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Induction of the multixenobiotic/multidrug resistance system in HeLa cells in response to imidazolium ionic liquids

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The multixenobiotic/multidrug resistance (MXR/MDR) system controls transport of foreign molecules across the plasma membrane as a preventive measure before toxicity becomes apparent. The system consists of an efflux pump, ABCB1, and/or a member of the ABCC family. Ionic liquids are broadly used solvents with several unique properties such as wide liquid range, negligible vapor pressure, good thermal and chemical stability and extraordinary dissolution properties for organic and inorganic compounds. Ionic liquids containing imidazolium ring are frequently used as solvents in drug synthesis. Constitutive and induced amounts of ABCB1 and ABCC1 proteins were estimated here by Western blotting and quantified by flow cytometry in HeLa cells exposed to three homologous 1-alkyl-3-methylimidazolium and one benzyl ring substituted salts. Aliphatic substituents in position 1 of the salts caused a weak toxicity but 1-benzyl ring was strongly toxic. An 8-day long treatment with 10^{-4} M 1-hexyl-3-methylimidazolium chloride resulted in an about 1.5-fold increase of ABCB1 level and over 2-fold increase of ABCC1 level. The amounts of both investigated ABC-proteins were linearly dependent on the length of the imidazolium ring side chain. Such distinctive changes of the amount of MXR/MDR proteins measured in cultured cells may be a useful marker when screening for potential toxicity of various chemicals.

Keywords: multidrug resistance, multixenobiotic resistance, imidazolium ionic liquids, flow cytometry, ABCB1, ABCC1

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INTRODUCTION

Vast quantities of newly synthesized compounds have been released to the environment for decades without sufficient control or proper evaluation of possible delayed toxic effects. Ionic liquids which are frequently used as solvents in organic synthesis are representatives of such compounds. They form salts with a melting point below room temperature. Cationic-type ones comprise a bulky organic cation paired with various anions (Welton, 1999). The entire group is referred to as technological "green solvents" because of a negligent hazard coming from unintended release due to an extremely low vapor pressure at high temperatures (Swatloski *et al*., 2001). Nevertheless, they are acknowledged as toxic agents (Stock *et al*., 2004; Zhao *et al*., 2007). Since early 1990s, the properties and solvating potential of ionic liquids have been actively investigated but little attention was paid to their presence in ready-to-use products i.e., drugs. The detection and estimation of residual solvents is of particular importance in quality control due to potential risk for human health (Liu & Jiang, 2007). A search for specific biomarkers of ionic liquid-triggered effects and evaluation of the risk related to compound structure become now a challenge. A good biomarker to verify the effects of this group of compounds might be the response of the cell MDR/MXR system.

Originally related to drugs but later to general xenobiotics, the MDR/MXR system is a biological first line defense mechanism against foreign substances. It contains an efflux pump comprising ABCB1 (also denominated P-glycoprotein, P-gp) and/or other proteins from the ABCC subfamily (also denominated multiple resistance associated protein, MRP). ABC transporters are multidomain proteins comprising both transmembrane and intracellular nucleotide-binding structures. The hydrolysis of ATP delivers energy for ejection of compounds out of the cell (Higgins, 1992).

ABCB1 is a 1280-amino-acid (170 kDa) protein encoded by the *ABCB1* gene (Endicott & Ling, 1989). It is expressed in epithelial cells of the kidney, liver and pancreas, intestinal mucosa and in capillaries of the brain and testis (Cordon-Cardo *et al*., 1990). ABCB1 plays there a xenobiotic-controlling role and is inhibited by calcium channel blockers (Gottesman & Pastan, 1993). When expressed constitutively, it confers intrinsic drug resistance on cancer cells. ABCB1-associated resistance to drugs can be acquired during a contact with many other chemicals. A prolonged exposure to cytotoxic factors *in vitro* may lead to the selection of cells with *ABCB1* gene amplification. ABCB1 expression *de novo* may also be induced by a short-term exposure to UV irradiation (Uchiumi *et al*., 1993), chemotherapeutic drugs (Chaudhary & Roninson, 1993) and other stressors (Chin *et al*., 1990). Generally, substrates for ABCB1 have an amphipatic character and molecular mass ranges between 300 and 2000 Da (Seelig, 1998).

