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ABC transporters affect the detection of intracellular oxidants by fluorescent probes

JIŘINA PROCHÁZKOVÁ[1], LUKÁŠ KUBALA[2], HANA KOTASOVÁ[1], IVA GUDERNOVÁ[1], ZUZANA ŠRÁMKOVÁ[1], MICHAELA PEKAROVÁ[2], BALÁZS SARKADI[3] & JIŘÍ PACHERNÍK[1]

[1]*Department of Animal Physiology and Immunology, Institute of Experimental Biology, Faculty of Science, Masaryk University, Brno, Czech Republic,* [2]*Department of Free Radical Pathophysiology, Institute of Biophysics, Academy of Sciences of Czech Republic, v.v.i., Brno, Czech Republic, and* [3]*Membrane Biology Research Group, Hungarian Academy of Sciences and Department of Biophysics and Radiation Biology, Semmelweis University, Budapest, Hungary*

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Abstract

Intracellular production of reactive oxygen species (ROS) plays an important role in the control of cell physiology. For the assessment of intracellular ROS production, a plethora of fluorescent probes is commonly used. Interestingly, chemical structures of these probes imply they could be substrates of plasma membrane efflux pumps, called ABC transporters. This study tested whether the determination of intracellular ROS production and mitochondrial membrane potential by selected fluorescent probes is modulated by the expression and activity of ABC transporters. The sub-clones of the HL-60 cell line over-expressing MDR1, MRP1 and BCRP transporters were employed. ROS production measured by luminol- and L-012-enhaced chemiluminescence and cytochrome *c* reduction assay showed similar levels of ROS production in all the employed cell lines. It was proved that dihydrorhodamine 123, dihexiloxocarbocyanine iodide, hydroethidine, tetrachloro-tetraethylbenzimidazolocarbo-cyanine iodide and tetramethylrhodamine ethyl ester perchlorate are substrates for MDR1; dichlorodihydrofluoresceine, hydroethidine and tetramethylrhodamine ethyl ester perchlorate are substrates for MRP1; dichlorodihydrofluoresceine, dihydrorhodamine 123, hydroethidine and tetrachloro-tetraethylbenzimidazolocarbo-cyanine iodide are substrates for BCRP. Thus, the determination of intracellular ROS and mitochondrial potential by the selected probes is significantly altered by ABC transporter activities. The activity of these transporters must be considered when employing fluorescent probes for the assessment of ROS production or mitochondrial membrane potential.

Keywords: *Fluorescence, redox status, mitochondria, reactive oxygen species (ROS).*

Abbreviations: *$(DIOC6)_3$, 3,3'-dihexiloxocarbocyanine iodide; ABC, ATP-binding Cassette; BCRP, Breast Cancer Resistance Protein; CL, Chemiluminescence; CSA, Cyclosporine A; DCFH, 2',7'-dichlorodihydrofluoresceine; DHR123, Dihydrorhodamine 123; DMSO, Dimethyl Sulphoxide; HBSS, Hank's Balanced Salt Solution; FBS, Foetal Bovine Serum; FTC, Fumitremorgin C; HE, Hydroethidine; JC1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbo-cyanine iodide; MDR1, Multidrug Resistance 1; MRP1, Multidrug Resistance Protein 1; PMA, Phorbol 12-myristate 13-acetate; ROS, Reactive Oxygen Species; TMRE, Tetramethylrhodamine Ethyl Ester Perchlorate.*

Introduction

Intracellular reactive oxygen species (ROS) production and overall intracellular redox balance play a crucial role in the control of cell proliferation, differentiation and apoptosis (for review see [1]). Intracellular ROS production significantly regulates the fate of various cell types through redox interaction with signal transduction pathways (reviewed in [2]). Current data suggest the importance of ROS in both cancer and healthy adult stem cells for the maintenance of their undifferentiated

Correspondence: Jiřina Procházková, Department of Animal Physiology and Immunology, Institute of Experimental Biology, Faculty of Science, Masaryk University, Kotlářská 2, 611 37 Brno, Czech Republic. Tel: +420549496090. Fax: +420541211214. E-mail: jipro@sci.muni.cz

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status [3]. Thus, the determination of the actual intracellular ROS level in cells is crucial for understanding the ROS role of oxidative stress in the key processes governing differentiation, cellular death and carcinogenesis. Intracellular ROS can be produced from different sources, including non-phagocytic NADPH-oxidases and mitochondria [4]. Thus, mitochondria mass and membrane potential are often determined together with intracellular ROS production.

