Can the assessment of \textit{ABCB1} gene expression predict its function \textit{in vitro}?

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\textbf{Abstract}

Increased expression of the \textit{ABCB1} gene in cancer cells is usually connected with occurrence of multidrug resistance (MDR) and poor prognosis. However, the correlation between \textit{ABCB1} expression and MDR phenotype is difficult to prove in clinical samples. Most of the researchers believe that these difficulties are due to the poor reliability and sensitivity of assays for detection of \textit{ABCB1} expression in clinical samples. However, the complexity of P-gp mediated resistance cannot be reduced to the methodical difficulties only. Here, we addressed the question how widely used methods for detection of \textit{ABCB1} expression levels could predict its functional activity and thus its contribution to drug resistance in defined conditions \textit{in vitro}. The \textit{ABCB1} expression was assessed at the mRNA level by quantitative real-time polymerase chain reaction (qRT-PCR), and at the protein level by flow cytometry using UIC2 antibody. The \textit{ABCB1} function was monitored using a calcein AM accumulation assay. We observed that K562 cells have approximately 320 times higher level of \textit{ABCB1} mRNA than HL-60 cells without detectable function. In addition, resistant K562/Dox cells exhibited significantly higher \textit{ABCB1} mRNA expression than resistant K562/HHT cells. However, the functional tests clearly indicated opposite results. Flow cytometric assessment of P-gp, although suggested as a reliable method, contradicted the functional test in K562/Dox and K562/HHT cells. We further used a set of MDR cells expressing various levels of P-gp. Similarly here, flow cytometry not always corresponded to the functional analysis. Our results strongly suggest that an approach which exclusively relies on a simple correlation between \textit{ABCB1} expression, either at the mRNA level or protein level, and overall resistance may fail to predict actual contribution of P-gp to overall resistance as the data indicating transporter expression reflect its function only roughly even in well-defined \textit{in vitro} conditions.

\textbf{Key words} P-glycoprotein; drug resistance; transporter function; transporter expression; UIC2 antibody; mRNA level of \textit{ABCB1}; protein level of \textit{ABCB1}; correlation between \textit{ABCB1} expression and function

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The most likely best characterised mechanism responsible for the multidrug resistance (MDR) phenotype in cancer cells involves the expression of the \textit{ABCB1 (MDR-1)} gene product, P-glycoprotein (P-gp), an ATP-dependent membrane transporter (1,2). It was suggested that the P-gp mediated efflux decreases the drug concentration in cancer cells, which results in the failure of chemotherapy. A large number of reports documented positive correlation between reduced intracellular accumulation of a cytotoxic drug and increased expression of P-gp (2–4). In addition, the P-gp mediated resistance of cancer cells can be reversed by its inhibitors such as verapamil or cyclosporin A (5–7). The laboratory data obtained from \textit{in vitro} experiments are convincing and coherently suggest that P-gp can mediate MDR (2,8).

However, contradictory results exist, especially from \textit{in vivo} experiments. The significance of the P-gp expression for clinical drug resistance to chemotherapy of many cancers is still a completely controversial issue. Indeed, some studies
reported a lack of correlation between P-gp expression and chemotherapeutic resistance (9,10). Probably, the most disappointing were the results of clinical trials testing the P-gp inhibitors, which have mostly failed to show a significant improvement in the response rate. Even the third generation of P-gp inhibitors largely failed to demonstrate an improvement in therapeutic efficacy (11).

Explanation for such antagonistic results might have several reasons. At least two main reasons are usually mentioned. First, the MDR phenotype cannot be reduced only to the P-gp function as more than one mechanism of resistance may be responsible for the MDR phenotype (8). Second, the application of different approaches to assess expression and function of P-gp as well as the appropriateness, reproducibility and sensitivity of the methods routinely used may be responsible for discrepancies in published results (12,13). The later issue was already addressed in details by a workshop held in Memphis in 1994 (12) and by French research groups (13,14), and it is undoubtedly important.