ABCC1 is a member of the distinct C-subfamily of ABC proteins. Structurally, the complete molecule (190 kDa) has three transmembrane domains, each containing 17 peptide helices, followed by two nucleotide-binding domains (Cole *et al*., 1994). It is a polytopic membrane transporter of considerable clinical importance as it con-

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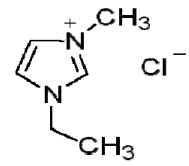
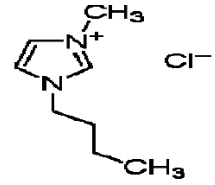
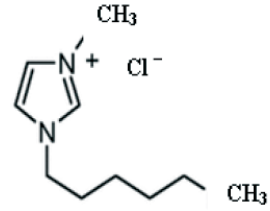
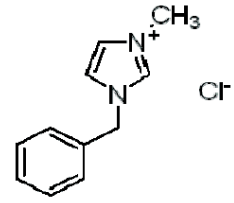
Abbreviations: ABC, ATP-binding cassette (generic); ABCB1, ATP-binding cassette (transporter) B1; ABCC1, ATP-binding cassette (transporter) C1; DMEM, Dulbecco modified Eagle's medium; IIL, imidazolium ionic liquids (general), for compounds BMIMCl, BeMIMCl, EMIMCl, HMIMCl see Table 1; MDR, multidrug resistance; MXR, multixenobiotic resistance; PBS, phosphate-buffered saline; TBS, Tris-buffered saline

Table 1. The imidazolium ionic liquids (IIL) investigated in this study

Systematic name	Abbreviation	Molecular formula	Molecular-mass	CAS Reg. No.	Chemical formula
1-ethyl-3-methylimidazolium chloride	EMIMCl	$C_6H_{11}ClN_2$	146.62	65039-09-0	
1-butyl-3-methylimidazolium chloride	BMIMCl	$C_8H_{15}ClN_2$	174.68	79917-90-1	
1-hexyl-3-methylimidazolium chloride	HMIMCl	$C_{10}H_{19}ClN_2$	202.73	171058-17-6	
1-benzyl-3-methylimidazolium chloride	BeMIMCl	$C_{11}H_{13}ClN_2$	208.69	36443-80-8	

fers multidrug resistance and reduces drug accumulation in tumor cells. It is also an efficient transporter of organic anions such as drugs conjugated with glutathione, sulfates or glucuronates, and anticancer drugs, such as anthracyclines or mitoxantrone (Deeley & Cole, 2006).

In this study, three homologues of 1-alkyl-3-methylimidazolium chloride together with 1-benzyl derivative (Table 1) were tested. These compounds are useful in medical and pharmaceutical applications (Mizuuchi *et al.*, 2008). We found that they have a high ability to induce the MDR/MXR system in cell culture.

MATERIALS AND METHODS

Cell lines, culturing and treatment. Cell lines: MCF7 (human breast cancer), HeLa (human cervical cancer) and HEK293 (human embryonic kidney) were cultivated in DMEM with 4.5 g glucose/l (Sigma, Germany). Medium contained 10% fetal bovine serum (Gibco, USA) and 1% PEN/STREP (Sigma, Germany). HEK293 were obtained from the American Type Culture Collection and the other lines were from the local cell bank (www.biology.pl/biomobil). Original lines were checked for the presence of mycoplasma. Cells were seeded in 150 mm dishes and cultured until about 70% confluence. Media were exchanged for fresh ones supplemented with 10^{-6}–10^{-4} M ionic liquids (EMIMCl, BMIMCl, HMIMCl, BeMIMCl, all from Merck, Germany; for explanation see Table 1). The incubation was continued for 3–8 days (depending on the experiment setup) with every second day exchange for fresh supplemented medium. Thereafter cells were scrapped in ice-cold PBS/proteases inhibitors cocktail, lysed in 0.1% Triton X-100 in PBS, centrifuged at 14000 r.p.m. for 5 min and the supernatant was subjected to Western blot analysis.

HeLa cell viability assay. The MTT assay was based on the protocol described by Park *et al.* (1987). MTT (5 mg/ml) was dissolved in PBS, sterilized by fil-

tration through a 0.22 μm Millipore® filter and stored at 4°C. Cells were seeded at a density of 10^4 cells/well in a 96-well plate in 100 μl of culture medium and allowed to grow for 24 h before adding ionic liquids at 10^{-6}–10^{-4} M final concentration. After 24–72 h of treatment, the cells were washed twice in PBS and then 100 μl of 0.5 mg/ml MTT in serum free medium was added into each well. The incubation was continued for 3 h at 37°C to allow MTT metabolism. The formazan produced was dissolved in 100 μl of acidified isopropanol and absorbance was measured at 570 nm using a BioTek®-ELx800 microplate reader from BioTek Instruments (Winooski, VT, USA). Results were presented as percentage of the control value for untreated cells.