A wide spectrum of fluorescent redox-sensitive probes for the determination of intracellular ROS production have been developed in the past few decades and employed in numerous studies [5,6]. Fluorescent probes are oxidized/reduced intracellularly and their emission spectra are shifted to a longer wavelength. The major advantage of these fluorescence probes is their high sensitivity, which enables measurement of an individual cell within a population. Both the high sensitivity and the reproducibility of results of probes for intracellular detection of ROS and mitochondrial mass and activity are directly connected with the preservation of the oxidized/reduced fluorescent probe inside the cells.

In this study, we addressed the effect of the efflux pump regarding the issue of the previously mentioned ROS detection by fluorescence probes. Most cell types have a physiological mechanism for the elimination of unwanted toxic and heterogenous chemical compounds from their intracellular space [7,8]. Members of the ATP-binding cassette (ABC) transporter family represent major and, thus, often studied enzymes responsible for these mechanisms [9]. ABC proteins are transmembrane ATP-dependent pumps involved in the transport of a broad spectrum of chemical compounds and metabolism products. The level and/or pattern of expression of ABC transporters may change during cell differentiation [10,11]. Importantly, some of the most studied cell types, such as stem and many tumour cells, express a high level of transmembrane pumps from the ABC transporter family (MDR1, MRP1, BCRP) responsible for a cell's drug resistance [8,12–15].

With respect to the facts mentioned above, it is of current interest to know how the MDR-associated ABC transporter activity affects the fluorescence intensity of the ROS-sensitive probes in different cell types. We tested whether commonly used fluorescent probes are substrates of ABC transporters on the model of the human leukaemia HL-60 cell line and its clones over-expressing multidrug resistance protein 1 (MDR1/ABCB1), multidrug resistance associated protein 1 (MRP1/ABCC1) and breast cancer resistance protein (BCRP/ABCG2). HL-60 cells exhibit a naturally low level of expression of ABC transporters. The presence of a particular ABC transporter on a cell membrane prevents the permeation of dye into the cell and results in a decreased median of fluorescence intensity compared to cells lacking ABC transporters. The results show that commonly used ROS-sensitive or mitochondria-related fluorescent probes are substrates for ABC transporters.

Materials and methods

Cell culture

The ability of ABC transporters to exclude studied dyes was tested using a set of promyelocytic leukaemic HL-60 cells and their clones containing over-expressed levels of a particular ABC transporter—MDR1, MRP1 or BCRP, here mentioned as cMDR, cMRP and cBCRP [16]. Cells from passage 7–30 were harvested, counted (Coulter counter, Beckman Coulter, Brea, CA, USA) and transferred into a suspension of $2 \times 10^*6$ cells/ml in fresh RPMI 1640 supplemented with 10% foetal bovine serum (FBS), streptomycin (0.1 mg/ml), penicillin (100 U/ml) and L-glutamin (3.3 mM) (all from PAN Biotech, Aidenbach, Germany) [17].

For further studies we used murine myoblasts C2C12 (a kind gift of P. Kašpar, Institute of Molecular genetics, Academy of Science of the Czech Republic) and murine leukaemic macrophages RAW 264.7, which were cultivated in DMEM media supplemented with 10% FBS, streptomycin (0.1 mg/ml) and penicillin (100 U/ml) (all from PAN Biotech). These cells were processed in the same way as the HL-60-based cell lines for all the experiments. Murine embryonic stem cell lines (designated D3, R1, GWT, O, W17, X47) were incubated and processed as described in Vesela et al. [20].

Staining by fluorescent probes

The cell suspensions were aliquoted 100 µl per tube and cells were stained with the following dyes: Hydroethidine (HE, 20 µM), 2',7'-dichlorodihydrofluoresceine (DCFH, 40 µM), Dihydrorhodamine 123 (DHR, 10 µM), 3,3'-dihexiloxocarbocyanine iodide [$(DiOC_6)_3$, 0.5 µM], 5,5',6,6'-tetrachloro-1-,1',3,3'-tetraethylbenzimidazolocarbo-cyanine iodide (JC1, 0.25 µg/ml), Mitosox (2 µM), Tetramethylrhodamine ethyl ester perchlorate (TMRE, 100 µM), Calcein acetoxymethyl ester (Calcein, 0.25 µM) and Hoechst 33342 (5 µg/ml) (all from Invitrogene, Carlsbad, CA, USA). Samples were incubated for 15 min at 37°C, washed in HBSS and analysed using FACScalibur (Becton Dickinson, Franklin Lakes, NJ, USA). An argon laser (488 nm) was used to excite the fluorescence of the dyes. Fluorescence emission was then measured with a 530/30 (FL-1) filter (DCFH, DHR, JC1, $(DiOC_6)_3$, Calcein) and a 585/42 BP filter (HE, Mitosox, TMRE). Hoechst 3342 (Hoechst) fluorescence was measured using a FACS Aria II (Becton Dickinson Bioscience, Franklin Lakes, NJ, USA) using 355 nm excitation

