmol of each specific gene primer set and 0.3 U of DyNAzyme II DNA polymerase (Finnzymes OY, Finland) in the buffer provided by the manufacturer. After treating the samples at 94°C for 3 min to inactivate the reverse transcriptase, the samples were subjected to 30 cycles of denaturation (95°C, 30 s), annealing (57°C, 30 s for P-gp; 57.2°C, 30 s for nestin; and 58°C, 30 s for GAPDH), extension (72°C, 90 s for P-gp and GAPDH; and 72°C, 120 s for nestin) and a final extension at 72°C for 10 min. The PCR products were separated on a 1.7% agarose gel (Invitrogen, Life Technology, Slovakia), and the gel was visualized using ethidium bromide with a Typhoon 9210 imaging system (GE Healthcare, USA, formerly Amersham Biosciences).

The sequences of the primers employed in this study are as follows: GAPDH, F: 5′-TGA ACG GGA AGC TCA CTG G-3′, R: 5′-TCC ACC ACC CTG TTG CTG TA-3′, which yielded a 291 bp product; P-gp, F: 5′-AGA CAT GAC CAG GTA TGC-3′, R: 5′-CTC CTG TCG CAT TAT AGC-3′, which yielded a 429 bp product; nestin, F: 5'-ACA TAC AGG ACT CTG CTG GAG G-3', R: 5'-TGA GGA CAG GGA GCA CAG AT-3', which yielded a 154 bp product.

Results and Discussion

Adaptation of MOLM-13 and SKM-1 cells to vincristine (see Materials and Methods) yields MOLM-13/VCR and SKM-1/VCR cell variants with reduced sensitivity to vincristine (Fig. 1). VCR resistance was documented by an increase of the vincristine LC_{50} value from 0.82 ± 0.07 nmol/l for MOLM-13 to 20.05 ± 0.85 nmol/l for MOLM-13/VCR, and from 1.99 ± 0.22 nmol/l for SKM-1 to 97.13 ± 11.05 nmol/l for SKM-1/VCR (expressed as the value obtained from nonlinear regression of data according to Eq. (1) ± SD for 9 degrees of freedom).

Massive protein bands with a relative molecular weight of approximately 150–180 kDa were detected by Western blotting using the anti-P-gp antibody (c219) on the crude membrane fraction isolated from SMK-1/VCR and MOLM-13/VCR cell variants (Fig. 2A). In contrast, similar protein

