Analytical Biochemistry 399 (2010) 246–250

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

ATP-binding cassette transporters as pitfalls in selection of transgenic cells

Dirk Theile, Bianca Staffen, Johanna Weiss *

Department of Clinical Pharmacology and Pharmacoepidemiology, University Hospital of Heidelberg, D-69120 Heidelberg, Germany

ARTICLE INFO

Article history:
Received 16 October 2009
Received in revised form 30 November 2009
Accepted 9 December 2009
Available online 14 December 2009

Keywords:
Puromycin
Hygromycin
Geneticin
G418
ABC transporters
Selection

ABSTRACT

Puromycin, hygromycin, and geneticin (G418) are antibiotics frequently used to select genetically engineered eukaryotic cells after transfection or transduction. Because intrinsic or acquired high expression of ATP-binding cassette (ABC) transporters, such as P-glycoprotein (Pgp/ABCB1) and multidrug resistance-associated proteins (MRP/ABCC1), can hamper efficient selection, it is important to know whether these antibiotics are substrates and/or inducers of efflux transporters. Therefore, we investigated the influence of these antibiotics on drug transporter expression by quantitative real-time polymerase chain reaction in the induction model cell line LS180. Moreover, we assessed whether ABC transporters influence the growth inhibitory effects of these antibiotics by proliferation assays using Madin–Darby canine kidney II (MDCKII) cells overexpressing the particular transporter. The results obtained indicate that puromycin and G418 are substrates of several ABC transporters, mainly Pgp/ABCB1. In contrast, hygromycin seems to be no good substrate for any of the ABC transporters investigated. Puromycin induced ABCC1/MRP1, whereas G418 suppressed ABCB1/Pgp, at the messenger RNA (mRNA) level. In contrast, hygromycin had no effect on ABC transporter mRNA expressions. In conclusion, this study emphasizes the significance of ABC transporters for the efficacy of selection processes. Consciousness of the results is supposed to guide the molecular biologist to the right choice of adequate experimental conditions for successful selection of genetically engineered eukaryotic cells.

Analysis of gene function can be performed by either overexpression or knockdown of the respective gene and protein. The vectors frequently used contain antibiotic resistance gene sequences that allow subsequent selection of successfully genetically engineered cells by treatment of cells with the corresponding selection antibiotic (e.g., puromycin, hygromycin, geneticin [G418]).[1] This approach premises distinct antibiotic resistance differences between successfully and nonsuccessfully transduced or transfected cells. However, in daily lab life, selection is a crucial step that can be hampered by unpredictable alterations of selection antibiotics' efficacy or by side effects leading to undesired properties of cell clones [1,2]. For instance, there is a report on isolated nontransfected G418-resistant carcinoma subclone that became resistant in the course of the transfection experiment [3]. Besides, mechanisms leading to attenuated drug uptake could lead to insufficient cytotoxicity of the selection antibiotic [4].

ATP-binding cassette (ABC) transporters, such as P-glycoprotein (Pgp/ABCB1), are membrane-located proteins using the hydrolysis of ATP to translocate chemically diverse substances across biological membranes [5]. In our laboratory, in many cases we have observed that epithelial cells with high Pgp/ABCB1 expression are extremely resistant to puromycin and/or G418, making it nearly impossible to eradicate them. Thus, the question arises whether ABC transporters might be responsible for diminished drug uptake and subsequent reduction in the effectiveness of antibiotics. For puromycin, data suggested that it is at least a substrate of the murine and hamster analogue of human Pgp/ABCB1 [6,7]. However, there are no data concerning the possible transport of puromycin by other ABC transporters or for transport of G418 and hygromycin.