and a 424/44 emission filter. Exclusion of the dye by ABC transporters was confirmed by the inhibition of dye efflux by specific inhibitors of MDR1 (Cyclosporine A (CSA), 50 μM), MRP1 (MK571, 50 μM) and BCRP (Fumitremorgin C (FTC), 5 μM) (all from Sigma Aldrich, St. Louis, MO, USA). The addition of transport inhibitors preceded fluorescent probes by 5 min.

Detection of ROS production by non-fluorescent probes

Both intracellularly and extracellularly produced ROS were determined by luminol- and L-012-enhanced chemiluminescence (CL), as described previously [17,18]. In brief, the reaction mixture consisted of 100 μl of cell suspension, 1 mM luminol (Sigma-Aldrich) (stock solution of 10 mM luminol in 0.2 M borate buffer) or 40 μM L-012 (8-Amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4(2H,3H)dione) (Wako Pure Chemical Industries, Osaka, Japan). The total reaction volume of 130 μl was adjusted with HBSS. The assays were run in duplicate. The CL emission was measured using a microplate luminometer LM-01T (Immunotech, Marseille, France) for 90 min at 37°C. The integral value of the CL reaction, which represents total cell ROS production, was evaluated.

The extracellular production of superoxide by HL-60 cells was determined via spectrophotometric analysis of cytochrome *c* reduction (final concentration 100 μM). Cells were incubated with cytochrome *c* for 3 h. The reduction was monitored as the increase in cytochrome *c* absorbance at 550 nm [19]. The reduction of cytochrome *c* was blocked by superoxide dismutase from 95–97%, which indicated that cytochrome *c* reduction was almost fully superoxide-dependent. The concentration of superoxide was calculated using the extinction coefficient of reduced cytochrome *c*.

qRT-PCR

Total RNA was extracted by the RNeasy Mini Kit (QIAGEN, Venlo, Netherlands). Complementary DNA was synthesized according to the manufacturer's instructions for M-MLV reverse transcriptase kit (Sigma-Aldrich). qRT-PCR was performed on a Light-cycler 480 (Roche, Basel, Switzerland), using the following program: initial activation step at 95°C for 5 min, followed by 45 cycles at 95°C for 10 s, annealing temperature (see below) for 10 s and 72°C for 10 s. Gene expression for each sample was expressed in terms of the threshold cycle normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as we described previously [20]. Primers and PCR conditions were as follows (primers sequence/annealing temperature):

- Human MDR1/ABCB1
  5′-CACCCGACTTACAGATGATG-3′,
  5′-GTCCAAGAACAGGACTGATG-3′/55°C,
- Human MRP1/ABCC1
  5′- GAGGCTTTGATCGTCAAGTC-3′,
  5′-TTGATGAGCAACTTTAAGATCTG-3′/57°C,
- Human BCRP/ABCG2
  5′-ATAAATGGAGCACCGCGACC-3′,
  5′-TTCCAACCTTGGAGTCTGCC-3′/57°C,
- Murine MDR1a /ABCB1a
  5′-TGGAAGAAGCTAAAAGGCTG-3′,
  5′-CACGGAAAAGAAGACAGTGA-3′/56°C,
- Murine MDR1b/ABCB1a
  5′-AATCAAAGTGGACCCAACAG-3′,
  5′-CACCAAAGTGAAACCTGGAT-3′/56°C,
- Murine MRP1/ABCC1
  5′-TTCCTGTGCAACCATGTATC-3′,
  5′-CCAAATATTGCTGCACCTTG-3′/58°C, and
- Murine BCRP/ABCG2
  5′-CCATAGCCACAGGCCAAAGT-3′,
  5′-GGGCCACATGATTCTTCCAC-3′/58°C.

