

## Induction of the multixenobiotic/multidrug resistance system in various cell lines in response to perfluorinated carboxylic acids<sup>★</sup>

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Received: 11 March, 2008; revised: 28 April, 2008; accepted: 04 June, 2008  
available on-line: 14 June, 2008

The multixenobiotic resistance (closely related to multidrug resistance) system controls transport across the plasma membrane as a defense against toxic molecules. Multixenobiotic resistance system consists of an efflux pump, ABCB1 (also named P-glycoprotein, P-gp), and/or a molecule of the ABCC family (also named multiple resistance associated protein, MRP). ABCB1 is able to increase efflux of many low-molecular foreign molecules. Measuring system induction may be used as a biomarker of cell/organism exposure to foreign substances. Various established cell lines were tested for constitutive and induced multixenobiotic resistance proteins by Western blotting immunodetection. The pumping function was indirectly assayed with Rhodamine B by visualization of cell fluorescence in the presence of verapamil. Changes in ABC proteins were measured by flow cytometry after exposition to various perfluorinated carboxylic acids. MCF7 and HeLa cells were found to contain the highest constitutive level of both ABCB1 and ABCC1. HEK293 exhibited much less ABCB1 and no activity of pumping out Rhodamine B. The pumping activity was found to be related to the amount of the cell-type specific 170 kDa ABCB1 protein. An 8-day exposure to  $10^{-4}$  M perfluorononanoic acid resulted in about 2–2.5-fold increase of ABCB1 level. That was confirmed also for short times by flow cytometry of cells exposed to perfluorinated acids and its natural congeners. Both ABCB1- and ABCC1-related fluorescence increased along with the carbon chain in acids from C<sub>6</sub> up to C<sub>9</sub>, and decreased for C<sub>10</sub>. Measuring of multixenobiotic resistance changes *in vitro* induced by chemicals may be a convenient test for screening for their potential toxicity.

**Keywords:** multixenobiotic resistance, multidrug resistance, perfluorinated carboxylic acids, flow cytometry, ABCB1, ABCC1

### INTRODUCTION

The general mechanism of treatment of foreign substances by organisms involves detoxification by enzymatic modification or cleavage, alteration of accessibility to targets, decreased permeability of the cell membrane and active toxin extrusion by membrane transporters. The latter system contains an efflux pump comprising ABCB1 (also named P-glyco-

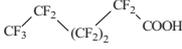
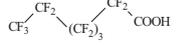
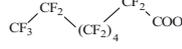
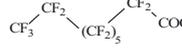
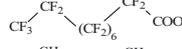
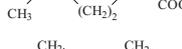
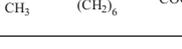
protein, P-gp) and/or other proteins from the ABCC family (also named multiple resistance associated protein, MRP). The presence of the multixenobiotic resistance (MXR), analogous to the multidrug resistance (MDR) of tumor cells (Juliano & Ling, 1976; Endicott & Ling, 1989; Gottesman & Pastan, 1993), was described for the first time in some aquatic organisms (Kurelec, 1992). The activity of a plasma membrane ATP-hydrolysing ABCB1-containing pump-

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<sup>★</sup>Preliminary report on the same subject was presented by the authors during the 41st Meeting of the Polish Biochemical Society, Białystok, 12–15 September 2006.

**Abbreviations:** ABC, ATP-binding cassette (generic); ABCB1, ATP-binding cassette (transporter) B1; ABCC1, ATP-binding cassette (transporter) C1; DMEM, Dulbecco modified Eagle's medium; MDR, multidrug resistance; MXR, multixenobiotic resistance; PBS, phosphate-buffered saline; PFA, perfluorinated (carboxylic) acids (general); PFHxA, PFHpA, PFOA, PFNA, PFDA (see Table 1).

**Table 1. Perfluorinated and natural carboxylic acids used in this study**

Common name	Molecular formula	Structural formula	MW	Abbreviation
Perfluorohexanoic acid	C <sub>6</sub> HF <sub>11</sub> O <sub>2</sub>		314	PFHxA
Perfluoroheptanoic acid	C <sub>7</sub> HF <sub>13</sub> O <sub>2</sub>		364	PFHpA
Perfluorooctanoic acid	C <sub>8</sub> HF <sub>15</sub> O <sub>2</sub>		414	PFOA
Perfluorononanoic acid	C <sub>9</sub> HF <sub>17</sub> O <sub>2</sub>		464	PFNA
Perfluorodecanoic acid	C <sub>10</sub> HF <sub>19</sub> O <sub>2</sub>		514	PFDA
Hexanoic acid	C <sub>6</sub> H <sub>12</sub> O <sub>2</sub>		116	HxA
Decanoic acid	C <sub>10</sub> H <sub>20</sub> O <sub>2</sub>		172	DA

ing system was confirmed by biochemical (Kurelec & Pivčević, 1989), molecular (Kurelec *et al.*, 1992; Toomey & Epel, 1993; Minier *et al.*, 1993; Cornwall *et al.*, 1995), physiological (Kurelec & Pivčević, 1991; Minier & Moore, 1996; Galgani *et al.*, 1996) and toxicological (Waldmann *et al.*, 1995) methods. The wide taxonomic distribution of MXR points to its universal role in biological defense (Epel, 1998; Kurelec, 1997; Eufemia & Epel, 2000) that rescues the owner by pumping the potentially toxic substances out of the cell. Some sensitizing agents can inhibit this system (Smital & Kurelec, 1997).