Western blot analysis. Anti-Mdr (G-1; Santa Cruz Biotechnology-13131) monoclonal antibodies directed to a highly conserved epitope of human ABCB1 and monoclonal anti-human MRP1 directed against an epitope of ABCC1 (QCRL-1; Santa Cruz Biotechnology-18835) were used. Total protein lysate (10 μg) was loaded onto each lane of a 6% SDS/polyacrylamide gel and electrophoresis was performed as described by Laemmli (1970). Wide-range molecular mass protein markers 24–180 kDa (Sigma, Germany) were used as a reference. After the transfer onto PVDF membrane and blocking with 5% not-fat dry milk in PBS overnight at 4°C, the blots were incubated with anti-Mdr in PBS (1:300) or anti-human MRP1 antibodies in TBS (1:300), overnight at 4°C. Secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology, USA; 1:3000) and BM Chemiluminescence Western Blotting Kit (Roche Diagnostic, Germany) were used to develop images on autoradiography film (Kodak X-Omat; AR, USA). The loading control was made by immunodetection of α-actin (1A4, Santa Cruz Biotechnology-32251).

Flow cytometry. Cultured HeLa cells were incubated with various ionic liquids in three different concentrations as described above. Cells were harvested by trypsinization and fixed for 10 minutes in 2% formaldehyde (diluted from 37 % stock solution; Sigma, Germany). Ice-cold methanol (POCh, Poland) was added and the suspension was kept on ice for 30 minutes to bring about permeabilization. Then cells were washed and antibodies against ABCB1 (anti-Mdr, 1:100) or ABCC1 (anti-human MRP1, 1:100) were added and samples were incubated for 1 h at room temperature. After washing the cells with incubation buffer (Roth, Germany; 0.5% BSA in PBS), secondary antibodies labeled with AlexaFluor 488 fluorochrome (Molecular Probes, USA; 1:400) were added, followed by 1 h incubation in the dark. Finally, cells were washed, resuspended in PBS and analyzed by flow cytometry (Beckton Dickinson LSR II).

Statistical analysis. The data from MTT test were expressed as means of three independent experiments conducted in triplicates for each concentration. The results from Western blotting are representatives of 2–7 independent experiments. The data from flow cytometry are means of 5-time repetition of independent experiments conducted in triplicates for each concentration. On this base, standard deviation (S.D.) was calculated. Data were analyzed by two-way ANOVA with appropriate *post-hoc* tests using Statistica PL software where $P < 0.05$ was assumed as statistically significant.

RESULTS

1-Alkyl-3-methyl-imidazolium ionic liquids are weakly toxic to HeLa cells

A weak, statistically significant, dose-independent decrease of cell viability was observed upon 72 h incubation with various IIL in the 10^{-6}–10^{-4} M range (Fig. 1). The elongation of the side carbon chain from C_2 to C_6 strengthened this effect. Surprisingly high decrease of cell viability was found after incubation with BeMIMCl, containing a benzene ring.

ABCB1 and ABCC1 are constitutively present in cancer and non-cancer cell lines

Different levels of the 170 kDa ABCB1 were detected in the cell lines studied (Fig. 2). MCF7 had the highest level, HeLa showed a lower but well visible level and in embryonic HEK293 the protein was undetectable at these conditions and it could only be detected when higher concentration of the primary antibody and