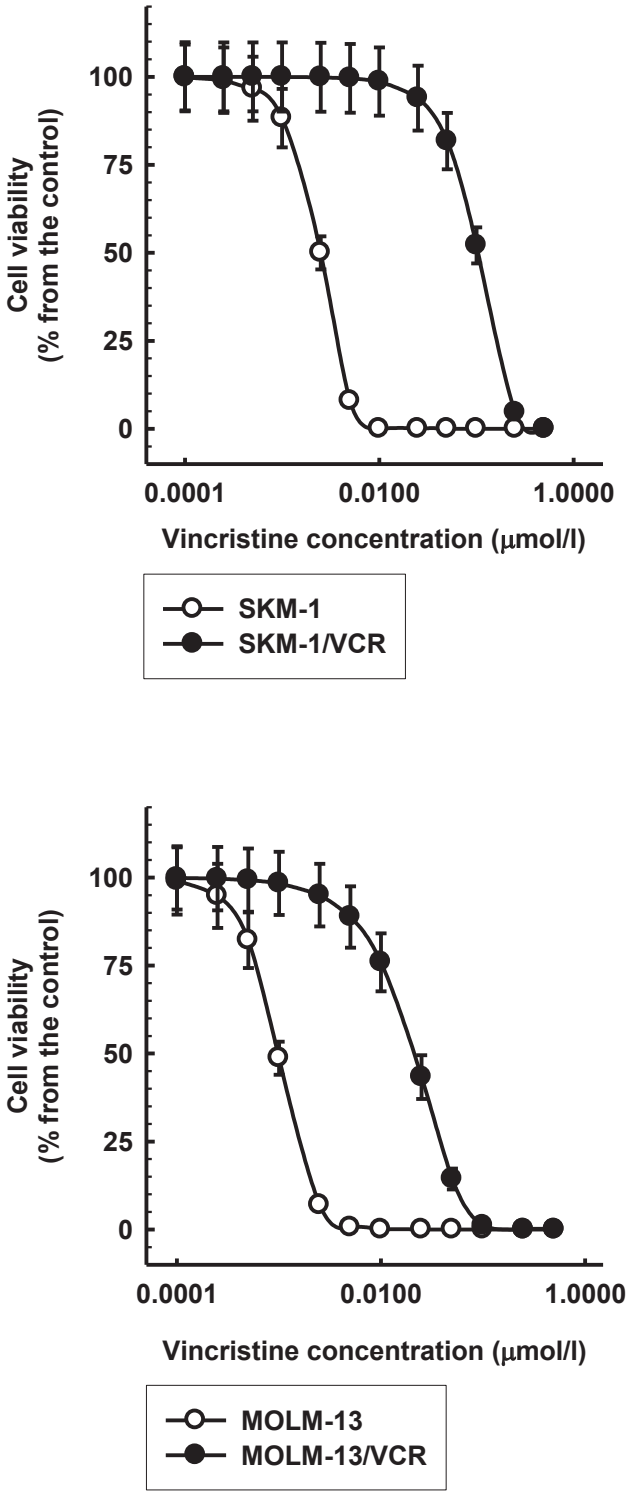

Figure 1. Reduced cell sensitivity of SKM-1/VCR and MOLM-13/VCR cells to VCR in comparison to sensitive parental SKM-1 and MOLM-13 cells. Cells were cultured as is described in Materials and Methods. Data represents the mean ± SD of six independent measurements. Dose-response curves were fit by non-linear regression using Eq. (1).

bands were not detected on Western blots from crude membrane fractions isolated from the parental counterpart cells. Thus, adaptation to VCR results in P-gp induction in both AML cell lines.

To determine whether P-gp, which is present in membranes from SMK-1/VCR and MOLM-13/VCR cells, is causing the transport activity, we used the calcein retention assay. In its esterified form, non-fluorescent calcein/AM, an intracellular calcium indicator, represents a suitable substrate for P-gp, and active P-gp blocks its intracellular entry (Eneroth et al. 2001). The AM ester groups are cleaved by esterases in the intracellular space, and the resulting calcein is able to bind calcium, which induces fluorescence. The liberated calcein is not a substrate of P-gp