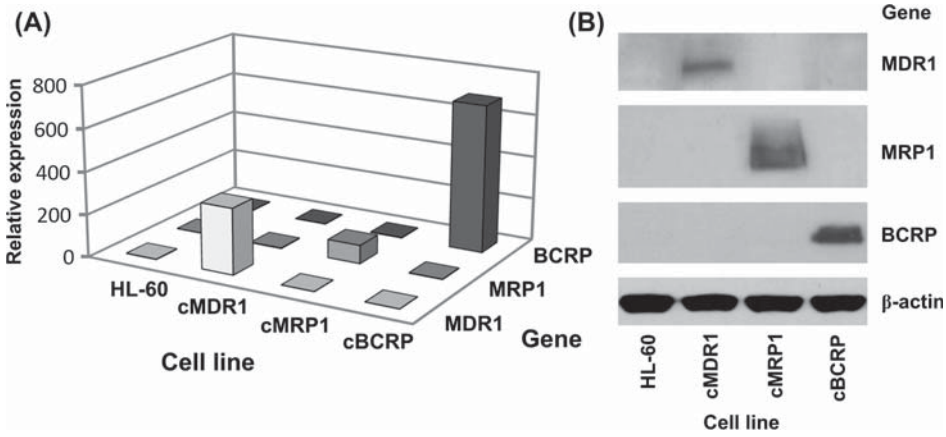

Figure 1. (A) qRT-PCR analysis of the expression of mRNA for MDR1, MRP1 and BCRP in HL-60 cells and their sub-clones (cMDR1, cMRP1 and cBCRP cells). Data are the results from two independent RNA isolations and qRT-PCR analyses presented as means. (B) Western blotting detection of the total protein level in HL-60, cMDR1, cMRP1 and cBCRP cells. Western blots are typical representatives of three independent experiments.

Western blotting

The total amount of ABC transporters in HL-60 cells and their sub-clones was assessed by Western blotting, as described in detail in Prochazkova et al. [21]. Standard 8% polyacrylamide gel electrophoresis was followed by wet blotting (100 V/70 min). Anti-MDR1 (P7965) antibody and secondary antibody (A6782) were produced by Sigma-Aldrich; anti-MRP1 (sc-59607) and anti-BCRP (sc-58224) were produced by Santa Cruz Biotechnology, Santa Cruz, CA, USA.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Groups were compared using a paired Student's *t*-test. Values of p less than 0.05 were considered significant.

Results

Expression of particular ABC transporter in HL-60 derived cell lines

First, we determined the mRNA expression of MDR1, MRP1 and BCRP in HL-60 cells and their sub-clones over-expressing a particular ABC transporter. Using qRT-PCR, we confirmed the expected high mRNA levels of MDR1, MRP1 and BCRP in cMDR, cMRP and cBCRP sub-clones, respectively. The transcription of non-over-expressed ABC transporters in sub-clones was inconsiderable. Maternal HL-60 cells also had a low expression of the mentioned ABC transporter (Figure 1A). These data are consistent with Western blotting analysis of the total ABC transporter expression (Figure 1B).

Assessment of total ROS production in HL-60 derived cell lines

Next, we analysed the basal total (both extra- and intracellular) ROS production by a luminescence method using two different luminophores, luminol and L-012 [18]. The detected CL signal was weak; however, it was detectable in all the tested cell lines. Importantly, the CL signal collected for 90 min had the same intensity in HL-60 cells and all the sub-clones (Figures 2A and B).

To avoid any doubt regarding the ability of luminophores to cross the membranes of ABC-rich cells, we employed the extracellular ROS-induced cytochrome *c* reduction assay, which was measured colourimetrically. This methodological approach confirmed that all the studied HL-60 sub-clones produce ROS to the same basal extent (Figure 2C).