Exposure of aquatic organisms to a polluted environment induces the expression of both MXR activity and protein titer (Kurelec *et al.*, 1996). The activity of the transporter also correlates with the concentration of well-identified pollutants (Eufemia & Epel, 1998). Therefore, measuring the induction of MXR in an individual or population may be used as a biomarker of exposure (Kurelec, 1997). It is suggested that measuring the MXR *in vitro* to evaluate the potential risk for toxicity has a low range error. The reliability of the MXR as a biomarker must be checked for various exposure conditions, and also background information on seasonal, geographical, stressor and population differences of its components must be taken into consideration (Kurelec *et al.*, 2000).

The important feature of the MXR mechanism is its low specificity towards chemicals which can be good substrates for the transporter without being obviously toxic. Less toxic agents can compete with the more toxic ones and effectively inhibit the pumping process, thus circumventing the MXR defense system. A variety of such MXR inhibitors or competitive substrates are present in polluted waters and significantly less in non-polluted ones (Smital & Kurelec, 1997). The competitive substrates

enhance accumulation and therefore strengthen the effects of xenobiotics on aquatic organisms.

The central ABCB1 protein is encoded by a gene of the *ABCB* gene family. The *ABCB1* gene seems to be functionally linked to the development of the MXR phenotype of the cell. Its product (170 kDa) consists of 1280 amino acids, forming two symmetrical homologous halves in the native state, each with six putative transmembrane helices and an intracellular nucleotide binding domain (Endicott & Ling, 1989). ABCB1 is present in some tissues, such as epithelial cells of the kidney, liver, pancreas, intestinal mucosa and in capillaries of the brain and testis. It plays there a physiological role, promoting the excretion of xenobiotics, preventing their absorption and also acting as a calcium channel. A consequence of its activity in cancer cells derived from such tissues constitutively expressing ABCB1 is an intrinsic drug resistance. In most cases, however, ABCB1-associated resistance is acquired during a contact with extracellular factors. 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 short term exposure to cytotoxic agents such as UV light (Uchiumi *et al.*, 1993), chemotherapeutic drugs (Chaudhary & Roninson, 1993) and inducers of the stress response (Chin *et al.*, 1990; Vilaboa *et al.*, 2000).

ABCC1 (another member of the multidrug resistance protein family) is a polytopic membrane transporter of considerable clinical importance that confers multidrug resistance on tumor cells by reducing drug accumulation. It is also an efficient transporter of conjugated organic anions. Like other ABC-proteins, including ABCB1, ABCC1 (190 kDa) has a core structure consisting of two membrane-spanning domains (MSDs), each followed by a nucleotide binding domain (NBD). Unlike ABCB1 and

most other ABC superfamily members, ABCC1 contains a third MSD with five predicted transmembrane segments and an extracytosolic NH<sub>2</sub>-terminus. Moreover, the two NBDs in ABCC1 are considerably more divergent than those found in ABCB1 (Rosenberg *et al.*, 2001).

In this study we tested five homologs of perfluorinated carboxylic acids varying in chain length (PFAs, see Table 1). They are technical substances broadly used in industry, public sector and in the household. Industrialized regions may soon envisage problems with negative effect of PFAs on living organisms (Guruge *et al.*, 2006). PFAs are highly thermostable and environmentally persistent pollutants and the balance between lipo- and hydrophilicity makes them easily penetrable through many cellular membranes. Reproductive and developmental toxicity of the most commonly used compounds — PFOA and PFOS (perfluorooctanoic acid and perfluorooctanesulphonate) — was proved in the mouse (Lau *et al.*, 2006). PFOS has been recently reported a danger (Brooke *et al.*, 2004) and introduced on the list of banned substances in the European Union. We hypothesize that as anionic xenobiotics, they also can induce expression of genes encoding components of the MXR/MDR system. This study verifies this hypothesis positively.