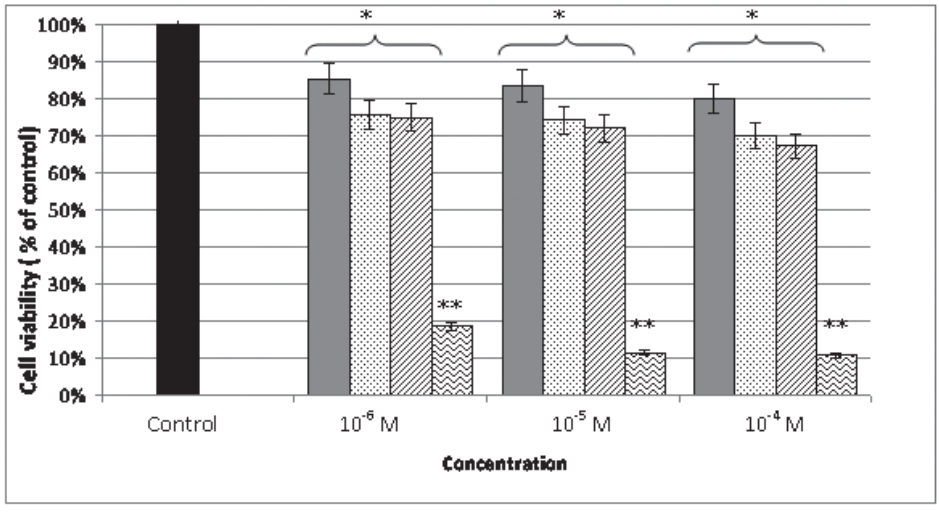


Figure 1. Viability of HeLa cells after 72–h incubation with imidazolium ionic liquids
Control (black); EMIMCl (grey); BMIMCl (dots); HMIMCl (slanted lines); BeMIMCl (wavy lines). All data represent the mean ± S.D. of three independent experiments conducted in triplicates for each ionic liquid concentration. Differences in viabilities were statistically significant comparing with control (*$p<0.05$ or **$p<0.01$).

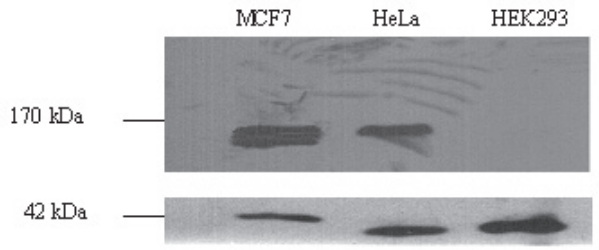


Figure 2. Constitutive presence of ABCB1 (170 kDa) in various cell lines
Lanes loaded with cell extracts from: **1**, MCF7; **2**, HeLa; **3**, HEK293. Aliquots containing 10 µg of protein were electrophoresed in 6% SDS/polyacrylamide gel, transferred on Immobilon-P and incubated with anti-Mdr antibodies (1:300) in PBS. Actin band of 42 kD was used as loading control. Figure is representative of at least five experiments.

longer time of film exposure were used (Rusiecka & Składanowski, 2008). Earlier, a very strong signal of the 190 kDa ABCC1 was also found in cultured MCF7 and HeLa cells (Rusiecka & Składanowski, 2008).

Exposure of cells to IIL induces both ABCB1 and ABCC1 genes

Induction of genes coding for ABCB1 and ABCC1 upon prolonged incubation with IILs was checked in HeLa cells. The lower level of constitutive ABCB1 expression in HeLa cells compared to MCF7 cells could allow for its incremental induction. Indeed, 8-day incubation with 10^{-4} M HMIMCl resulted in about 3-fold increase of the ABCB1 170 kDa band (Fig. 3A). A similar experiment performed with anti-human MRP1 antibodies showed a band at 190 kDa corresponding to ABCC1 at an 8-fold higher level than in the control cells (Fig. 3B).