However, the P-gp mediated resistance is a very complex issue which evaluation could not be limited to the methodological difficulties only. In this context, it is surprising that there are attempts to find the simple correlation between P-gp expression, either at the mRNA or protein level, and the cell resistance or clinical outcome. Although such simplified approach is frequently used (15–20), it is uncertain whether it is sufficient to predict the actual contribution of P-gp to the MDR phenotype.

Here, we show that the ABCB1 expression detected at the mRNA level might be misleading in relationship to the P-gp function and/or to the cell resistance even if it is assessed in well-defined in vitro conditions. Similarly, the flow cytometric assessment of moderate or high levels of P-gp expression may fail to indicate the P-gp function and/or the cell resistance in well-defined in vitro conditions.

Materials and methods

Cell culture

Human chronic myelogenous leukaemia K562 cells, obtained from ECACC, were cultured in the RPMI-1640 medium supplemented with a 10% calf foetal serum and antibiotics in 5% CO2 atmosphere at 37°C. Human promyelocytic HL-60 and human histiocytic lymphoma U937 cells were cultured in RPMI-1640 medium supplemented with a 10% calf foetal serum and antibiotics in 5% CO2 atmosphere at 37°C. K562/Dox cells were subsequently obtained by a limited dilution of transfected K562/HHT cells (23, 24).

Human promyelocytic HL-60 and human histiocytic lymphoma U937 cells were cultured in RPMI-1640 medium supplemented with a 10% calf foetal serum and antibiotics in 5% CO2 atmosphere at 37°C. HL-60 and U937 cells were obtained from ECACC.

The cell density and viability was determined using electronic particle counter ViCell (Beckman Coulter). Vi-CELL determines cell viability utilising the trypan blue dye exclusion method (25).

QRT-PCR P-gp mRNA expression analysis

Total RNA was isolated from cells using RNA isolation kit (Sigma-Aldrich, St. Louis, MO, USA) according to manufacturer’s instructions. Contaminating DNA was removed using DNA-free kit (Ambion, Austin, TX, USA).

cDNA was synthesised from 1 μg of total RNA by two-step RT-PCR with a mixture of anchored-oligo(dT) and random hexamer primers as suggested by the manufacturer (Roche, Basel, Switzerland). cDNA served as a template for subsequent assessment of relative expression of P-gp by real-time PCR (LightCycler 480 SYBR Green I Master kit). Reactions were performed in 10 μL mixture. Following protocol was used: initial denaturation at 95°C for 10 min followed by 45 cycles of denaturation at 95°C for 10 s, annealing at 55°C for 10 s and extension at 72°C for 20 s. Condition of melting cycle was from 55 to 97°C. Following primers were used: ABCB1 + 5’-GTC TGG ACA AGC ACT GAA A-3’, ABCB1- 5’-AAC AAC GGT TCG GAA GTT T-3’, GAPDH+ 5’-ACC TCA ACT ACA TGG TTT AC-3’, GAPDH- 5’-GAA GAT GGT GAT GGG ATT TC-3’ (synthesised by EastPort). GAPDH gene was used as internal control.

The 2ΔΔCT method was used to calculate relative gene expression in different cell lines (26).

Western blot analysis of P-gp expression

Western blot analysis of P-gp expression was performed as described previously (24) using monoclonal anti-P-gp (ABCB1) antibody produced in mouse, clone F4 (1: 1000; Sigma-Aldrich) and polyclonal anti-α-tubulin (1 : 2000; Cell Signaling Technology, Danvers, MA, USA). The signal was detected using a horseradish peroxidase-conjugated secondary antibody (1 : 3000; Dako, Glostrup, Denmark). Products were visualised using an enhanced chemiluminescence (ECL; Amersham, Little Chalfont, UK).
Flow cytometric analysis of P-gp expression