## MATERIALS AND METHODS

**Cell lines and culture conditions.** Cell lines: MCF7 (human breast cancer), HeLa (human cervical cancer), A375 (human melanoma) and HEK293 (human embryonic kidney) were cultivated in DMEM with 4.5 g glucose/L (Sigma, Germany) and WM9 (human melanoma) in RPMI-1640 (Sigma, Germany). Both media contained 10% fetal bovine serum (Gibco, USA) and 1% PEN/STREP (Sigma, Germany). A375 and HEK293 were obtained from American Type Culture Collection and all other lines were from the local cell bank ([www.biology.pl/biomobil](http://www.biology.pl/biomobil)).

**Measurement of MXR activity.** The procedure was performed according to Minier and Moore (1996) with small modification. Cells were incubated with 1  $\mu$ M Rhodamine B (Sigma, Germany, made out of 1 mM stock) for 60 min with or without 20  $\mu$ M verapamil (Sigma, Germany, made out of 20 mM stock) added 20 min after Rhodamine B. Specimens were taken, fixed in 4% formaldehyde and visualized in a fluorescence microscope (Axiovert S-200, Zeiss, Germany). Cells actively pumping out the dye emitted residual light only. ABCB1-negative cells or cells in the presence of a competitive substrate, verapamil, emitted intensive fluorescence at 551 nm.

**Localization of ABCB1 *in situ*.** Cells were seeded at  $1 \times 10^5$  per 22 mm well (12-well plate) 24 h prior to fixing in a dish containing 20 mm cover slip. The cover slips were sterilized by immersing in 75% ethanol and dried under tissue culture hood. After 24 h 60–80% confluent culture was washed with PBS and treated with 400  $\mu$ l of 4% formaldehyde in PBS for 15 min. Then cells were washed twice with PBS and treated with 0.1% Triton X-100 for 1 min and washed again with PBS. Consecutively, cells were blocked with blocking solution at room temp. for a minimum 30 min and immediately incubated either for 60 min with anti-Mdr (G1) sc-13131 antibodies 1 : 100 (Santa Cruz Biotechnology, USA) in blocking solution or for 15 min with PKH26 (Sigma, Germany) according to the instruction of the manufacturer. Cells were then washed with PBS and incubated with secondary goat anti-mouse antibodies conjugated with Alexa Fluor 488 (Molecular Probes, USA; 1 : 100) for 1 h. Consecutively, cells were washed with PBS and incubated with 1  $\mu$ M Hoechst 33342 (Sigma, Germany), washed again with PBS and mounted on a glass plate using Vectashild mounting medium (Vector Laboratories), placed up-side down on a superfrost glass plate (Roth, Germany) and glued at the edges with a nail polish.

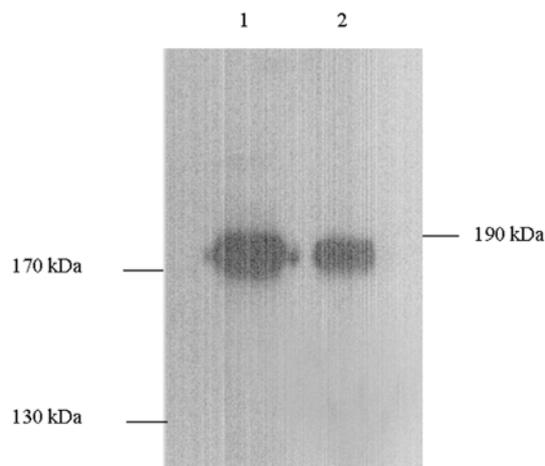
**Western blot analysis.** Western blotting was applied for the detection of the MXR components. Anti-Mdr (G1) sc-13131 monoclonal antibodies (Santa Cruz Biotechnology, USA) directed to a highly conserved epitope of human ABCB1 and monoclonal anti-human MRP1 directed to an epitope of ABCC1 were used. The MRP1 antibodies did not cross-react with other human ABCC gene products. Total protein lysate (10  $\mu$ g) was loaded onto each lane and electrophoresis was performed in 12% or 6% polyacrylamide gel as described by Laemmli (1970). The 6% gel was used in the few cases when we focused on proteins of high molecular weight. Wide-range molecular mass protein markers 24–180 kDa (Sigma, Germany) were used as a reference. After the transfer onto a 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 1:100) or anti-human MRP1 antibodies (Santa Cruz Biotechnology, USA; 1 : 300) in TBS 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).

**Exposure of cells to toxic substances: Cd<sup>2+</sup> ions and PFAs.** Cells were seeded in a 150 mm dish and cultured until about 70% confluence. Media were changed then for those containing either  $10^{-6}$  M CdCl<sub>2</sub> (Sigma, Germany) or  $10^{-4}$  M perfluorinated

organic acid (PFHxA, PFHpA, PFOA, PFNA, PFDA, all from Merck, USA) and incubation was continued for 3–15 days depending on the substance. The media were changed for fresh ones each 2nd day of incubation. Thereafter cells were scraped in ice-cold PBS/protease inhibitors cocktail, lysed in 0.1% Triton X-100 in PBS, spun down at 14 000 r.p.m. for 5 min and subjected to Western blot analysis.

