

Review

YEAST ABC PROTEINS INVOLVED IN MULTIDRUG RESISTANCE

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Abstract: Pleiotropic drug resistance is a complex phenomenon that involves many proteins that together create a network. One of the common mechanisms of multidrug resistance in eukaryotic cells is the active efflux of a broad range of xenobiotics through ATP-binding cassette (ABC) transporters. *Saccharomyces cerevisiae* is often used as a model to study such activity because of the functional and structural similarities of its ABC transporters to mammalian ones. Numerous ABC transporters are found in humans and some are associated with the resistance of tumors to chemotherapeutics. Efflux pump modulators that change the activity of ABC proteins are the most promising candidate drugs to overcome such resistance. These modulators can be chemically synthesized or isolated from natural sources (e.g., plant alkaloids) and might also be used in the treatment of fungal infections. There are several generations of synthetic modulators that differ in specificity, toxicity and effectiveness, and are often used for other clinical effects.

Key words: ABC proteins, PDR subfamily, *Saccharomyces cerevisiae*, Multidrug resistance, Regulation of ABC proteins, Transcription factors, P-glycoprotein, Modulators of ABC proteins, Flavonoids, Phenothiazines

YEAST ABC PROTEINS

ATP-binding cassette (ABC) transporters are widespread among prokaryotes and eukaryotes. They are localized mainly in the mitochondrial, vacuolar and peroxisomal membranes and the plasma membrane (Fig. 1) [1].

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Abbreviations used: ABC – ATP-binding cassette; AR – activation region; BRCP – breast cancer resistance protein; MDR – multidrug resistance; MRP – multidrug resistance protein; NBD – nucleotide-binding domain; PDR – pleiotropic drug resistance; Pgp – P-glycoprotein; TET – tetrandrine; TMD – transmembrane domain; YRE – Yap responsive element

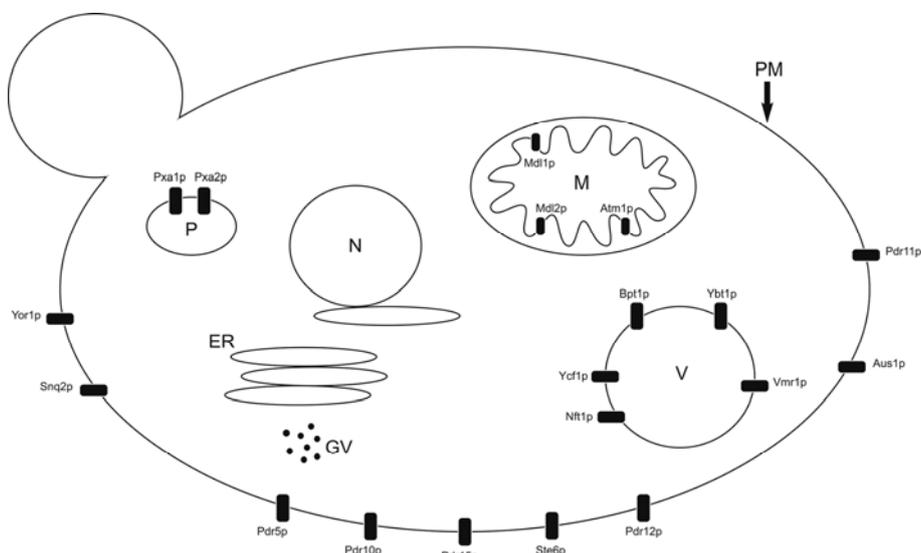


Fig. 1. Localization of the ABC transporters in the *Saccharomyces cerevisiae* cell. N – nucleus; V – vacuole; M – mitochondrion; P – peroxisome; PM – plasma membrane.