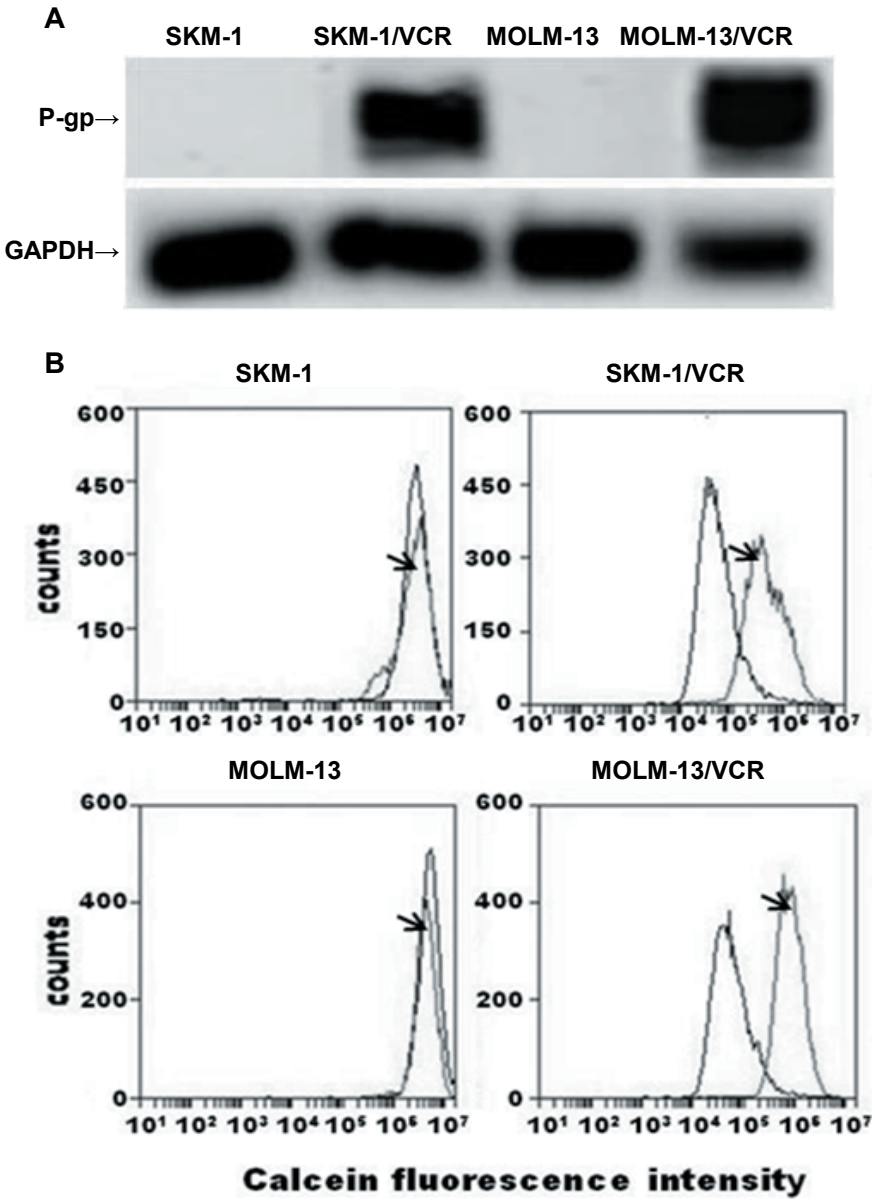

Figure 2. Expression and efflux activity of P-gp in SKM-1/VCR and MOLM-13/VCR cells. **A.** Detection of P-gp in SKM-1/VCR and MOLM-13/VCR cell variants by Western blotting according to the protocol described in chapter 2.3. GAPDH was used as an internal standard. The data are representative of three independent experiments. **B.** Estimation of P-gp efflux activity by the calcein/AM retention assay according to the protocol described in Materials and Methods. Arrows indicate the presence of verapamil at a concentration 10 μmol/l, which was used to eliminate the P-gp efflux activity in another P-gp positive cell variant L1210/VCR (Sulova et al. 2005). FACS histograms are representative of three independent experiments.

(Versantvoort et al. 1995). Thus, in P-gp-positive cells, the efflux activity of this transporter blocks calcein/AM entry and antagonizes the intracellular retention of fluorescent calcein. The decrease in calcein retention has often been used as a measure of drug efflux activity of transporters present in the plasma membrane of MDR cells (Glavinas et al. 2011; Szeremy et al. 2011), and in the case of P-gp, may be antagonized by P-glycoprotein inhibitors such as verapamil, cyclosporine A and ketoconazole (Eneroth et al. 2001; Orlicky et al. 2004). Depression of calcein retention within the intracellular space is visible in both VCR-resistant cell lines (MOLM-13/VCR and SKM-1/VCR) compared with VCR-sensitive cells (MOLM-13 and SKM-1) (Fig. 2B). In resistant cell variants, calcein retention to values similar to sensitive cells could be obtained by P-gp inhibitors, such as verapamil (Fig. 2B), cyclosporine A and ketoconazole (not shown). Therefore, it was concluded that adaptation of both cell lines to vincristine induced expression of active P-gp, and P-gp is involved in the decrease in sensitivity of MOLM-13/VCR and SKM-1/VCR cells to VCR. A typical feature of P-gp expression in leukemia cells is reduced sensitivity to a large group of structurally unrelated substances (reviewed in Breier et al. 2005, 2013a). Both resistant cell variants exert cross-resistance to doxorubicin and mitoxantrone (not shown), which have been shown to be P-gp substrates (Drobna et al. 2002; Shen et al. 2009). Vincristine could induce P-gp expression through activation of the pregnane X receptor (Huang et al. 2006), which binds to the DR4 motif in the upstream enhancer region of the gene encoding P-gp and induces transcription (Geick et al. 2001).