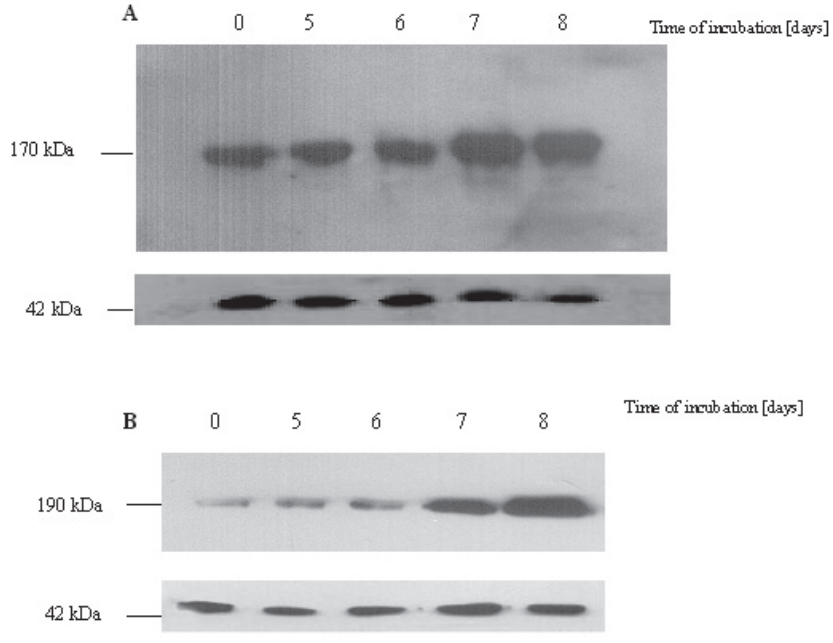


Figure 3. Changes of ABCB1 and ABCC1 in HeLa cells upon incubation with 10^{-4} M HMIMCl.
Aliquots containing 10 µg of protein were electrophoresed in 6% SDS/polyacrylamide gel, transferred on Immobilon-P and incubated with anti-Mdr antibodies (1:300) in PBS **(Panel A)** or anti-human MRP1 (1:300) in TBS **(Panel B)**. Actin band of 42 kDa was used as loading control. Figure is representative of at least five experiments.

Induction of ABCB1 and ABCC1 in HeLa cells depends on IIL structure

Three homologous IILs together with BeMIMCl were tested by immunoflow cytometry for their impact on the synthesis of the ABC transporters. Significantly elevated fluorescence signals of ABCB1 (Fig. 4A) and ABCC1 (Fig. 4B) were observed in HeLa cells scanned after an 8-day exposure. The increase was better pronounced at higher concentrations of IIL. An evident correlation with the carbon side chain length was observed for homologous IIL. Whereas the level of ABCB1 after incubation with 10^{-4} M EMIMCl was at the control value, the longer alkyls enhanced ABCB1 expression, up to 2.25-fold with 10^{-4} M HMIMCl. ABCB1 induction by BeMIMCl containing an aromatic ring was observed only at the 10^{-5} M concentration (Fig. 4A).

The increase of fluorescence specific for ABCC1 in HeLa cells after incubation with IIL was even more significant (Fig. 4B). The induction reached 2.1, 2.6 and 3.1-fold after incubation with 10^{-4} M EMIMCl, BMIMCl and HMIMCl, respectively. The level achieved by BeMIMCl was lower than that for HMIMCl but still high. A dose-effect dependence in the 10^{-6}–10^{-4} M range was observed for all compounds tested.

Summing up, a clear relationship between the amount of ABCB1 or ABCC1 induced and the length of the side chain in homologous IIL was observed. The aromatic BeMIMCl had a similar effect to HMIMCl (at least at 10^{-5} M), however, the much higher toxicity of BeMIMCl distinguished it from the other homologues.

DISCUSSION

HeLa culture seems to be a convenient model to study induction of genes coding for ABCB1 and ABCC1 of the MDR/MXR system. Earlier we checked the activity of the ion pump in HeLa cells (Rusiecka & Składanowski, 2008). The intracellular retention of Rhodamine B (a cationic tracer dye, substrate of the ion pump) in the presence of Verapamil (a calcium channel inhibitor and the pump blocker) proved constitutive functionality of this transport. Positively charged ionic liquids are probably transported into the cell by the same mechanism as is Rhodamine B.

However, the constitutive levels of ABCB1, a molecular equivalent of the pump, in various cells were significantly different. Cancerous lines, MCF7 and HeLa, showed much more pronounced signals than the non-cancerous HEK293. The latter one had a minute induction potential for ABCB1 and only after long-time incubation with the toxic compounds. The upregulated ABCB1 protein rapidly returned to the initial level after removal of the inducer (Rusiecka & Składanowski, 2008). This suggests a self-limiting system in assuring cell homeostasis.