The structure of ABC transporters is conserved across organisms. The major unit is composed of two homologous moieties containing a transmembrane domain (TMD) with several (usually six) α -helices spanning the membrane, and a nucleotide-binding domain (NBD). The NBD couples ATP hydrolysis with substrate transport and can be localized C- or N-terminally relative to the TMD [2]. Each NBD is about 200 amino acids long and contains conserved regions, which are Walker A and Walker B motifs (separated by 90-120 aa) and a signature site with the consensus sequence LSGGQ (ABC signature, motif C) before Walker B. The crystal structure of the bacterial ABC transporters shows that the two domains interact with each other: Walker A and B of one domain interact with motif C of the other domain. Some ABC proteins contain no transmembrane domain and do not participate in substrate transport, but couple ATP hydrolysis with other crucial cellular processes, such as DNA repair or translation [3, 4]. ATP hydrolysis is the key activity of ABC proteins.

The ABC transporters are common determinants of resistance to antibiotics, fungicides and herbicides, so understanding their functioning is very important [5]. Mutations in genes encoding ABC proteins in humans cause numerous severe diseases, including cystic fibrosis and hyperbilirubinemia [6, 7]. Another important issue is the resistance of cancer cells to chemotherapy, due to overexpression of the *MDR1* gene, which encodes P-glycoprotein. Full understanding of the mechanisms of action and regulation of the ABC transporters is fundamental in overcoming the phenomenon of multidrug resistance. *Saccharomyces cerevisiae* is an excellent model organism to study these proteins, mainly thanks to the structural and functional similarities of its ABC transporters to those of mammals and pathogenic fungi [1].

The first yeast ABC transporter identified was Ste6p. The NBD domain sequence of this protein was used as a template to identify 30 genes encoding putative ABC transporters (22 proteins with NBD and TMD domains and eight proteins with NBD only) in the yeast genome [3, 5, 8].

One of the roles of ABC transporters is efflux of a wide variety of xenobiotics: anticancer drugs, cytotoxic compounds, fungicides, antibiotics, and other growth inhibitors. This transport is normally non-specific and the substrate spectrum of a given pump is very wide, which can contribute to the development of multidrug resistance (MDR).

The classification of ABC proteins includes six subfamilies: MDR, MRP/CFTR, ALDP, RLI, YEF3 and PDR, or, according to Human Genome Organization (HUGO) classification, ABCB to ABCG [3]. The functions of the main yeast ABC transporters are summarized in Table 1.

Table 1. ABC proteins in *Saccharomyces cerevisiae*.

Gene/protein	Subfamily	Function	Localization	Reference
<i>STE6/Ste6p</i>	MDR (ABCB)	Pheromone a export	Plasma membrane	8
<i>MDL1/Mdl1p</i>	MDR (ABCB)	Transport of protein degradation products	Inner mitochondrial membrane	9, 10
<i>MDL2/Mdl2p</i>	MDR (ABCB)	Unknown	Inner mitochondrial membrane	9, 10
<i>ATM1/Atm1p</i>	MDR (ABCB)	Transport of iron-sulfur clusters	Inner mitochondrial membrane	9, 13
<i>YOR1/Yor1p</i>	MRP/CFTR (ABCC)	Multidrug efflux pump	Plasma membrane	15
<i>YCF1/Ycf1p</i>	MRP/CFTR (ABCC)	Heavy metal transport	Vacuolar membrane	16
<i>YBT1/Ybt1p</i>	MRP/CFTR (ABCC)	Bile salt transport	Vacuolar membrane	18
<i>BPT1/Bpt1p</i>	MRP/CFTR (ABCC)	Bilirubine, glutathione and glucuronic acid transport	Vacuolar membrane	19, 20
<i>VMR1/Vmr1p</i>	MRP/CFTR (ABCC)	Multidrug efflux pump	Vacuolar membrane	21
<i>NFT1/Nft1p</i>	MRP/CFTR (ABCC)	Unknown	Vacuolar membrane	22
<i>PXA1/Pxa1p</i>	ALDP (ABCD)	Fatty acid transport	Peroxisomal membrane	23, 24
<i>PXA2/Pxa2p</i>	ALDP (ABCD)	Fatty acid transport	Peroxisomal membrane	23, 24
<i>PDR5/Pdr5p</i>	PDR (ABCG)	Multidrug efflux pump, lipid translocation, quorum sensing	Plasma membrane	35-38
<i>SNQ2/Snq2p</i>	PDR (ABCG)	Multidrug efflux pump	Plasma membrane	42
<i>PDR15/Pdr15p</i>	PDR (ABCG)	Cell detoxification during general stress response	Plasma membrane	48
<i>PDR12/Pdr12p</i>	PDR (ABCG)	Transport of weak organic acids	Plasma membrane	50
<i>PDR11/Pdr11p</i>	PDR (ABCG)	Sterol transport	Plasma membrane	53
<i>AUS1/Aus1p</i>	PDR (ABCG)	Sterol transport	Plasma membrane	53
<i>PDR18/Pdr18p</i>	PDR (ABCG)	Plasma membrane sterol incorporation	Plasma membrane	54, 55