P-gp expression was measured using UIC2 (Pgp, Beckman Coulter, Miami, USA) monoclonal antibody conjugated with phycoerythrin (UIC2-PE) according to the manufacturer’s instruction. The fluorescence of the cells was measured and analysed by flow cytometry. Protein expression was quantified as the median fluorescence intensity shift (MFI). Phycoerythrin conjugated isotype IgG2a was used as a control. The fluorescence of the cells was analysed by flow cytometry (Cytomics FC500, Beckman Coulter). ABCB1 expression was determined by the ratio of the mean fluorescence intensity (MFI) shift of UIC2-PE antibody to isotype control (UIC2-PE/IgG2a-PE). For each sample, 10 000 events were collected.

Functional assay of P-glycoprotein

Calcein accumulation as a fluorometric functional assay of P-gp was used (27). Cells (2 × 10^5 cells/mL) were incubated in a standard growth medium containing 0.25 μM calcein AM (Molecular Probes, Eugene, OR, USA) for 15 min at 37°C. Cell fluorescence was then analysed by flow cytometry at excitation and emission wavelengths 488 and 525 nm, respectively, with a band width of 39 nm.

Assay for determination of intracellular daunorubicin level

Cells at density 5 × 10^5 cells/mL were incubated in growth medium with various concentration of daunorubicin (DRN) for 4 h (to achieve steady-state level) at 37°C (27). Afterwards cells were centrifuged through silicone oil and resulting pellet was extracted with 1% formic acid (w/v) in 50% methanol/water (v/v)). DRN content in cell extract was quantified using liquid chromatography (HPLC) coupled a low-energy collision tandem mass spectrometer as described previously (28).

ABCB1 SNPs

Primers and amplicon sizes of ABCB1 gene regions covering the examined SNPs are described in Table 1. The following ABCB1 SNPs were selected from our previous study on associations between SNPs, gene expression and clinicopathological data (29): rs2214102, rs1128503, rs2032582 and rs1045642. Other ABCB1 SNPs were adopted from (30); rs9282564, rs1128501, rs2229109 and rs1202183.

High resolution melting analyses

Real-time PCR and HRM analysis of ABCB1 SNPs (rs2214102, rs1128503, rs2032582, rs2032583 and rs1045642) in genomic DNA samples were carried out using the RotorGene 6000 (Corbett Research, Sydney, Australia) as described in detail in our recent study (31). Results of HRM analysis were confirmed by direct sequencing.

DNA sequencing

ABCB1 SNPs rs9282564, rs1128501, rs2229109 and rs1202183 were estimated using direct sequencing. Primers were designed by help of Primer3 program (32). Primer sequences and sizes of amplicons of optimised PCR conditions are listed in Table 1. PCR products were generated using 50 ng of genomic DNA in a 25 μL final reaction volume containing 2.5 μL of 10× reaction buffer consisting of 1.6 mM MgCl2 (0.8 mM MgCl2 for rs9282564 and 3.2 mM MgCl2 for rs1128501), 0.2 mM dNTP, 0.4 μM of each primer (Table 1), and 0.5 μL of Taq DNA polymerase, 1 U/μL (all chemicals from Top-Bio, Vestec, Czech Republic). Samples