Both resistant cell variants (MOLM-13/VCR and SKM-1/VCR) contain P-gp gene transcripts that were detected by reverse transcription PCR (Fig. 3). In contrast, parental VCR-sensitive cells (MOLM-13 and SKM-1) did not contain any detectable P-gp mRNA. Moreover, expression of P-gp in resistant cell variants is associated with expression of nestin (Fig. 3). Thus, coexpression of P-gp and nestin may occur not only in neural stem/progenitor cells (Yamamoto et al. 2009) but also in leukemia cells when it could be induced by selective pressure from anticancer drugs like VCR. The mechanism of this coexpression is currently unclear. According to the NCBI gene database, P-gp (position q21.12 on chromosome 7) and nestin (position q23.1 on chromosome 1) are located on different sites in the human genome, and they cannot be induced by a common promoter. Expression of P-gp is predominantly controlled by the pregnane X receptor and constitutive and rostane nuclear receptors (Cervenyet al. 2007). These two receptors could be classified as adopted orphan receptors with a specific ligands (including VCR Breier et al. 2013b) identified in recent years (Shi 2007) and are dimerization partners of retinoid X receptors (Saeki et al. 2010) with known ligand 9-cis retinoic acid (Brtko and Thalhamer 2003; Brtko and Dvorak 2011). Interestingly, the role of regulatory pathway of nuclear receptors for retinoids in development of P-gp-mediated MDR could take part (Sulova et al. 2008, 2012; Breier et al. 2014). Nestin was assumed to be transcriptionally controlled by the orphan receptor Nurr1 (Kappen and Yaworsky 2003), which the ligands are currently unknown (Shi 2007). To determine the mechanism that VCR induces coexpression of P-gp and nestin in MOLM-13 and SKM-1 cells as well as to determine the role for this coexpression in the development of P-gp-mediated multidrug resistance, further research is necessary.

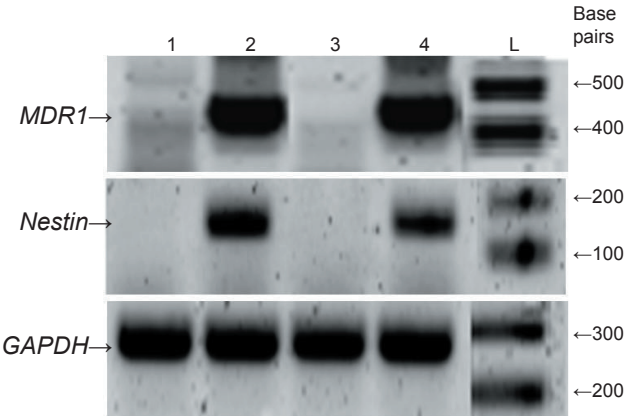

Figure 3. Detection of P-glycoprotein and nestin gene transcripts in SKM-1/VCR and MOLM-13/VCR cells using reverse transcription PCR according to the protocol described in Materials and Methods. GAPDH was used as an internal standard. Line 1 – SMK-1; line 2 – SMK-1/VCR; line 3 – MOLM-13; line 4 – MOLM-13/VCR, L – base pair ladder. The data are representative of three independent experiments.

Acknowledgments. This research was supported by grants from the Slovak grant agencies: APVV grant agency No. APVV-02-90-10, VEGA grant agency Vega 2/0100/12, Vega 2/0182/13 and by a project of the Development of Competence Centre for research and development in molecular medicine, ITMS 26240220071. Paper was edited for proper English language, grammar, punctuation, spelling, and overall style by one or more of the highly qualified native English speaking editors at American Journal Experts.

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Received: February 14, 2014
Final version accepted: May 27, 2014
First published online: June 26, 2014