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Research article

Drug-selected cell line panels for evaluation of the pharmacokinetic consequences of multidrug resistance proteins

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ABSTRACT

Through the selection with five chemotherapeutics of diverse chemical structures and modes of action (*cis*-diamminedichloroplatinum, doxorubicin, etoposide, methotrexate and vincristine), four multidrug-resistant cell line panels were developed. Cancer cell lines of different species (human and murine) as well as tissue/organ (skin, colon) origin, characterized by low endogenous expression of multidrug resistance (MDR) proteins and high sensitivity to anticancer agents, were used as parental cell lines. A selection process resulted in the up-regulation of several ABC transporters (ABCB1/Abcb1a, ABCC1/Abcc1 and ABCG2/Abcg2), which was confirmed by a number of molecular and cell biology methods. The MDR protein expression pattern seemed to be mainly dependent on the drug used for the selection and not on the species or tissue origin of the cell line. We postulate that such cell panels can be used as a research model to assess the role of MDR proteins in the pharmacokinetics of novel drugs or drug formulations.

1. Introduction

Multidrug resistance (MDR) proteins are members of the vast ABC (ATP-binding cassette) protein superfamily and, in physiological conditions, they constitute a first-line defence system of a cell exposed to xenobiotics (Kunjachan, Rychlik, Storm, Kiessling, & Lammers, 2013). As active transporters, they expel xenobiotic molecules or their metabolites outside the cell – either back to the extracellular milieu or to the bile or urine to dispose of it from the body. Therefore, they are important factors affecting the bioavailability and distribution of toxins and, what is even more essential, of drugs. Major drug regulatory agencies, e.g. the U.S. Food and Drug Administration (2012), or European Medicines Agency (2012), require in their guidance documents that the role of major MDR proteins is taken into account when analysing drug–drug interactions of novel pharmaceutical substances. Thus, there is a need for efficient tools that allow the analysis of the role of specific multidrug resistance proteins in the pharmacokinetics of therapeutic substances.

In the molecular biology era, it is easy to construct a transfected cell, stably or transiently overexpressing a given protein – either wild type or mutated. Thus, research models of this kind are nowadays very popular and widely used in studies on the biology and pharmacology of MDR proteins (a simple query: '(ABCC* OR ABCB* OR ABCG2) AND transfected AND (cell line)' in PubMed returns approx. 400 records). The main benefit resulting from such an attitude is the presence of a novel and well-defined gene product in (usually) otherwise unchanged cells. However, there is also a significant drawback to these kinds of models – they hardly mimic the real-life situations where cells attune their entire metabolism to face xenobiotics. Therefore, we decided to come back to the roots of multidrug resistance protein science and, like pioneers in this field, to employ selection with drugs to produce MDR cells. We wanted to determine the relation between the selection agent (recruited from commonly used chemotherapeutics) and the resulting phenotype as well as to check whether the tissue/organ origin (skin and colon) is important for the final MDR profile. Finally, we were interested in the universality of the observed pattern and thus we compared murine and human cells.

2. Materials and methods

2.1. Reagents

The highest-purity reagents available were used to perform the experiments described below. Inorganic salts, acids and bases were

Abbreviations: ABC proteins, ATP-binding cassette proteins; cDDP, *cis*-diamminedichloroplatinum(II); DOX, doxorubicin; EC*n*, effective concentration at which *n*% of control is active; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Ko143, (3S,6S,12aS)-1,2,3,4,6,7,12,12a-octahydro-9-methoxy-6-(2-methylpropyl)-1,4-dioxopyrazino[1′,2′:1,6]pyrido[3,4-b]indole-3-propanoic acid 1,1-dimethylethyl ester; MDR, multidrug resistance; MK571, 5-(3-(2-(7-chloroquinolin-2-yl)ethenyl)phenyl)-8-dimethylcarbamyl-4,6-dithiaoctanoic acid; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; MTX, methotrexate; qPCR, quantitative polymerase chain reaction; RR, resistance ratio; SD, standard deviation; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; VINC, vincristine; VP16, etoposide.