| Target       | Primers (5’–3’)                      | Amplicon size (bp) |
|--------------|--------------------------------------|--------------------|
| rs2214102    | Forward: CTTACTGCTCTCTGGCTTCG        | 200                |
|              | Reverse: TTCAGAGCTGGAGCCTAGAA        |                    |
| rs9282564    | Forward: CTTACTGCTCTCTGGCTTCG        | 251                |
|              | Reverse: TTCAGAGCTGGAGCCTAGAA        |                    |
| rs120183     | Forward: GAGGAAAAGCAAATCTTCCAGA      | 177                |
|              | Reverse: CACACAGCTCATGAGAGGAA        |                    |
| rs2229109    | Forward: TCACCTTTACGAGCTTCACAAGA     | 334                |
|              | Reverse: GGCAATTCACAGACACAGA         |                    |
| rs1128501    | Forward: CCTACACTCAAAAAAGCGTCCAAGA  | 202                |
|              | Reverse: CACAGGTTAGCTTCCAACCCAGA    |                    |
| rs1128503    | Forward: TGTGTCTGTAATGCTTG          | 181                |
|              | Reverse: CACATCCACATCCCTGT          |                    |
| rs2032582    | Forward: TGTGTCTGGAAGCAAAGCATGA     | 182                |
|              | Reverse: AAAAAATGTTTGGGAAGAATGG     |                    |
| rs2032583    | Forward: TGTGTCTGGAAGCAAAGCATGA     | 182                |
|              | Reverse: AAAAAATGTTTGGGAAGAATGG     |                    |
| rs1045642    | Forward: TCCTGAAGTTGACCTGTA         | 233                |
|              | Reverse: AGTGAACCTGATAAGGCCA        |                    |
were denatured by 5 min incubation at 94°C, followed by 35 cycles of denaturation for 45 s at 94°C, annealing for 45 s at 62°C (rs2214102), 64°C (rs2032582), 58°C (rs1128503 and rs1045642), 59°C (rs9282564, rs2229109, and rs1202183) or 65°C (rs1128501) and extension for 45 s at 72°C. The PCR products were resolved and analysed on 3% agarose gel containing ethidium bromide and visualised by ultraviolet light. All samples containing PCR products were then cleaned by ExoSAP-IT according to protocol supplied by the manufacturer (USB Corp., Cleveland, OH, USA). Sequencing reactions were performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with 5 ng of PCR product and 2 pmol of the sequencing primer (reverse for rs2214102, rs2032582 and rs1128503 and forward for rs1045642, rs9282564, rs1128501, rs2229109 and rs1202183, Table 1) in a 10 μL final reaction volume. PCR conditions for sequencing reactions recommended by the producer (Applied Biosystems) were used. Separate sequencing reaction included a control template pGEM-3Zf(+) under the same conditions as above. Sequencing products were purified by EDTA/sodium acetate/ethanol precipitation. DNA sequencing was performed on ABI PRISM 310 Genetic Analyzer, and the results were evaluated by Sequencing Analysis Software v5.2 (Applied Biosystems).

**Results**

Expression level of the *ABCB1* gene is often analysed at the mRNA level using quantitative real-time PCR (qRT-PCR) because of its high specificity and accuracy. However, results obtained using qRT-PCR must be interpreted with a caution. For example, K562 cells exhibited 320 and 213 times higher levels of *ABCB1* mRNA than HL-60 and U937 cells, respectively (Fig. 1a). Importantly, K562 cells exhibited neither detectable ABCB1 mediated transport measured by two independent methods (Fig. 1b,c) nor measurable ABCB1 expression at the protein level (Fig. 1d). Flow cytometric analysis also failed to indicate significant expression level of P-gp in K562 cells (not shown).

**Figure 1** Analysis of *ABCB1* expression and function in K562, HL-60 and U937 cells. Panel (A) qRT-PCR analysis of *ABCB1* mRNA expression. * denotes significant change in *ABCB1* mRNA expression (P < 0.05) between HL-60 cells and U937 or K562 cells. Panel (B) Analysis of ABCB1 function using calcein accumulation assay. Calcein uptake, expressed as the mean fluorescence intensity (MFI), was analysed using flow cytometry. Cells incubated with 0.25 μM calcein AM alone (white columns); 0.25 μM calcein AM+ 3 μM CsA (grey columns); 0.25 μM calcein AM+ 5 μM CsA (dark grey columns). The experimental points represent mean values from three replicate experiments, with standard deviations. Panel (C) Intracellular level of DRN. Cells were incubated with 0.3 μM DRN for 3 h, and then intracellular DRN was determined. Panel (D) Western blot analysis of ABCB1 expression. Lane 1 - K562 cells, lane 2 - U937 cells, lane 3- HL-60 cells. (i) Picture represents a typical result. β-Actin was used to verify equal loading of the protein samples on the gel.
We further compared expression levels of \textit{ABCB1} mRNA in resistant K562/Dox and K562/HHT cells. Our results indicated that K562/Dox cells express a significantly higher level of \textit{ABCB1} mRNA than K562/HHT cells (Fig. 2a). Functional analysis using calcein accumulation indicated, however, higher level of transport activity in K562/HHT cells (Fig. 2b). Similar results were obtained also for drugs that serve as \textit{ABCB1} substrates such as DRN (Fig. 2c).