**Viability test.** MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] test was used to check the effect of PFAs. Cells were seeded at a density of  $1 \times 10^4$  cells/well in a 96-well plate in 100  $\mu$ l of culture medium and allowed to grow 24 h before adding PFAs (out of  $10^{-3}$  M stock). Following 24 h incubations with various acids, 100  $\mu$ l of MTT solution (5 mg/ml in PBS, pH 7.4) was added and incubated for the next 3–4 h. Supernatants were removed followed by the addition of 100  $\mu$ l of a solution containing 4% HCl and 96% propan-2-ol. The  $A_{570}$  of each well was determined with an automated plate reader. Survival was calculated relative to the staining value of an untreated control (100%).

**Flow cytometry.** MCF7 and HeLa cells were exposed to various perfluorinated acids in three different concentrations ( $10^{-6}$ ,  $10^{-5}$ ,  $10^{-4}$  M). Cells were harvested by trypsinization and fixed for 10 min in 2% formaldehyde (Sigma, Germany). Thereafter ice-cold methanol (POCh, Poland; final concentration 90%) was added for 30 min on ice to permeabilize cells. Antibodies against ABCB1 (anti-Mdr, sc-13131, 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), second-



**Figure 2. Presence of ABCC1 detected by anti-human MRP1 antibodies in MCF7 (lane 1) and HeLa (lane 2) cell lysates.**

Cell extract (10  $\mu$ g) was electrophoresed in 6% SDS/polyacrylamide gel, transferred on Immobilon-P and detected with anti-MRP1 antibodies (1 : 300) in TBS. Figure is a representative of minimum five experiments.

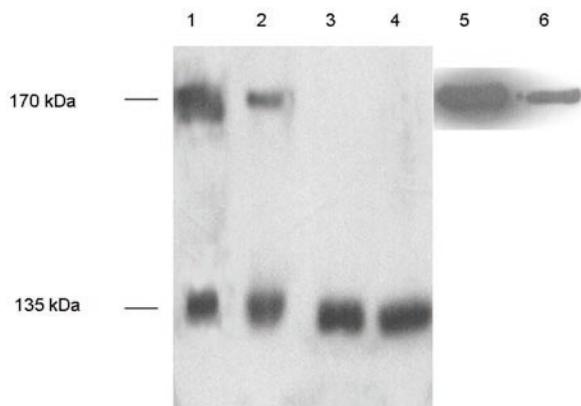
ary antibodies labeled with fluorochrome Alexa 488 (Molecular Probes, USA; 1 : 400) were added, followed by 1 h incubation in the dark and thereafter cells were washed, suspended in PBS and analyzed by flow cytometry (Beckton Dickinson LSR II).

**Statistics.** The data are means of three independent experiments conducted in triplicates for each acid. The percentage of fluorescence from cells was calculated from five independent experiments.

