Inhibitors of ABC Transporters and Biophysical Methods to Study their Activity

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Multidrug resistance caused by the presence and overproduction of ABC transporters makes serious problems in cancer treating. The drugs administered during therapy are pumped outside the cell using the energy obtained from ATP hydrolysis. The augmented dosage of drugs to overcome the multidrug resistance is not sufficient. Thus knowledge of the structure of ABC proteins is necessary to understand the rules of their action. It could be also helpful to understand how the multidrug resistance could be overcome. One of the strategies involves the treatment of cancer cells with a mixture of anticancer drugs and inhibitors of ABC transporters. The yeast *Saccharomyces cerevisiae*, whose PDR pumps are analogues of mammalian MDR proteins responsible for multidrug resistance, is a suitable research model. Biophysical methods with different fluorescent dyes seem to be very suitable for the measurement of the efflux pump activity. This review describes some known inhibitors of ABC proteins and biophysical methods which could be used for measuring the ABC transporters activity.

Key words: ABC Transporters, Inhibitors, *Saccharomyces cerevisiae*

Introduction

Multidrug resistance, which is caused by the presence and overexpression of ABC transporters, represents a serious problem in cancer treatment. Drugs used during anticancer therapy are pumped out of the cell, the export being fuelled by the energy obtained from ATP hydrolysis. The use of increased doses of anticancer drugs to overcome resistance is not efficient. Elucidation of the structure of the ABC transporters is therefore necessary for understanding their activity and ultimately overcoming the multidrug resistance. Simultaneous application of anticancer drugs and inhibitors of ABC pumps is one of the strategies to surmount the multidrug resistance. A convenient model for this kind of investigation is the yeast *Saccharomyces cerevisiae*. Its PDR pumps are analogues of mammalian multidrug proteins, which are responsible for the resistance. Some compounds that inhibit the activity of yeast ABC pumps and methods used to study the activity of these transporters are presented here.

Yeast ABC Transporters

ABC transporters are present in membranes of many living organisms from microorganisms to man and are involved in the transport of a large spectrum of substrates, such as ions, sugars, lipids, antibiotics, and larger molecules, such as oligopeptides or oligosaccharides (Biemans-Oldehinkel *et al.*, 2006).

These ATP-dependent pumps or channels have a modular architecture: two cytosolic, nucleotide-binding domains (NBDs) with a highly conservative amino acid sequence of the ATP-binding region called nucleotide-binding fold (NBF), designated also as ABC domain, and two transmembrane domains (TMDs) form a functional transporter (Schmitt and Tampe, 2002). A hydrophilic NBD has in its structure the glycine-rich Walker A and hydrophobic Walker B motifs. Both motifs play a crucial role as ATP-binding site commonly occurring in nucleotide-binding proteins such as: P-, F-, V-ATPases or phosphofructokinase (Lage, 2003). The third consensus sequence is the ABC

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signature, also named C motif, which is unique for the structure of ABC transporters and is situated between Walker A and Walker B motifs (Del Sorbo *et al.*, 2000). A "full-ABC transporter" contains two transmembrane TMDs and two NBD domains $[TMD\text{-}NBD]_2$. In eukaryotic ABC transporters the whole structure is a single polypeptide, in prokaryotes there are a few polypeptides. In "half-transporters" with one transmembrane domain and NBD these proteins accumulate in homo- or heterodimers (Lage, 2003).

The ABC transporter activity encompasses several steps. First the substrate and ATP bind to the transporter and then the NBDs undergo dimerization. During the second stage ATP hydrolysis dissociates the NBD dimer and the binding site reorientates from an outward- to an inward-facing conformation, and the substrate is translocated across the membrane. ADP and inorganic phosphate are released and the ABC transporter returns to the initial state (Biemans-Oldehinkel *et al.*, 2006; Jasinski and Figlerowicz, 2006).

Cells of the model eukaryotic organism *Saccharomyces cerevisiae* synthesize 30 ABC transporters divided into five different families: PDR (pleiotropic drug resistance), MRP (multidrug resistance-associated protein), MDR (multidrug resistance), ALDp (adrenoleukodystrophy protein), and YEF3/RLI (Jungwirth and Kuchler, 2006, Decottignies and Goffeau, 1999; Taglicht and Michaelis, 1998; Bauer *et al.*, 1999; Wolfger *et al.*, 2000).