These results indicated that mRNA analysis of \textit{ABCB1} expression might fail to fit the functional data.

Monoclonal antibodies may be used for the measurement of P-gp expression using various immunochemical assays. Here, two variants were used, either flow cytometry in combination with the UIC2 antibody, which was suggested as a convenient and advantageous technique for evaluation of the \textit{ABCB1} mediated MDR phenotype (12,13), or Western blot analysis in combination with the F4 antibody, which is less frequently used. Analysis of the \textit{ABCB1} expression at protein level using Western blot indicated a significantly higher expression level of the drug transporter in K562/HHT cells.
in comparison with K562/Dox cells (Fig. 2d). These results corresponded to the functional tests (Fig. 2b,c). However, flow cytometric analysis of ABCB1 expression using UIC2 antibody indicated a significantly higher expression of ABCB1 in K562/Dox cells in comparison to K562/HHT cells (Fig. 2e), that is completely opposite results.

Owing to the fact that single nucleotide polymorphisms (SNPs) of the ABCB1 gene might influence the gene function and protein folding, which in turn affect the drug transport efficiency and the interaction between the UIC2 antibody and ABCB1, the main SNPs were analysed in K562/Dox and K562/HHT cells. Results indicated no differences in SNPs between K562/Dox and K562/HHT cells (Table 2).

We further addressed the question how reliable is flow cytometry utilising UIC2 antibody for evaluation of P-gp function in cells with various expression levels of ABCB1 in vitro. For this study, K562/HHT cells were transfected with a plasmid targeting the ABCB1 gene, and then cells with decreased expression of ABCB1 levels were selected. We established subclones of K562/HHT cells K562/HHT1-3 with distinct expression levels of ABCB1 as judged from flow cytometry (Fig. 3a). The assessment of transporter function corresponded to the transporter expression levels except the subclones K562/HHT2 and K562/HHT3 (Fig. 3b, c). Calcein accumulation assay indicated significantly higher efflux in K562/HHT2 in comparison with K562/HHT3 (Fig. 3b). Similar results were obtained for intracellular DRN levels (Fig. 3c). We also determined the ABCB1 expression using Western blot (Fig. 3d) and found a better agreement with the functional assay (compare Fig. 3b–d).

These results demonstrated that suggested the flow cytometric assessment of ABCB1 transporter expression might not fit the transporter function reliably. Importantly, the measured expression levels of P-gp were far from threshold level.

Discussion

P-gp mediated MDR may contribute to chemotherapy failure in the treatment of cancer, including haematologic malignancies. However, the exact prognostic significance of the ABCB1 gene expression for MDR mechanism is still unclear in clinical samples (33). Such findings call the relevance of expression of ABCB1 in the clinical resistance of human cancer into question. Some researchers even believe that the ABCB1 expression does not indicate mechanism of resistance but a marker of a more aggressive cancer phenotype (10,11). Vast majority of researches, however, hold less decided opinion and believe that the major problem is methodical, largely caused by insufficient reliability and accuracy of methods used for assessment of the ABCB1 expression and function. Indeed, there is no doubt that assays for evaluation of ABCB1 are important and may fundamentally affect the conclusion.