## RESULTS

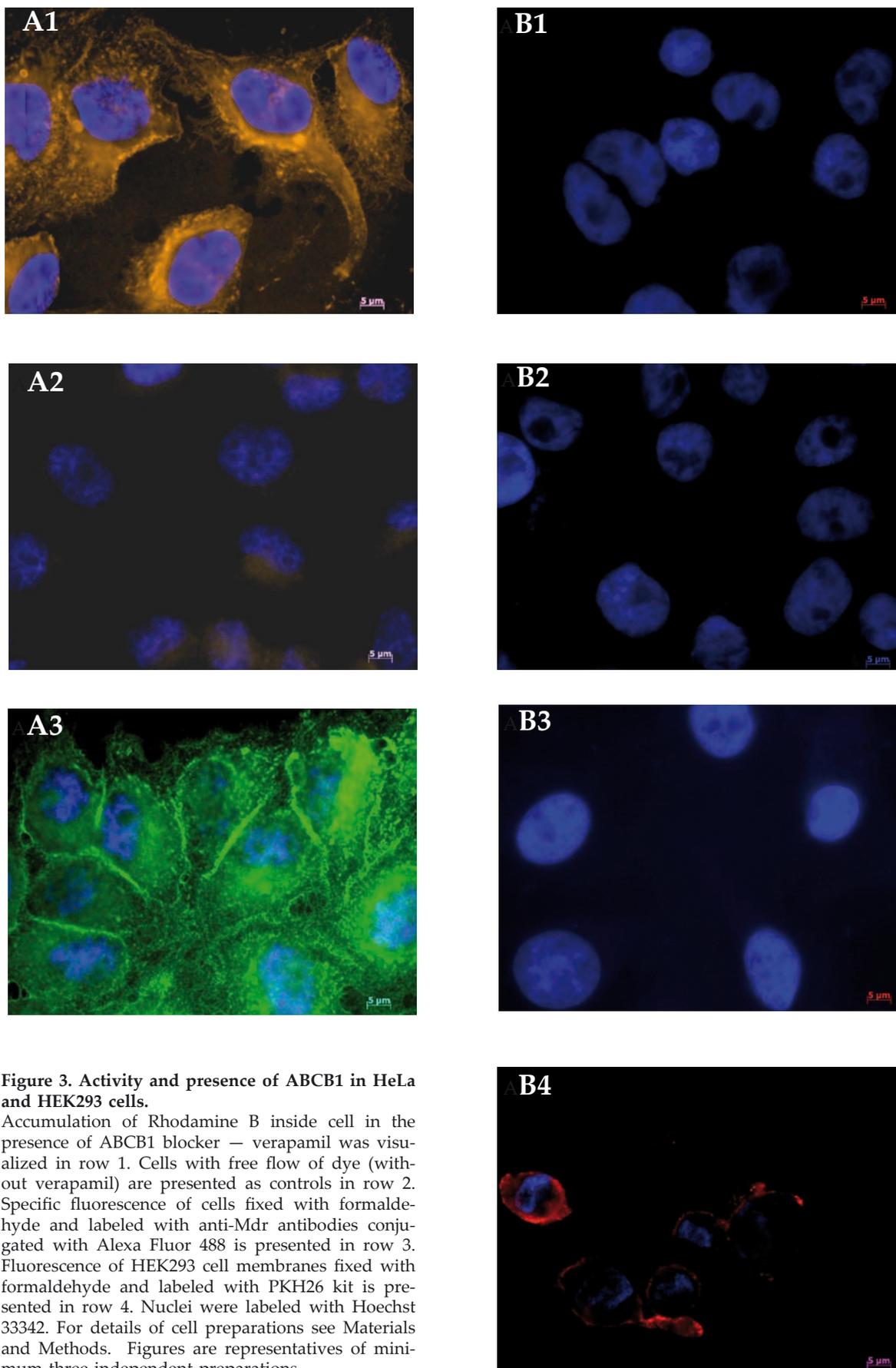
### ABCB1 and ABCC1 presence in different cell lines

Different levels of ABCB1 components were noticed in various cells (Fig. 1). MCF7 contained the highest level of the 170 kDa ABCB1 protein, HeLa showed a lower level, and in the melanomas WM9 and A375 the typical ABCB1 was virtually absent (Fig. 1). The non-specific second band resulted from the cross-reactivity of the anti-Mdr mAb with an unrelated protein of 135 kDa. Similar non-specific bands ranged from 135 to 170 kDa were found earlier on immunoblots of plasma membrane preparations from multidrug-resistant mouse, hamster or human cell lines (Jette *et al.*, 1995; Kurelec *et al.*, 2000). HEK293 cells contained a several-fold lower level of ABCB1 than HeLa, which could be only detected in more stringent conditions – higher concentrations of antibody and longer time of film exposure (Fig. 1, lanes 5 and 6).



**Figure 1. Presence of ABCB1 (170 kDa) in cell lysates.**

Lanes: 1, MCF7; 2 and 5, HeLa; 3, WM9; 4, A375; 6, HEK293). Cell extract (10  $\mu$ g) was electrophoresed in 12% (lanes 1–4) or 6% (lanes 5 and 6) SDS/polyacrylamide gel, transferred onto Immobilon-P and detected with monoclonal anti-Mdr sc-13131 antibodies at 1 : 300 (lanes 1–4) or 1 : 100 (lanes 5 and 6) dilution in PBS. Standard exposition on an autoradiography film for lanes 1–4 was extended to 15 min for lanes 5 and 6. Figure is a representative of minimum five experiments.



**Figure 3. Activity and presence of ABCB1 in HeLa and HEK293 cells.**

Accumulation of Rhodamine B inside cell in the presence of ABCB1 blocker – verapamil was visualized in row 1. Cells with free flow of dye (without verapamil) are presented as controls in row 2. Specific fluorescence of cells fixed with formaldehyde and labeled with anti-Mdr antibodies conjugated with Alexa Fluor 488 is presented in row 3. Fluorescence of HEK293 cell membranes fixed with formaldehyde and labeled with PKH26 kit is presented in row 4. Nuclei were labeled with Hoechst 33342. For details of cell preparations see Materials and Methods. Figures are representatives of minimum three independent preparations.

Another component of the MXR/MDR system, ABCB1, was also found in two cell lines: HeLa and MCF7. They showed a very strong signal at the level of 190 kDa (Fig. 2).