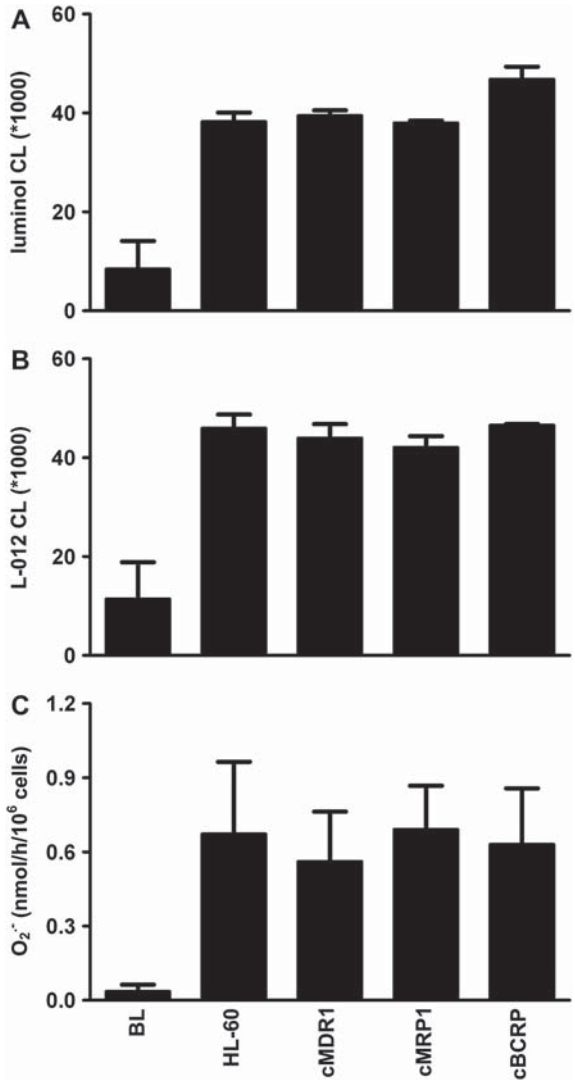

Figure 2. Analysis of ROS production in HL-60 cells and their sub-clones (cMDR1, cMRP1 and cBCRP cells). The spontaneous ROS production was determined by enhanced chemiluminescence (A, B) and cytochrome *c* reduction assay (C). Data represent results from three independent experiments presented as means and standard errors.

Measurement by fluorescent ROS-sensitive and mitochondria-related probes, inhibition of ABC transporter function

In the previous part, we showed that there was no difference in basal ROS production in the cell lines employed here. However, the employed cell lines differed significantly in their expression of ABC transporters. The efficiency of various probes' exclusion from a particular HL-60 sub-clone was analysed by flow-cytometry (Figure 3). All of these results were related to the HL-60 cells as a control (100%), which did not express any ABC transporter (Figure 1). Generally, we found out that each tested probe was a substrate for at least one studied ABC transporter. Excluding DCFH, all tested probes were substrates for MDR1, as was documented by the low