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purchased from Avantor Performance Materials Poland (Gliwice, Poland) or Chempur (Piekary Śląskie, Poland), while organic compounds and primary monoclonal antibodies F4 and AC-74 were obtained from Sigma-Aldrich (Saint Louis, MO, USA). qPCR reagents were purchased from Roche (Basel, Switzerland). Primary monoclonal antibodies C219 and MRPr1 were acquired from Enzo Life Sciences Inc. (the Alexis brand, Farmingdale, NY, USA), and BXP9 and BXP21 from Abcam (Cambridge, UK).

All buffers and aqueous solutions were prepared using Milli-Q water (Milli-Q Integral water station, Millipore, Billerica, MA, USA).

2.2. Cell lines

Parental cell lines: A-431 (human epidermoid carcinoma), SW620 (human colorectal adenocarcinoma), B16-F10 (murine melanoma) and CT26.WT (murine colon carcinoma) were purchased from American Type Culture Collection and cultured in standard cell culture conditions (37 °C, 5% CO_2, 95% relative humidity). The cells were grown in high-glucose Dulbecco's Modified Eagle Medium buffered with HEPES, supplemented with Glutamax-I and 10% v/v foetal bovine serum (Thermo Fisher Scientific Inc., Waltham, MA, USA). Care was taken to avoid cross-contamination between the cell lines (handling one cell line at a time, using anti-aerosol tips and single-use equipment were the minimum safety conditions). The cells were tested every 3 months for *Mycoplasma* contamination with a MycoProbe® Mycoplasma Detection Kit by R&D (Minneapolis, Minnesota, USA).

2.3. Selectant cell lines

The sensitivity of parental cell lines towards five standard chemotherapeutics: cisplatin (cDDP), doxorubicin (DOX), etoposide (VP16), methotrexate (MTX) and vincristine (VINC) was tested with MTT assay (see below). Then, 10^5 cells of a given line were seeded into a well of a standard Nunc 6-well plate in a final volume of 2 mL of a complete medium supplemented with a selective concentration of a specific chemotherapeutic agent: EC10 (for most cell line and drug combinations) or EC25 (for A-431 and all drugs; B16-F10 and cDDP and vincristine; CT26.WT and cDDP, doxorubicin and vincristine). The medium supplemented with a selective agent was carefully replaced every 3 days until single clones of resistant cells could be observed. Single colonies were transferred in a droplet of trypsin/EDTA solution to separate wells of a 96-well plate and allowed to grow until confluency under the constant selection pressure. The cells were subsequently transferred to larger dishes until a stably growing daughter cell line was obtained. The respective drug sensitivity of these lines was tested again (the cells were allowed to grow for 3 passages, (approximately 7 days), without the selection pressure before the assay) and the most resistant clones were selected for further manipulations. The procedure was repeated twice for SW620 and CT26.WT selectants. We failed to obtain the second-round resistant clones in the case of A-431 and B16-F10 selectants.

2.4. Viability assay

The cell line drug sensitivity was determined according to a modified MTT-reduction assay (Carmichael, DeGraff, Gazdar, Minna, & Mitchell, 1987). Cells suspended in 100 μL of a complete medium were seeded on 96-well plates at a density of 10^4/well (human cell lines) or 5×10^4/well (murine cell lines). The cells were allowed to attach for 24 h and then the drug was added at the desired concentration. Stock solutions were prepared in dimethyl sulphoxide/phosphate-bufferred saline and the solvent concentration was maintained constant in all wells, including the controls. The final dimethyl sulphoxide concentration did not exceed 0.1% v/v and was determined to be non-toxic to the cells. After 70 h of incubation, MTT was added to the medium to a final concentration of 1.1 mM. After a further 2 h, the medium was removed and the formazan crystals were dissolved in 100 μL of dimethyl sulphoxide. The absorbance was measured at 580 nm analytical wavelength and 720 nm reference wavelength. The results were turned into percentage of controls and the EC50, EC25 and EC10 values for each cell line and substance were calculated with the GraphPad Prism 5.02 software (GraphPad Inc.) using a four-parameter nonlinear logistic regression.