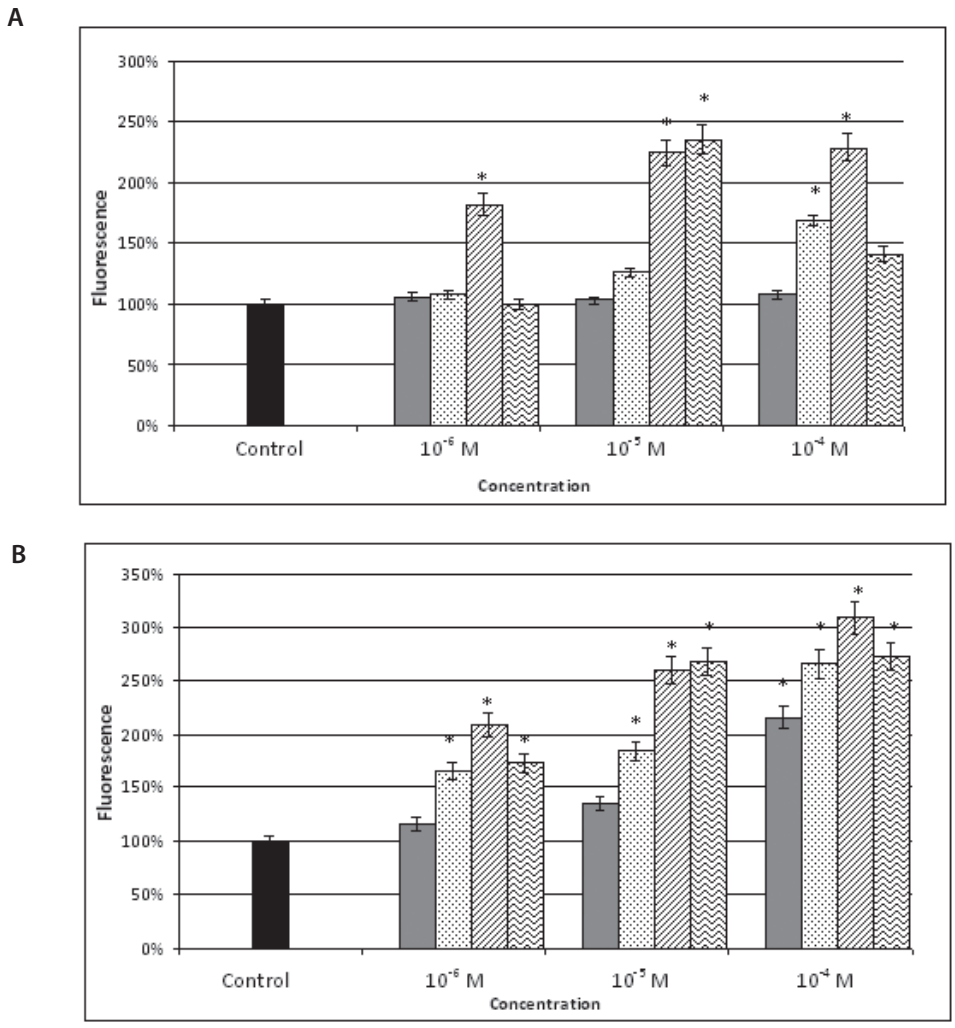


Figure 4. Effect of 8-day incubation of HeLa cells with various imidazolium ionic liquids on the fluorescence of ABCB1 (Panel A) and ABCC1 (Panel B) in flow cytometry
Cells incubated with specific antibodies in the absence of effectors were used as controls (black); EMIMCl (grey); BMIMCl (dots); HMIMCl (slanted lines); BeMIMCl (wavy lines). All data represent the mean ± S.D. of five independent experiments conducted in triplicates for each ionic liquid concentration. Differences in fluorescence were statistically significant comparing with control (*P<0.05).

It was confirmed in this paper that both the total amounts of ABCB1 and ABCC1 and their domains accessible to antibodies in the cell membrane were increasing during 8-day incubation, in particular with HMIMCl. The amounts indicated on Western blots (Fig. 3) were in good agreement with the immunoflow cytometry data (Fig. 4) for ABCB1 (3 and 2.3-fold increase) but in the case of ABCC1 the agreement was poor (8 and 3-fold, respectively, for these two techniques).

We defined the induction potential of the MDR/MXR system for individual toxic compounds. The increase in the ABCB1/ABCC1 protein level was related to the IIL toxicity ranked according to the structure-activity relationship (SAR). Our data refer to the toxic properties of IIL based on theoretical SAR considerations and on experiments estimating their biological activity (Jastorff *et al*., 2003; Ranke *et al*., 2004; Stepnowski *et al*., 2004; Stock *et al*., 2004; Składanowski *et al*., 2005). All those studies indicated a simple relationship with the elongation of n-alkyl chain of IIL increasing their toxicity (Swatloski *et al*., 2004).

The presented data also show induction of genes coding for the ABCB1/ABCC1 transporters under the influence of homologous compounds based on 1-substituted imidazolium ring. Naturally, these results do not preclude using various IILs in drug synthesis. They rather draw attention to their potential toxicity and indicate necessity of monitoring their presence in the final products. One should pay attention to the induction of cell resistance to drugs contaminated with IILs *in vivo* as it might reduce the effectiveness of therapy. Our data propose HeLa cells as a system of choice in assays for induction of MDR/MXR.

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