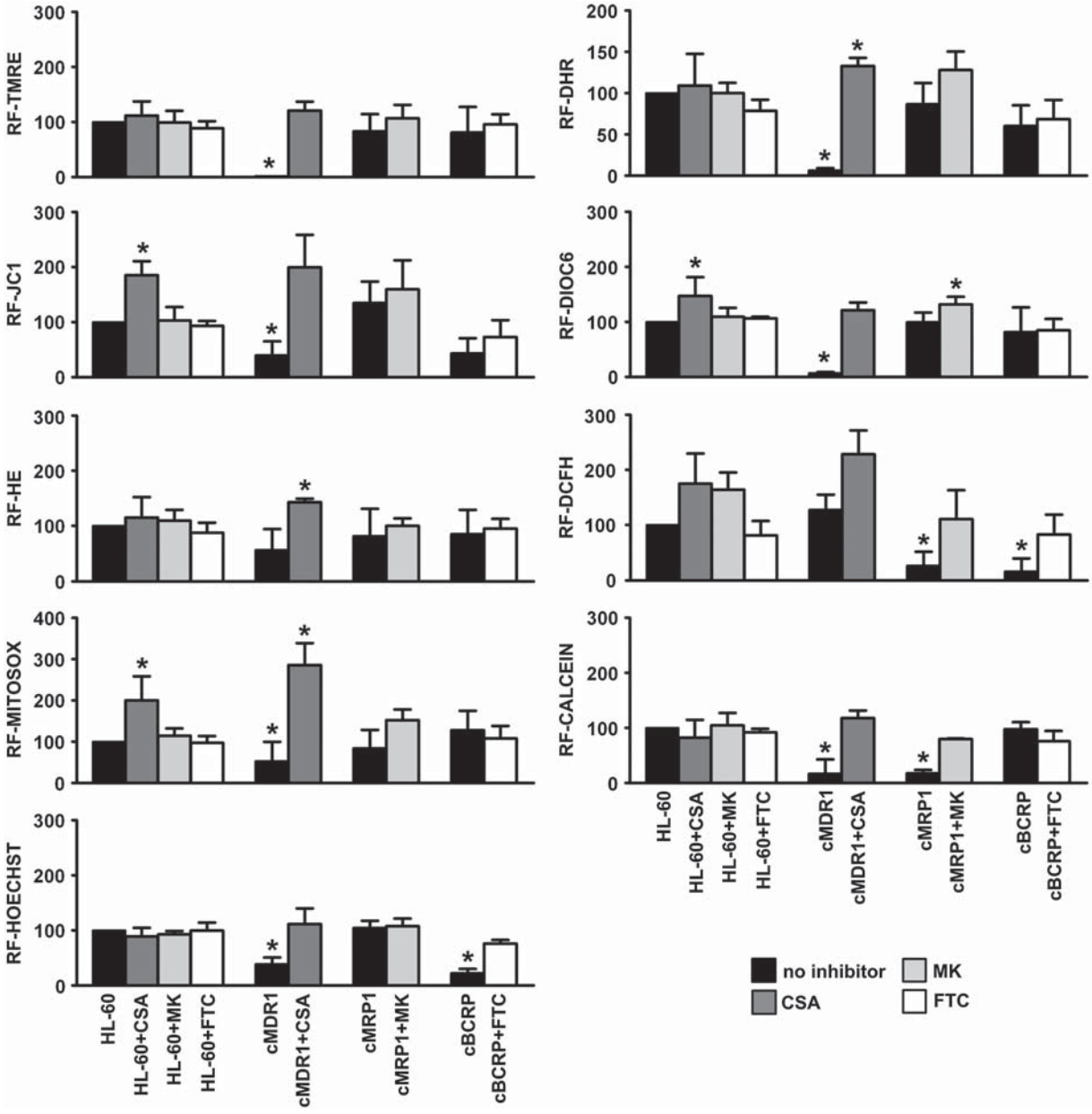

Figure 3. Dye exclusion assay of fluorescent ROS- and mitochondrial activity dependent probes in HL-60 cells and their sub-clones (cMDR1, cMRP1 and cBCRP cells). Calcein AM and Hoechst 33342 dyes were used as positive controls. Black bars represent untreated cells; shaded bars represent particular inhibitors of ABC transporter activity (CSA, cyclosporine A; MK, MK571; FTC, fumitremorgin C). Intensity of fluorescence is expressed as a percentage of fluorescence median of untreated HL-60 cells (100%) and presented as the mean median of fluorescence of three independent experiments and standard errors. *Significant difference between marked sample and HL-60 cells without any inhibitor ($p < 0.05$).

fluorescence signal detected in MDR1 over-expressing cells. The fluorescence signals of DCFH and Calcein, a well known substrate of MRP1, were significantly lower in MRP1 over-expressing cells; HE, MitoSOX and TMRE were also slightly excluded from these cells. The BCRP over-expressing cells predominantly excluded DCFH, JC1 and the commonly used substrate for BCRP, Hoechst. However, the intracellular levels of DHR and HE were also partly reduced in these BCRP over-expressing cells. The observed ability of ABC transporters to efflux fluorescent probes is summarized in Table I.

To confirm the role of ABC transporters in the decreased fluorescence of probes, we employed specific inhibitors of the ABC transporters cyclosporine A (inhibits MDR1), MK571 (inhibits MRP1) and Fumitremorgin C (inhibits BCRP) [22,23]. The inhibition of ABC transporter function resulted in an increased median of fluorescence, caused by a decreased efflux of the probes. In the majority of combinations, the fluorescence intensity reverted at least to the level of un-treated control HL-60 cells. In BCRP over-expressing cells, the reversion was achieved only partially, although significantly in statistical terms (Figure 3).

Table I. Summary of ABC transporters' ability to efflux studied probes.

ABC transporter	Probe								
	DCFH	DHR	DIOC6	HE	JC1	MitoSOX	TMRE	Calcein	Hoechst
MDR1	−	+++	+++	+	+++	+	+++	+++	+++
MRP1	+	−	−	+	−	+	+	+++	−
BCRP	+++	+	−	+	+	+	−	−	+++

+++, very strong substrate; +, weak substrate; −, no substrate.