2.5. Evaluation of gene expression by RT-qPCR

Total cellular RNA was isolated from 5×10^6 cells using Chomczynski and Mackey's method (Chomczynski & Mackey, 1995). Reverse transcription reaction was carried by the protocol of Wiame, Remy, Swennen, and Sagi (2000) using a Maxima™ Reverse Transcriptase (Thermo Fisher Scientific) kit. The following primers were used for evaluation of specific gene expression: human genes (forward/reverse pair) – *ABCB1*(TGACATTTATTCAAAGTTAAAAGCA/TAGACACTTTATGCAAACATTTCAA), *ABCC1*(AGTGGAACCCCTCTCTGTTTAAG/CCTGATACGTCTTGGTCTTCATC), *ABCG2*(CCGCGACAGTTTCCAATGACCT/GCCGAAGAGCTGCTGAGAACTGTA), *HPRT1*(TGACACTGGCAAAACAATGCA/GGTCCTTTTCACCAGCAAGCT) and *GAPDH*(CCCTTCATTGACCTCAACTA/GCCAGTGAGCTTCCCGTTCA); murine genes: *Abcb1*(AGCATTACTAATCAAAGTGGACCC/CTATGCAGCACACCAGCACCA), *Abcc1*(TGTGGACCTAGAGACAGATTAACCT/ACAATCACCCGCGTGTAGT), *Abcg2*(AAGTCTTCGTTGCTAGATGTC/GTCATCTTGAACCACATAACCTG), *Hprt*(TGATTAGCGATGATGAACCAG/AAGTCTTTCAGTTCCTGTCCA) and *Gapdh*(GGAGAGTGTTTCCTCGTCCC/GCAACAATCTCCACTTTGCCA). qPCR of 20 ng of cDNA was performed using FastStart Taq DNA Polymerase (Roche) at CFX96 system (Bio-Rad, Hercules, CA, USA). Additionally, the expression of 42 different human ABC genes in A-431 and SW620 cell line sets was analysed using a commercial RealTime Ready® ABC Transporter Panel (Roche) according to the manufacturer's instructions.

2.6. Western blotting

Cells were detached using a scraper and suspended in an ice-cold 100 mM TrisHCl pH 7.4 supplemented with Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific) and washed three times ($100 \times g$, 10 min, 4 °C) in this buffer. The cell pellet was lysed by freeze-thaw method. The lysate protein content was assayed using bovine serum albumin as a standard (Bradford, 1976) and lysates were diluted to protein concentration of 2 mg/mL and mixed with equal amount of 2× Laemmli buffer supplemented with 2-mercaptoethanol. The samples were heat-denaturated (99 °C, 1 min) and loaded into gel. SDS-PAGE was performed by Laemmli procedure (Laemmli, 1970) with 5% stacking gel and 10% separating gel at a constant voltage of 110 V for 1.5 h at room temperature using Mini-Protean® Tetra Cell TM (Bio-Rad). Membranes isolated from Sf9 cells transfected with human *ABCB1*, *ABCC1* or *ABCG2* genes (Solvo Biotechnology, Szeged, Hungary) were used as respective positive controls, while membranes of Sf9 cells transfected with human β-galactosidase gene were used as a negative control. Control samples were treated exactly as lysate samples. SeeBlue® Pre-Stained Standards in a range between 4 and 250 kDa (Thermo Fisher Scientific) were used for molecular weight calibration. Proteins were transferred to activated ImmobilonP™ membrane (Millipore) according to Towbin, Staehelin, and Gordon (1979) at a constant amperage of 360 mA for 90 min at 4 °C. Unspecific protein binding was blocked by overnight incubation of membrane in 5% solution of bovine serum albumin in TBST buffer (0.05% Tween 20, 150 mM NaCl, 50 mM TrisHCl, pH 8.0) at 4 °C. Incubation with primary antibodies (directed towards a specific protein and β-actin as an internal standard) was carried out for 60 min at room temperature, followed by a triple rinsing of the membrane with TBST. Incubation with a secondary antibody lasted for another 60 min at room temperature. The membrane was rinsed three times with TBST followed by water and placed in Super Signal West Dura Chemiluminescent Substrate