In addition, if selection antibiotics are substrates of ABC transporters, induction of these efflux transporters would further lead to diminished selection efficacy. However, studies demonstrating possible inducing effects of selection antibiotics are sparse and data are conflicting. Puromycin was demonstrated to induce mdr1a, the murine analogue to human Pgp/ABCB1, in rat brain endothelial cell lines in vitro [8]. In addition, higher expression of Pgp/ABCB1 after puromycin treatment was also demonstrated in a human brain endothelial cell line. However, these findings were at least in part attributed to selective cell death of low-expressing subpopulations [9]. In contrast, Schuetz and coworkers showed

* Corresponding author. Fax: +49 6221 56 4642.
E-mail address: johanna.weiss@med.uni-heidelberg.de (J. Weiss).

[1] *Abbreviations used:* G418, geneticin; ABC, ATP-binding cassette; Pgp/ABCB1, P-glycoprotein; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; FCS, fetal calf serum; PCR, polymerase chain reaction; cDNA, complementary DNA; QPCR, quantitative PCR; BCRP/ABCG2, breast cancer resistance protein; MRP/ABCC, multidrug resistance-associated protein; MDCKII, Madin–Darby canine kidney II; RF, resistance factor; RT–PCR, reverse transcriptase PCR; GU, glucuronidase β; RNAi, RNA interference.

0003-2697/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2009.12.014

failure of puromycin to induce Pgp/ABCB1 in human hepatocytes in vitro [10].

We hypothesized that intrinsic or acquired high expression levels of ABC transporter can lead to diminished selection efficacy and, therefore, aimed to systematically investigate the potential interference between common selection antibiotics and ABC transporters possibly hindering successful selection of transfected or transduced cells.

Materials and methods

Materials

Culture media, medium supplements such as antibiotics (penicillin/streptomycin) and glutamine, dimethyl sulfoxide (DMSO), and phosphate-buffered saline (PBS) were purchased from Sigma–Aldrich (Taufkirchen, Germany). Puromycin was obtained from Invitrogen (Karlsruhe, Germany). Crystal violet, Triton X-100, and rifampicin were purchased from AppliChem (Darmstadt, Germany). Methanol and hygromycin were obtained from Roth (Karlsruhe, Germany). G418 and fetal calf serum (FCS) were obtained from PAA (Coelbe, Germany). MK571 was purchased from Biomol Research Laboratories (Plymouth Meeting, PA, USA), and LY335979 (zosuquidar) was purchased from Eli Lilly (Bad Homburg, Germany). Cell culturing bottles were supplied by Greiner (Frickenhausen, Germany), and 96-well microtiter plates were supplied by Nunc (Wiesbaden, Germany). Collagen R was obtained from Serva (Heidelberg, Germany). An RNeasy Midi-Kit was obtained from Qiagen (Hilden, Germany). Materials for real-time polymerase chain reaction (PCR) were purchased from Roche Applied Science (Mannheim, Germany). A RevertAid H Minus First Strand cDNA (complementary DNA) Synthesis Kit was purchased from Fermentas (St. Leon-Rot, Germany). An Absolute QPCR (quantitative PCR) SYBR Green Mix was obtained from ABgene (Hamburg, Germany).

Cell lines

LS180 cells (available at American Type Culture Collection [ATCC], Manassas, VA, USA), a cell line derived from human colon adenocarcinoma, were used to investigate changes of drug transporter mRNA expression by induction [11]. Cells were cultured under standard cell culture conditions as described previously [11] and were seeded 3 days before the assay.

As an in vitro model for human Pgp/ABCB1, breast cancer resistance protein (BCRP/ABCG2), and multidrug resistance-associated proteins (MRP/ABCC) 1, 2, and 3, we used Madin–Darby canine kidney II (MDCKII) cells overexpressing the corresponding transporter. All cell lines were generated by stable cDNA transfection into MDCKII cells and were provided by Piet Borst and Alfred H. Schinkel (Netherlands Cancer Institute, Amsterdam) [12–16]. Parental MDCKII/Par cells served as a control. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate.