PDR proteins in *Saccharomyces cerevisiae* are analogues of MDR proteins in mammals (Bauer *et al.*, 1999). Five ABC transporters in the yeast plasma membrane belong to the PDR family: Pdr5p, Pdr12p, Snq2p, Yor1p and Ycf1p (Lage, 2003; Del Sorbo *et al.*, 2000). Overexpression of Pdr5p with high homology to human glycoprotein P causes multidrug resistance of cells to many clinically important antibiotics, cytostatics, fungicides as well as herbicides and mycotoxins (Kolaczkowski *et al.*, 1996, 1998). Pdr12p contributes to the resistance of cells to food preservatives such as sorbic, propionic or benzoic acid (Piper *et al.*, 1998; Holyoak *et al.*, 1999). Snq2p is responsible for cell resistance to mutagens – *N*-oxide-4-nitroquinolines, 2,3,5-tris(ethylenoimino)benzoquinone and other reagents such as phenanthroline (Servos *et al.*, 1993). Yor1p and Ycf1p, which are analogues of the human CFTR transporter (cystic fibrosis transmembrane conductance regulator) and MRP1 (multidrug-resistance related protein 1), cause resistance against oligomycin and cadmium (Del Sorbo *et al.*, 2000; Katzmann *et al.*, 1995). Moreover, Yor1p and Ycf1p efflux a broad spectrum of compounds which are also substrates of Pdr5p (Decottignies *et al.*, 1998; Rebbeor *et al.*, 1998; Katzmann *et al.*, 1999).

ABC Transporter Inhibitors

The strategy to overcome the MDR involves *e.g.* the treatment of cancer cells with a mixture of anticancer drugs and inhibitors of ABC transporters. Chemosensitizers, named also MDR modulators, are compounds which inhibit ABC pumps and hence reduce the resistance of cells to cytostatics (Choi, 2005; Ozben, 2006).

The first known chemosensitizer was verapamil, which blocks calcium channels (Tsuruo *et al.*, 1981). The group of first-generation chemosensitizers contains for example: antagonists of calmodulin, steroids, inhibitors of kinase C, indole alkaloids and detergents (Thomas and Coley, 2003; Ford and Hait, 1990). The first-generation modulators are characterized by low activity and high toxicity, while the second-generation chemosensitizers, *e.g.*, cyclosporin D, diarylimidazole, valspodar, have a low affinity to ABC transporters (Ozben, 2006).

Inhibitors can influence ABC transporters in three ways: through specific interactions with proteins, by changing the intracellular ATP level which is the source of energy for ABC pumps, or by influencing membrane phospholipids and increasing the membrane permeability for ions that decrease the activity of ABC transporters.

Some modulators of human glycoprotein P also influence the yeast pump Pdr5p. This effect was observed for flavonoids (Conseil *et al.*, 2000), immunosuppressant FK506 (Egner *et al.*, 1998), or protein kinase C effectors (Conseil *et al.*, 2001).

Effective and specific inhibitors for the Pdr5p pump are eniatins B, B1 and D isolated from *Fusarium* sp. Y-53. They are neutral ionophores forming complexes with ions which increase the membrane permeability (Hiraga *et al.*, 2005). A similar specific inhibitor for Pdr5p is isonitrile [3-(3'-isocyano-cyclopent-2-enylidene)-propionic acid] isolated from *Trichoderma* sp. P24-3. This compound is nontoxic and, in contrary to eniatins, it is not a substrate for the Pdr5p pump

but is inactivated in the presence of nickel ions (Yamamoto *et al*., 2005).

The most effective modulators of multidrug resistance are phenothiazines which change the asymmetry of rearrangements of aminophospholipids in the erythrocyte membrane. This suggests a mechanism of modulator activity through influence of membrane lipids (Kołaczkowski *et al*., 2003).

Some biophysical methods presented in this review are used to determine the activity of ABC transporters.

Determination of ABC Transporter Activity

Quenching of rhodamine G6 or rhodamine 123 fluorescence

Rhodamine G6 and rhodamine 123 are substrates for the Pdr5p transporter. Preparations of membranes isolated from strains with overexpression of Pdr5p indicate ATP-dependent quenching of the rhodamine fluorescence. This quenching is competent inhibited by many anticancer drugs, such as vinblastine, vincristine, taxol or verapamil, and ionophoric proteins (*e.g.* valinomycine, nigericin, gramicidin D) and steroids (progesterone, deoxycorticosterone, β-estradiol). The noncompetent inhibition of the Pdr5 pump was observed for the anticancer drug colchicine or the antiarrythmic drug chinidine (Kolaczkowski *et al.*, 1996).