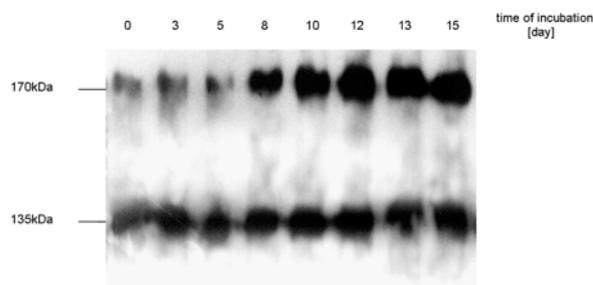
For further investigation we chose HeLa cells, characterized by high expression of both ABCB1 and ABCC1.

#### Active pump is present in non-exposed ABCB1-positive cells

The pumping function of the MXR/MDR system was verified using HeLa and HEK293 cells containing different levels of the 170 kDa ABCB1 protein. The method of Rhodamine B retention upon ABCB1 active site saturation with verapamil was used earlier with single cells, such as eggs and 1–2 cell embryos from numerous marine invertebrates (Toomey & Epel, 1993), the blood cells of a mussel (Minier & Moore, 1996), and gill tissue of bivalves which can be treated as single-cell sheets (Cornwall *et al.*, 1995; Kurelec *et al.*, 2000). In our hands, the ABCB1-positive cells pumped the dye effectively out to the medium. Addition of the competitor verapamil affected the efflux so that the dye was not extruded from the cells (Fig. 3A1). In the absence of verapamil (Fig. 3A2) accumulation of the dye inside cells did not occur. HEK293 did not retain Rhodamine B regardless of the presence of verapamil (Fig. 3B1 and B2). The pumping activity of HeLa cells correlated with their high fluorescence after labeling with anti-Mdr antibodies conjugated with Alexa Fluor 488 (Fig. 3A3). Although the ABCB1 fluorescence did not have a single localization, it was the most intensive at the plasma membrane. ABCC1 was also found in HeLa plasma membranes (using anti-human MRP1 antibodies, not shown). At the same conditions no fluorescence was found in HEK293 cells (Fig. 3B3). Controlling the status of HEK293, we did not find substantial signs of disintegration as checked with a red fluorescent cell linker, PKH26 (Fig. 3B4).

#### Exposure of cells to Cd<sup>2+</sup> induces ABCB1 protein

HeLa cells were tested for exposure-related changes in MXR/MDR system components through 3–15 day-long incubation with xenobiotics. Cadmium chloride, well known for its toxic properties, was a reference for further experiments. Changes of the ABCB1 band intensity are presented in Fig. 4. An about 5-fold increase of the specific 170 kDa ABCB1 protein was detected after 8 days of incubation of the cell culture with 2.5  $\mu$ M Cd<sup>2+</sup>. The well known effect (Achard *et al.*, 2004) of Cd<sup>2+</sup> on the expression of genes encoding



**Figure 4.** Changes of ABCB1 in HeLa cell lysates after 3–15 days of incubation with 10<sup>-6</sup> M CdCl<sub>2</sub>.

Cell extract (10  $\mu$ g) was electrophoresed in 12% SDS/polyacrylamide gel, transferred on Immobilon-P and detected with monoclonal anti-Mdr antibodies (1 : 300) in PBS. Figure is representative of minimum five experiments.

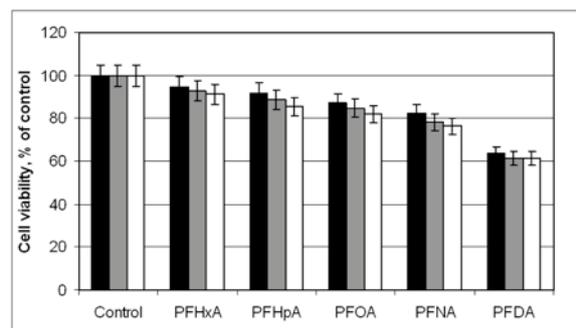
the MXR system components was thus confirmed for mammalian cells.

#### Perfluorinated carboxylic acids are weakly toxic for HeLa cells

A weak, dose-independent cytotoxic effect was observed upon 24 h incubation with various perfluorinated carboxylic acids in the range 10<sup>-6</sup>–10<sup>-4</sup> M (Fig. 5). Elongation of the carbon backbone in the tested compound resulted in a decrease of cell viability. PFDA exerted the most pronounced effect. Longer incubation (up to 72 h) resulted in a higher percentage of cellular death, but not exceeding 40% at 10<sup>-4</sup> M PFDA (not shown).