However, there exists another problem which might result in contradictory results. We believe that contradictory results might be brought about by oversimplification of evaluation

| Polymorphism   | HGVS description1 | MAF2 | K562 | K562/Dox | K562/HHT |
|----------------|-------------------|------|------|----------|----------|
| rs2214102      | NC_000007.13:g.87229501T>C   | 0.09 | TT   | TT       | TT       |
| rs1128503      | NC_000007.13:g.87179601A>G   | 0.45 | GG   | GG       | GG       |
| rs1045642      | NC_000007.13:g.87138645A>G   | 0.43 | AA   | AA       | AA       |
| rs2032582      | NC_000007.13:g.87160618A>C NC_000007.13:g.87160618A>T | 0.47 | CC   | CC       | CC       |
| rs2032583      | NC_000007.13:g.8716061A>G    | 0.15 | AA   | AA       | AA       |
| rs9282564      | NC_000007.13:g.87229440T>C   | 0.10 | TT   | TT       | TT       |
| rs2229109      | NC_000007.13:g.87179809C>A   | 0.03 | CC   | CC       | CC       |
| rs1128501      | NC_000007.13:g.87195534C>A   | NA   | CC   | CC       | CC       |
| rs1202183      | NC_000007.13:g.87214983T>C   | 0.0 | TT   | TT       | TT       |

1Checked by Mutalyzer v2.0 (https://mutalyzer.nl/snp).
2MAF, minor allele frequency according to HapMap CEU sample; NA, unknown.
of the MDR phenotype in malignant cells, including leukemias. Indeed, a single detection method for ABCB1 expression could hardly provide relevant data for the MDR phenotype evaluation. Despite this principle is not novel, there still exist a large number of reports relying on a simple correlation between ABCB1 expression and clinical outcome in haematological malignancies as well as in solid tumours (19,20,33–38).

Distinctive examples are given here. QRT-PCR represents a sensitive and robust technique for precise assessment of the ABCB1 expression at the mRNA level. However, the mRNA level of ABCB1 gene, even if correctly assessed, provides neither relevant information about its protein level nor any knowledge about its function. For example, K562 cells express a very high level of the ABCB1 mRNA when compared to HL-60 or U937 cells (Fig. 1). Based on these data one could expect a significant ABCB1-mediated resistance in K562 cells. However, K562 cells do not express a detectable function of the ABCB1 transporter and also level of ABCB1 gene at protein level is below detection limits (Fig. 1). The reason for such contradictory results might be due to different sensitivity of used methods. The expression levels of ABCB1 mRNA are low in all cases as judged from cycle threshold (ct) values of real-time PCR for ABCB1 (ctK562 = 31.1, ctU937 = 38.1, and ctHL-60 = 37.4) and GAPDH (ctK562 = 18.0, ctU937 = 17.4, and ctHL-60 = 16.0). These expression levels are detectable by qRT-PCR (Fig. 1a) but undetectable for functional assay (Fig. 1b, c) or for immunodetection (Fig. 1d). However, studies that rely on qRT-PCR evaluation of ABCB1 gene expression just compare their relative mRNA levels without defining any threshold level (14). In fact, sensitive cells are usually used as a control, that is they define the threshold level. Unfortunately, different studies use different type of controls, and thus, results are compared to the different threshold levels (e.g. 14,39).

Another important issue is what type of reference gene is used. In general, different reference genes might provide dif-
different results, especially when the *ABCB1* gene expression is evaluated in different cell types. Probably the best solution would be to use the mean value from several reference genes. However, to our knowledge, there are no strict rules which reference gene should be used. Different laboratories use different single reference genes (12,14), albeit β2-microglobuline and GAPDH are used very frequently (14, 40). In this study, we used β2-microglobuline in addition to GAPDH as a reference gene. We obtained similar results with β2-microglobuline (not shown).