In the *S. cerevisiae* mutant with Pdr5 deletion accumulation of rhodamine in cells was observed. The same effect was noted in the case of cells with Pdr5, but the efflux of rhodamine was interfered by compounds with inhibitory activity (Hiraga *et al.*, 2005; Yamamoto *et al.*, 2005). The excitation wavelength of rhodamine G6 is 529 nm and the emission wavelength is 553 nm; for rhodamine 123 it is accordingly 505 nm and 534 nm (Kolaczkowski *et al*., 1996). The quenching or accumulation of these fluorescent probes can be observed by fluorescent microscopy, spectrofluorimetry or flow cytometry.

The fluorescent dye diS-C_3(3) as a marker of the ABC transporter activity

The carbocyanine dye diS-C_3(3) undergoes efflux from *S. cerevisiae* cells by some ABC transporters, *e.g*. Pdr5p and Snq2p. In low concentration (10^{-8} M) diS-C_3(3) is nontoxic for cells (Cadek *et al*., 2004). The excitation wavelength of diS-C_3(3) is 531 nm, its fluorescence range is 560–590 nm. The binding of the dye to molecules (nucleic acids, proteins, lipids) in a cell causes a red-shift to λ_{max} = 582 nm (Gaskova *et al.*, 1999, 2001). The $\Delta\psi$-dependent uptake of the dye into the cells is accompanied by a red-shift of the fluorescence maximum and an increase in the fluorescence intensity. At the same time the probe is actively exported from the cells by PDR pumps, and the extent of pump action can be determined by comparing the intracellular concentration of the probe in a pump-free and a pump-competent strain (Gaskova *et al.*, 2002). If a tested compound inhibits the pump activity in a pump-competent strain a shift of the fluorescence maximum to longer waves (red-shift) is observed.

Similar properties were observed for the fluorescent dye $DiOC_5$ (3,3'-dipentyloxacarbocyanine iodide) (Prudencio *et al.*, 2000).

Acetoxymethyl derivatives as fluorescent markers determinate the ABC pump activity

Acetoxymethyl calceine (calceine-AM) is hydrolyzed by esterase to a form with the ability of fluorescence. The derivative of calceine-AM can be translocated into the cells and is a substrate for ABC pumps. The fluorescence of the calceine derivative is pH-dependent and the fluorescence intensity increases with the pH value.

High fluorescence intensity of calceine is in the visible range (excitation, 485 nm; emission, 520 nm). Hence the changes in the transport of calceine can be easily detected. Calceine is used as a marker for the detection of the ABC transporters activity (Prudencio *et al*., 2000; Hollo *et al*., 1994).

Acetoxymethyl derivatives of another fluorescent marker, fura-2, fluo-2, indo-1, BCECF, also undergo efflux from cells by ABC transporters and can be used for determining their activity (Hollo *et al*., 1994).

In a recently described method for the determination of the ABC pump activity, the fluorescent dye Hoechst 33342 was used. Excitation wave length for Hoechst 33342 is 365 nm, emission wave length 450 nm. In this case the fluorescence increases when Hoechst 33342 intercalates to DNA or interacts with membranes; hence, the fluorescence intensity is higher inside than outside the cells.

Transport of this dye is inhibited by ABC transporter inhibitors causing an increase of the fluorescence intensity in cells. The method with

Hoechst 33342 determines the specifity of ABC pump inhibitors. If the modulator influences the binding site of Hoechst 33342 in the protein, the fluorescence intensity of this fluorochrome increases (Muller *et al.*, 2007, 2008).

Bauer B.-E., Wolfger H., and Kuchler K. (1999), Inventory and function of yeast ABC proteins: about sex, stress, pleiotropic drug and heavy metal resistance. Biochim. Biophys. Acta **1461**, 217–236.

Biemans-Oldehinkel E., Doeven M.-K., and Polman B. (2006), ABC transporter architecture and regulatory roles of accessory domains. FEBS Lett. **580**, 1023–1035.