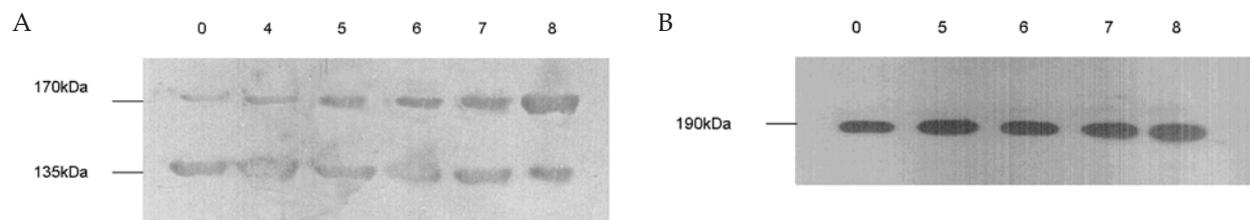
#### Exposure of cells to PFAs induces both ABCB1 and ABCC1 proteins

HeLa cells were also checked for PFA-mediated induction of genes coding for transporting proteins. We chose HeLa line as having a less pro-



**Figure 5.** Viability of HeLa cells measured with MTT after 24 h incubation with perfluorinated acids of various chain lengths.

Concentrations were 10<sup>-6</sup> M (black), 10<sup>-5</sup> M (gray) or 10<sup>-4</sup> M (white). Bars represent S.D.  $\pm$  5% (n = 3).



**Figure 6.** Changes of proteins reacting with anti-Mdr and anti-human MRP1 antibodies in HeLa lysates after 4–8 days of incubation with  $10^{-4}$  M PFNA.

(A) The 170 kDa band represents ABCB1 and (B) the 190 kDa — ABCC1. The conditions were analogous as in Fig. 2 for ABCC1 and in Fig. 4 for ABCB1. Figure is representative of minimum five experiments.

nounced constitutive ABCB1 protein than MCF7 that so small changes could be easier to detect. Incubation of HeLa with  $10^{-4}$  M perfluorononanoic acid (PFNA) resulted in about 3-fold increase of the ABCB1 170 kDa molecular component with the non-specific band virtually at the same level (Fig. 6A). The same experiment was performed with the use of antibodies against ABCC1 (anti-human MRP1). When HeLa cells were incubated with  $10^{-4}$  M PFNA for 8 days, they showed a smaller but robust 1.5–2-fold increase of the ABCC1 intensity (190 kDa) (Fig. 6B).

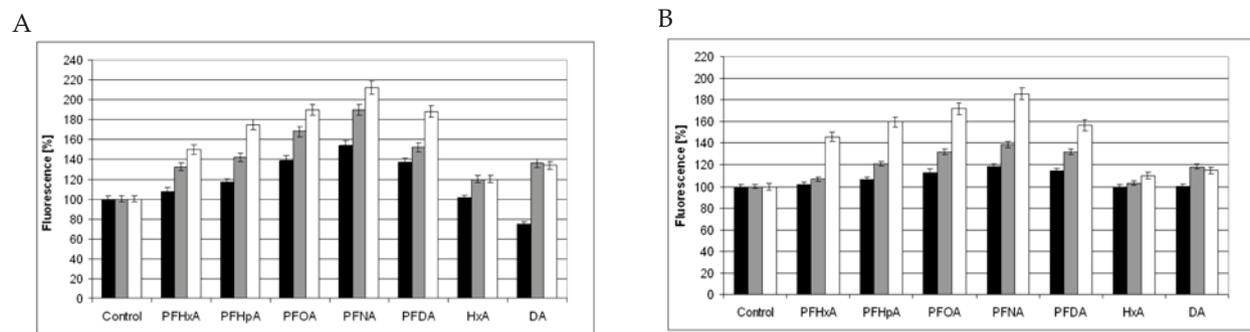
#### Induction of ABCB1 and ABCC1 by PFAs is carbon chain length-related

Induction of the 170 kDa ABCB1 in the cells exposed to PFNA could be detected using immunochrometry of cell lysates (Fig. 6A). To realize our aim of finding structure–activity relationship for induction by homologous PFAs, we used a sensitive cytoimmunofluorescence method. Five different PFAs were tested by immunoflow cytometry for their influence on ABCB1 expression in HeLa cells. Normal carboxylic acids, hexanoic (HxA) and decanoic (DA), were used as controls. We observed significantly elevated ABCB1 (Fig. 7A) as

well as ABCC1 (Fig. 7B) in HeLa cells after exposure.

A significant increase in intensity of specific fluorescence coming from antibody-labeled ABCB1 was observed after 8 days of incubation with  $C_6$ – $C_{10}$  perfluorinated acids. Only slight increases in the presence of its natural congeners were detected (Fig. 7A). The increase was more pronounced at higher concentrations of PFAs and a clear correlation with the chain length of the tested compounds was visible. PFHxA at  $10^{-4}$  M increased the level of ABCB1 on cells by 50% *vs* control and compounds with longer chains made this effect stronger until a maximum 2.1-fold increase was achieved with the  $C_9$ -long  $10^{-4}$  M PFNA. Its  $C_{10}$  congener caused a 1.9-fold increase only. A similar correlation was observed at lower concentrations of PFAs ( $10^{-5}$  and  $10^{-6}$  M).