Similarly, apparent discrepancy between *ABCB1* expression at the mRNA level and its function is demonstrated in Fig. 2a–c. Also here, relying only on qRT-PCR analysis, one could expect the higher transporter function in K562/Dox cells. However, this prediction would be misleading. Explanation for these disparate findings could be found in the report by Yague and co-workers who observed that P-gp expression is regulated at two distinct steps, mRNA stabilisation and initiation of translation (41). The complex regulation of *ABCB1* expression may cause that the relationship between *ABCB1* expression at mRNA and protein levels might not be simply proportional. Therefore, the level of mRNA of *ABCB1* gene indicates the actual expression of P-gp very roughly.

Although the measurement of *ABCB1* mRNA by qRT-PCR enables its precise quantitation, here we show that such data might be completely unrelated to the transporter function.

Considering the fact that assessment of *ABCB1* expression at the mRNA level is vague, the detection of P-gp using monoclonal antibody (mAb) seems to be very attractive. Protein assays obviously include Western blot, flow cytometry, immunocytochemistry and immunohistochemistry. Flow cytometry was recommended as a suitable method for assessment of P-gp expression in haematological malignancies due to its sensitivity and simplicity. In addition, a number of P-gp-specific mAb is available (MRK16, MRK17, C219, JSB-1, HYB-241, HYB-612, HYB-195, 265/4F, 4E3, UIC2, F4 (42). Among them, the MRK16/17, 4E3, 265/4F and UIC2 are the most suitable ones as they recognise extra-cellular epitopes of P-gp, and the assay protocols are very simple. Here, we used the UIC2 antibody conjugated with phycoerythrin which is very frequently used for its convenient application. It was reported that measurement of high expression levels of P-gp is much more reliable than that of low expression levels (12). However, here we demonstrated that even measurement of high (Fig. 2) and moderate (Fig. 3) expression levels of P-gp might not correspond to the transporter function and thus that the prediction of MDR based solely on the flow cytometric evaluation of expression might be misleading. One could oppose that the binding of UIC2 antibody is affected by P-gp conformation (43) and therefore application of different antibody might provide somewhat different results. Indeed, some researchers prefer the MRK16 antibody. However, its application in prediction is still less reliable than functional analysis using rhodamine 123 (44). Similarly, usage of the JSB1 or C494 antibody is relatively frequent; however, their applications do not seem to be more reliable as these antibodies cross-react with pyruvate carboxylase (45,46). We achieved best results with Western blot analysis in combination with the F4 antibody (47). However, we do not want to suggest this application as favourable because the number of tested samples was not large enough in our work. In addition, this method is laborious and hardly applicable in solid tumours. In general, different antibodies might provide different results. Some of them might provide expression levels that fit better the functional data. However, as discussed above, probably none of the antibodies could provide reliable results that would always correspond to the functional data. Nevertheless, we do not want to deal with the methodical difficulties in this work as this issue was extensively addressed in other papers (12,13).

Owing to the fact that drug transporters exhibit broad substrate specificity [44,45], and thus, other efflux and influx transporters might account for the discrepancy between *ABCB1* expression at protein level and function, expression levels of other pharmacologically important drug transporters, including ABCC1, ABCG2 and hOCT1 (human organic cation transporter-I) were also measured. Undetectable expression levels of ABCC1 and ABCG2 transporters were observed in paternal K562 cells as well as in resistant cell variants (not shown). In contrast, the expression level of hOCT1 was detectable, and virtually, there were no differences among sensitive and resistant cells used (not shown). The possible involvement of other efflux or influx transporters (some members of ABCC subfamily and/or hOCT2 and hOCT3) was considered to be less probable, and therefore, we did not measure their expression levels.

Our results strongly suggest that an approach which exclusively relies on a simple correlation between the *ABCB1* expression either at the mRNA level or protein level and overall resistance may have compromised predictive value even in well-defined *in vitro* conditions. One of the main reasons is that it is not easy to establish correlation between the *ABCB1* gene expression and the transporter activity as the expression data reflect the transporter function only roughly.

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**Conflict of interest**

Authors declare that they are not in any conflict of interest.
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