Cadek R., Chladkova K., Sigler K., and Gaskova D. (2004), Impact of the growth phase on the activity of multidrug resistance pumps and membrane potential of *S. cerevisiae*: effect of pump overproduction and carbon source. Biochim. Biophys. Acta **1665**, 111–117.

Choi C.-H. (2005), ABC transporters as multidrug resistance mechanisms and the development of chemosensitizers for their reversal. Cancer Cell. Int. **4**, 5–30.

Conseil G., Decottignies A., Jault J.-M., Comte G., Barron D., Goffeau A., and Di Pietro A. (2000), Prenylflavonoids as potent inhibitors of the Pdr5p multidrug ABC transporter from *Saccharomyces cerevisiae*. Biochemistry **39**, 6910–6917.

Conseil G., Perez-Victoria J.-M., Jault J.-M., Gamarro F., Goffeau A., Hofmann J., and Di Pietro A. (2001), Protein kinase C effectors bind to multidrug ABC transporters and inhibit their activity. Biochemistry **40**, 2564–2571.

Decottignies A. and Goffeau A. (1999), Complete inventory of the yeast ABC proteins. Nat. Genet. **15**, 137–145.

Decottignies A., Grant A.-M., Nichols J.-W., de Wet H., McIntosh D.-B., and Goffeau A. (1998), ATPase and multidrug transport activities of the overexpressed yeast ABC protein Yor1p. J. Biol. Chem. **273**, 12612–12622.

Del Sorbo G., Schoonbeek H., and De Waard M.-A. (2000), Fungal transporters involved in efflux of natural toxic compounds and fungicides. Fungal Genet. Biol. **30**, 1–15.

Egner R., Rosenthal F.-E., Kralli A., Sanglard D., and Kuchler K. (1998), Genetic separation of FK506 susceptibility and drug transport in the yeast Pdr5 ATP-binding cassette multidrug resistance transporter. Mol. Biol. Cell. **9**, 523–543.

Ford J.-M. and Hait W.-N. (1990), Pharmacology of drugs that alter multidrug resistance in cancer. Pharmacol. Rev. **42**, 155–199.

Gaskova D., Brodska B., Holoubek A., and Sigler K. (1999), Factors and processes involved in membrane potential build-up in yeast: diS-C_3(3) assay. Int. J. Biochem. Cell. Biol. **31**, 575–584.

Gaskova D., Cadek R., Chaloupka R., Plasek J., and Sigler K. (2001), Factors underlying membrane potential-dependent and -independent fluorescence responses of potentiometric dyes in stressed cells: diS-C_3(3) in yeast. Biochim. Biophys. Acta **1511**, 74–79.

Gaskova D., Cadek R., Chaloupka R., Vacata V., Gebel J., and Sigler K. (2002), Monitoring the kinetics and performance of yeast membrane ABC transporters by diS-C_3(3) fluorescence. Int. J. Biochem. Cell Biol. **34**, 931–937.

Hiraga K., Yamamoto S., Fukuda H., Hamanaka N., and Oda K. (2005), Enniatin has a new function as an inhibitor of Pdr5, one of the ABC transporters in *Saccharomyces cerevisiae*. Biochem. Biophys. Res. Commun. **328**, 1119–1125.

Hollo Z., Homolya L., Davis C.-W., and Sarkadi B. (1994), Calcein accumulation as a fluorometric functional assay of the multidrug transporter. Biochim. Biophys. Acta **1191**, 384–388.

Holyoak C.-D., Bracey D., Piper P.-W., Kuchler K., and Coote P.-J. (1999), The *Saccharomyces cerevisiae* weak-acid-inducible ABC transporter Pdr12 transports fluorescein and preservative anions from the cytosol by an energy-dependent mechanism. J. Bacteriol. **181**, 4644–4652.

Jasinski M. and Figlerowicz M. (2006), Plant ABC transporters – the family with tradition. Adv. Biochem. **52**, 296–302.

Jungwirth H. and Kuchler K. (2006), Yeast ABC transporters – a tale of sex, stress, drugs and aging. FEBS Lett. **580**, 1131–1138.

Katzmann D.-J., Hallstrom T.-C., Voet M., Wysock W., Golin J., Volckaert G., and Moye-Rowley W.-S. (1995), Expression of an ATP-binding cassette transporter-encoding gene (YOR1) is required for oligomycin resistance in *Saccharomyces cerevisiae*. Mol. Cell Biol. **15**, 6875–6883.