We observed an about 50% increase of fluorescence specific for ABCC1 on HeLa cells after incubation with  $10^{-4}$  M PFHxA (Fig. 7B). Longer chain PFAs elevated ABCC1 fluorescence even more significantly. The effects was better pronounced at high  $10^{-4}$  M concentration. Both transportation proteins, ABCB1 and ABCC1, exhibited maximum induction at the presence of  $C_9$  long PFNA.



**Figure 7.** Effect of 8-days incubation of HeLa cells with various chain length perfluorinated or normal acids on the fluorescence of ABCB1 or ABCC1 transporters as detected by flow cytometry.

Panels represent ABCB1 (A) and ABCC1 (B). Concentrations were  $10^{-6}$  M (black),  $10^{-5}$  M (gray) or  $10^{-4}$  M (white). Cells incubated with specific antibodies only in the absence of effectors were used as controls. Bars represent S.D.  $\pm$  3% (n = 15).

## DISCUSSION

The HeLa cell line seems to be a good model to study changes in the ABCB1 and ABCC1 proteins of the MXR/MDR system upon exposure to PFAs. These cells were relatively unsusceptible to the toxic effects of PFAs and most of them remained viable up to 15 days in culture. Another cancer cell line checked recently, colon carcinoma HCT116, exhibited  $EC_{50}$  of 47.7  $\mu$ M for PFDA (Kleszczynski *et al.*, 2007). We checked the function of the cell membrane ion pump by Rhodamine B exclusion in combination with a specific inhibitor of calcium channel (verapamil). Retention of the dye with verapamil proved that Rhodamine can penetrate the cell. Our presumption was that the negatively charged PFAs are transported into the cell by the same mechanism as Rhodamine B and that verapamil can inhibit proteins of the ABC family in HeLa cells like it did in P388 leukemia *in vivo* and *in vitro* (Tsuruo *et al.*, 1981). Earlier we measured directly the PFDA gradient across HCT116 cell membrane *in vitro* (Kleszczynski *et al.*, 2007) and proved the plausibility of cross-membrane movement of PFAs. It seems that such amphipathic compounds can accumulate in the negatively charged cell membrane despite their own charge. They could also affect efflux transporters indirectly through plasma membrane effects. Plasma membranes may directly serve as reservoirs for slowly released chemicals binding then to the active sites of the transporter proteins. Alternatively, the chemicals might have a high affinity for specific sites of the transporters such as substrate binding sites or other functional sites from where they are only slowly released, with a resulting long-term inhibition (Ferte, 2000).

Our experiments suggest that the constitutive presence of the 170 kDa protein of the ABCB1-dependent MXR system is a prerequisite for the pumping function as indicated by comparison of HeLa and HEK293 cells. In the latter, a much lower amount of the 170 kDa band correlated with a virtual lack of pumping activity.

We checked the *in vitro* influence of potentially toxic compounds on ABCB1 and ABCC1 presence. As a reference, cadmium ions, well known to induce MXR proteins (in actual nomenclature ABCB1) in exposed aquatic organisms (Achard *et al.*, 2004), were used. A substantial increase of the ABCB1 signal after 8-day incubation of HeLa cells was observed. This confirms the existence of the same mechanism in neoplastic cell line realized through overproduction of one or more of ABC-proteins. HeLa responded also to exposure to perfluorodecanoic acid, a representative of the whole group of PFAs, although less strongly. Both ABCB1 and ABCC1 in HeLa exposed to PFDA were gradually increased with the

time of incubation. It can be concluded that PFAs stimulate expression of ABCB and ABCC genes synchronously.

Disclosing how this stimulation is dependent on structure of PFAs will help to understand the mechanism of the effect. Changes of ABCB1 fluorescence intensity after incubation with  $C_6$ – $C_{10}$  acids for 8 days were clearly visible. They correlated with the length of the alkyl chain in the tested compounds, the longer backbone generally exerting a stronger effect. It is suggested that the recruitment of components of an active complex targeting specific genes in the nucleus is dependent on the lipophilic potential of a xenobiotic. As we found that the longest,  $C_{10}$ -compound was a bit less stimulatory, this dependence has to be more complicated. A similar effect on ABCC1 expression suggests the same mechanism on various transport proteins whose genes can be under common control.

Testing for potential toxicity by measuring MXR/MDR protein changes *in vitro* can be done with relatively low error at a high diversity of inducing compounds. Data on structure–activity relationship (SAR) may help to formulate less toxic but still technologically effective chemicals for a common use. We suggest that such method after validation, may be useful and economically viable for toxicity screening.

## Acknowledgements

This study was supported by grant 2 P04G 118 29 from the Ministry of Science and Higher Education.

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