Endogenous expression of ABC transporters may affect the ROS analysis performed by fluorescent probes

First, we performed a search for appropriate cell lines with the endogenously increased expression of ABC transporters. Using qRT-PCR, we screened embryonic stem, C2C12 and RAW 264.7 cell lines with supposedly increased ABC transporters levels (Figure 4A). Based on these results, murine myoblasts C2C12 and murine leukaemic macrophages RAW 264.7 were chosen for further experiments. The level of MDR1b mRNA in RAW 264.7 is much higher than in C2C12 cells (relative expression is 5.14 and 0.03, respectively); the same trend was observed for MRP1 mRNA level (relative expression is 126.6 and 5.3, respectively). On the other hand, RAW 264.7 cells have a much lower level of the BCRP mRNA (relative expression is 4.11 and 130.3, respectively). Both cell lines have an inconsiderably low level of MDR1a mRNA (relative expression is 0.11 and 0.07, respectively).

Next we stimulated the production of ROS in these cells by 0.8 μM phorbol 12-myristate 13-acetate (PMA) for 90 min [24]. We compared the non-stimulated and PMA-stimulated ROS production assessed by DCFH, DHR123 and cytochrome *c* reduction (Figure 4B). Although cytochrome *c* reduction assay evidenced increased ROS production in both cell lines, the fluorescent probes did not. PMA-stimulated C2C12 cells had the same intensity of fluorescence as control cells. RAW 264.7 stimulated by PMA and analysed by DCFH showed a statistically significant increase in fluorescence intensity. However, when using DHR, RAW 264.7 cells failed to increase their fluorescence in reaction to PMA in contrast to C2C12 cells.

Discussion

In the presented study we discovered that each of the tested ROS and mitochondria sensitive fluorescent probes (DCFH, HE, DHR-123, $(DiOC_6)_3$, MitoSOX red, JC1 and TMRE) is a substrate for at least one MDR-associated ABC transporter. We also demonstrated that the ability of these probes to detect PMA-stimulated ROS production could be negatively affected in cell lines endogenously over-expressing ABC transporters.

First, we compared our results with previously published data in the context of the ability of ABC transporter efflux-tested fluorescent probes. Although widely used for the determination of intracellular ROS, particularly hydrogen peroxide, the DCFH probe has not yet been described as a substrate of ABC transporters [25]. Here, we observed a significant efflux of DCFH in cells expressing MRP1 and BCRP transporters. In contrast, HE (oxidized mainly by superoxide) was previously shown to be cleared from cells resistant to adriamycin, a substrate of MDR1 [26]. In our study, this was confirmed by observation that HE was most efficiently excluded by cells over-expressing the MDR1 transporter. Moreover, the HE efflux was also mediated by MRP1 and to a lesser extent by the BCRP transporter. MitoSOX red dye (derivate of HE) was newly evidenced in our

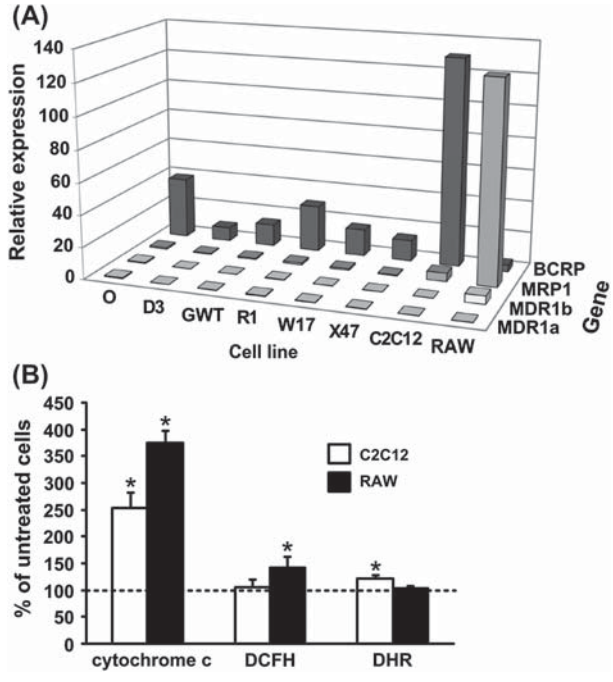

Figure 4. False negative assessment of ROS production by fluorescent probes. (A) Screening of murine embryonic stem cell lines (O, D3, GWT, R1, W17, X47), C2C12 and RAW 264.7 cells for endogenous ABC transporter expression performed by qRT-PCR. (B) C2C12 and RAW 264.7 cells were incubated with 0.8 μM PMA for 90 min before undergoing analysis of ROS production by cytochrome *c* reduction assay, DCFH and DHR assays. Results of PMA treated cells are expressed as a percentage of control cells untreated with PMA (100%) and presented as the mean of three independent experiments and standard errors. *Significant difference between marked sample and untreated control ($p < 0.05$).