Proliferation assay

Proliferation assays with MDCKII cells overexpressing one particular ABC transporter were conducted to evaluate substrate properties of the antibiotics investigated. Significantly different IC_{50} values between transporter overexpressing cells and parental cells are interpreted as the result of enhanced efflux of the particular drug indicating the investigated drug to be a substrate.

Cell proliferation was quantified by crystal violet staining [17]. In brief, cells were seeded onto collagen-coated 96-well microtiter plates and preincubated for 24 h. After the addition of test compounds, cells were incubated for another 48 h. Cells were then washed with PBS and stained with crystal violet (0.5%) as described previously [17]. Absorption was measured using a Multiskan RC photometer with 555 nm excitation. Proliferation was expressed as a proliferation index by calculating crystal violet absorption intensity as percentage relative to baseline (absorption intensity of medium-only wells [set to 0%]) and native proliferation (absorption intensity of untreated cells [set to 100%]). Each concentration was tested in octuplet, and each assay was performed three times.

Calculation of resistance factors

Resistance factor (RF) values were calculated either by division of IC_{50} of ABC transporter overexpressing cells with IC_{50} of parental cells (Fig. 1) or by division of IC_{50} of ABC transporter overexpressing cells or inhibitor-treated overexpressing cells with IC_{50} of parental cells (Fig. 2).

Thus, RF indicates increased resistance, equal sensitivity, or higher sensitivity if RF > 1, RF = 1, or RF < 1, respectively.

Evaluation of MRPs and Pgp/ABCB1 contribution to G418 resistance

Because MDCKII cells overexpressing Pgp/ABCB1, MRP1/ABCC1, MRP2/ABCC2, and MRP3/ABCC3 were generated by stable transduction of the corresponding cDNA with a pCMV-neo vector containing the neo gene (mediating G418 resistance) [12–14,16], we also performed proliferation assays in the presence of 50 μM MK571 and 5 μM LY335979 (zosuquidar), known inhibitors of MRPs [18,19] and Pgp/ABCB1 [20], respectively, to distinguish between drug transporter and neo gene-mediated G418 resistance.

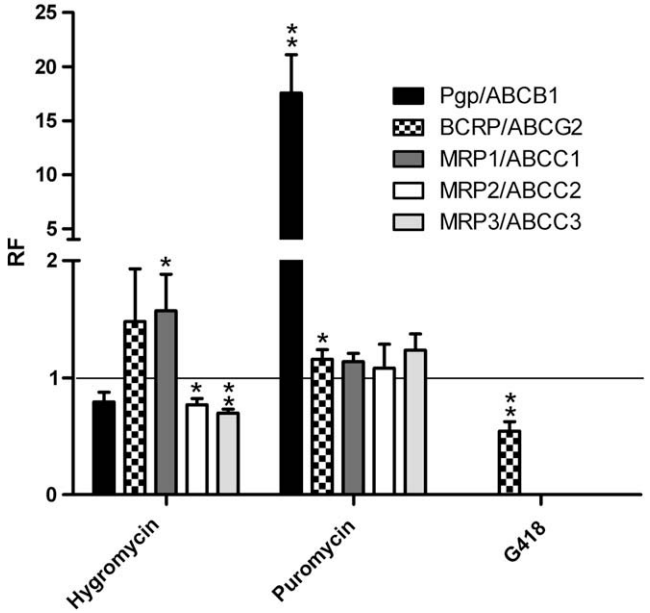

Fig. 1. Effect of hygromycin, puromycin, and G418 on proliferation of MDCKII cells overexpressing Pgp/ABCB1, BCRP/ABCG2, MRP1/ABCC1, MRP2/ABCC2, and MRP3/ABCC3. RF values were calculated by division of IC_{50} of ABC transporter overexpressing cells with IC_{50} of parental cells. RF values are means ± standard deviations of at least three independent experiments. Statistical significance was evaluated by two-tailed unpaired Student's *t* test compared with the corresponding parental cell line used as control. $^{*}P < 0.05$; $^{**}P < 0.01$.