Katzmann D.-J., Epping E.-A., and Moye-Rowley W.-S. (1999), Mutational disruption of plasma membrane trafficking of *Saccharomyces cerevisiae* Yor1p, a homologue of mammalian multidrug resistance protein. Mol. Cell Biol. **19**, 2998–3009.

Kolaczkowski M., van der Rest M., Cybularz-Kolaczkowska A., Soumillion J. P., Konings W. N., and Goffeau A. (1996), Anticancer drugs, ionophoric peptides, and steroids as substrates of the yeast multidrug transporter Pdr5p. J. Biol. Chem. **271**, 31543–31548.

Kolaczkowski M., Kolaczkowska A., Luczynski J., Witek S., and Goffeau A. (1998), *In vivo* characterization of the drug resistance profile of the major ABC transporters and other components of the yeast pleiotropic drug resistance network. Microb. Drug Res. **4**, 143–158.

Kołaczkowski M., Michalak K., and Motohashi N. (2003), Phenothiazines as potent modulators of yeast multidrug resistance. Int. J. Antimicrob. Agents **22**, 279–283.

Lage H. (2003), ABC-transporters: implications on drug resistance from microorganisms to human cancers. Int. J. Antimicrob. Agents **22**, 188–199.

Muller H., Klinkhammer W., Globisch C., Kassack M.-U., Pajeva I.-K., and Wiese M. (2007), New functional

assay of P-glycoprotein activity using Hoechst 33342. Bioorg. Med. Chem. **15**, 7470–7479.
Muller H., Pajeva I.-K., Globisch C., and Wiese M. (2008), Functional assay and structure-activity relationships of new third-generation P-glycoprotein inhibitors. Bioorg. Med. Chem. **16**, 2448–2462.
Ozben T. (2006), Mechanisms and strategies to overcome multiple drug resistance in cancer. FEBS Lett. **580**, 2903–2909.
Piper P., Mahe Y., Thompson S., Pandjaitan R., Holyoak C., Egner R., Mühlbauer M., Coote P., and Kuchler K. (1998), The Pdr12 ABC transporter is required for the development of weak organic acid resistance in yeast. EMBO J. **17**, 4257–4265.
Prudencio C., Sansonetty F., Sousa M.-J., Corte-Real M., and Leao C. (2000), Rapid detection of efflux pumps and their relation with drug resistance in yeast cells. Cytometry **39**, 26–35.
Rebbeor J.-F., Connolly G.-C., Dumont M.-E., and Ballatori N. (1998), ATP dependent transport of reduced glutathione on YCF1, the yeast orthologue of mammalian multidrug resistance associated proteins. J. Biol. Chem. **273**, 33449–33454.
Schmitt L. and Tampe R. (2002), Structure and mechanism of ABC transporters. Curr. Opin. Struct. Biol. **12**, 754–760.
Servos J., Haase E., and Brendel M. (1993), Gene SNQ2 of *Saccharomyces cerevisiae*, which confers resistance to 4-nitroquinoline-*N*-oxide and other chemicals, encodes a 169 kDa protein homologous to ATP-dependent permeases. Mol. Gen. Genet. **236**, 214–218.
Taglicht D. and Michaelis S. (1998), *Saccharomyces cerevisiae* ABC proteins and their relevance to human health and disease. Methods Enzymol. **292**, 130–162.
Thomas H. and Coley H.-M. (2003), Overcoming multidrug resistance in cancer: an update on the clinical strategy of inhibiting P-glycoprotein. Cancer Control. **10**, 159–165.
Tsuruo T., Iida H., Tsukagoshi S., and Sakurai Y. (1981), Overcoming of vincristine resistance in P388 leukemia *in vivo* and *in vitro* through enhanced cytotoxicity of vincristine and vinblastine by verapamil. Cancer Res. **41**, 1967–1972.
Wolfger H., Mamnun Y.-M., and Kuchler K. (2000), Fungal ABC proteins: pleiotropic drug resistance, stress response and cellular detoxification. Res. Microbiol. **152**, 375–389.
Yamamoto S., Hiraga K., Abiko A., Hamanaka N., and Oda K. (2005), A new function of isonitrile as an inhibitor of the Pdr5 multidrug ABC transporter in *Saccharomyces cerevisiae*. Biochem. Biophys. Res. Commun. **330**, 622–628.