study to be a good substrate of MDR1, and was also partially effluxed by MRP1. For the detection of intracellular ROS, non-fluorescent DHR is widely used, which is then oxidized to fluorescent rhodamine 123. DHR was established as the model compound for MDR1 mediated transport [27–29] and our data confirmed this observation. Importantly, we discovered that rhodamine 123 was also partially excluded by BCRP. $(DiOC_6)_3$, the fluorescent membrane dye used for detection of mitochondrial membrane potential in living cells [30], was demonstrated to be a substrate of MDR1 [31–33]. Our results also demonstrated that $(DiOC_6)_3$ had a high specificity for MDR1 and, thus, could be recommended as a reliable marker in studies of MDR1 activity. JC1, the cationic dye initially used for analysis of mitochondrial potential [34], was shown to be an even more effective substrate of MDR1 than classical DHR123 assay [35]. Here, we newly showed that JC1 is also a substrate for BCRP. TMRE, the membrane potential-dependent dye that selectively stains mitochondria with an intact membrane [36], was shown to be excluded from MDR1 over-expressing cells, but not from MRP1 and BCRP ones [37,38]. Our experiments confirmed TMRE as a substrate of MDR1; but, in contrast to Rajagopal and Simon [38], we observed that TMRE may also be slightly effluxed from cells by MRP1. Probes affinity to particular ABC transporters tested here are summarized in Table I. To demonstrate accuracy of the used model system we included two classic dyes generally used for the assessment of ABC transporters function. Our results are in concordance with the common knowledge that Calcein AM is a substrate of MRP1 and MDR1 [39] and Hoechst 33342 of BCRP and MDR1 [40].

To validate the function of particulate ABC transporters, we used the following specific inhibitors. Cyclosporine A inhibits MDR1 function [27] and prevents the mitochondrial permeability transition pore from opening [41]. Cyclosporine A inhibits MDR1 effectively (Figure 3); however, in samples stained by mitochondria-related dyes, it also increased the stainability, probably through the up-regulation of the mitochondrial potential. MK571 is a selective, competitive antagonist of the leukotriene D4 receptor, so it is not believed to affect ROS production in mitochondria [42]. Its ability to block MRP1 function is often used in ABC function assays [43]. We proved that, in MRP1 over-expressing HL-60 cells treated with MK571, fluorescence reverted to the level of HL-60 control. Fumitremorgin C (FTC) is a fungal toxin that causes cell cycle arrest [44]. Recently it was shown to be a potent and specific inhibitor of BCRP [45]. Although we inhibited BCRP function by a concentration of FTC widely used by other authors, the fluorescence intensity did not reach the level of control HL-60 cells. The incomplete blocking of FTC efflux could be explained by the inability of FTC to reach the BCRP protein that is present on the mitochondrial membrane [46].

Second, we aimed to show that ROS detection could be affected by the endogenous expression of ABC transporters. We showed that DCFH is a substrate of BCRP and, therefore, PMA-stimulated C2C12 myoblasts highly expressing BCRP revealed the same intensity of fluorescence as control cells. In contrast, RAW 264.7 with a relatively low expression of BCRP stimulated by PMA showed a significant increase in DCFH fluorescence intensity. However, when using DHR, a substrate of MDR1, the RAW 264.7 cells expressing high levels of MDR1b failed to increase DHR fluorescence in response to PMA in contrast to C2C12 cells. Thus, we proved that an inappropriate combination of selected dyes and cells, endogenously expressing a high amount of ABC transporter, could generate a false negative result when detecting ROS production or mitochondrial membrane potential.

In light of the presented data, the probes discussed here, and probably also other similar probes, must be used very carefully when analysing intercellular ROS and mitochondrial potential with regard to analysed cell type. Considering the described flaws of ROS and mitochondria-sensitive dyes, some results could possibly be affected by the combination of used cell type and dye [47–50]. In cancer and stem cells, the ROS production and mitochondrial potential need to be determined by at least two fluorescent probes and verified by different methodological approaches. A parallel analysis of the level and activity of the responsible ABC transporters should be presented. Substantial attention needs to be paid to experimental design with regard to the changing expression and activity of ABC transporters (e.g. by differentiation).

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper

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