The MDR (ABCB) subfamily includes Ste6p, which is localized in the plasma membrane, and Atm1p, Mdl1p and Mdl2p, which are localized in the inner mitochondrial membrane. Ste6p is responsible for pheromone transport (for mating type a cells), which is necessary for conjugation [8]. Mdl1p transports peptides (products of misfolded protein degradation, e.g., Yta12p) from the mitochondrial matrix to the periplasmic space. *MDL1* deletion causes sensitivity to oxidative stress [9, 10]. In spite of its large homology to Mdl1p, Mdl2p is not connected with peptide transport. The role of this protein is unknown, but *MDL2* deletion causes aerobic respiration arrest at high temperature, resistance to osmotic stress, and sensitivity to oleic acid [10-12]. Atm1p, similarly to Mdl1p and Mdl2p, is localized to the inner mitochondrial membrane. It is thought that this protein contributes to the maintenance and maturation of cytosolic iron-sulfur clusters by transporting them from the mitochondrial matrix to the cytoplasm [13]. Atm1p is thus believed to support mitochondrial genome stability. Its dysfunction causes iron ion accumulation, a signal of oxidative stress in the cell [9].

Another subfamily of ABC proteins is MRP/CFTR (ABCC), which consists of Yor1p, Ycf1p, Ybt1p, Bpt1p, Vmr1p and Nft1p. These proteins transport their substrates in the form of glutathione conjugates (e.g., cadmium ions), sulfates or glucuronates [3]. Yor1p is localized to the plasma membrane. *YORI* was first identified as a gene conferring resistance to oligomycin, but later data indicate that Yor1p reduces the toxic effects of many unrelated compounds that contain carboxyl groups [14, 15]. Ycf1p is found in the vacuolar membrane and is responsible for detoxification of cadmium, arsenic, lead, mercury and antimony by their transport from the cytoplasm to the vacuole [16, 17]. Ybt1p is a vacuolar transporter of bile salts [18]. Bpt1p, also a vacuolar protein, is responsible for vacuolar import of bilirubin (unconjugated or in the form of glutathione or glucuronate conjugates). *BPT1* also confers resistance to cadmium [1, 19, 20]. Vmr1p, which is localized to the vacuolar membrane, participates in the export of various growth inhibitors from the cell (cycloheximide, 2,4-dinitrophenole, cadmium, mercury) [21]. The role of Nft1p is unknown, but it has been proposed to negatively regulate Ycf1p; this possibility requires further study [22].

The ALDP (ABCD) subfamily is represented by two transporters: Pxa1p and Pxa2p (peroxisomal ABC transporter). Both proteins are found in the peroxisome membrane and form a heterodimer that translocates long-chain acyl-CoA esters. The expression of these proteins increases in the presence of oleic acid. Disruption of *PXA1* and *PXA2* arrests beta-oxidation of long-chain fatty acids. Pxa1p shows strong homology with the human peroxisomal proteins ALDP and Pmp70p, which are necessary for the correct biogenesis and functioning of peroxisomes [23, 24].

The RLI and YEF (ABCE and ABCF, respectively) subfamilies comprise proteins (Yef3p, Hef3p, Arb1p, Gcn20p, Rli1p and New1p) that do not function as transporters. Yef3p is present in the cytoplasm and ribosomes and is required

for cell functioning, because it plays a role in translation. Overexpression of *YEF3* increases cell sensitivity to inhibitors of translation, such as paromomycin and hygromycin B [25, 26]. Hef3p is a paralog of Yef3p, which arose from whole genome duplication [27]. Gcn20p, which regulates Gcn2p kinase, is one of the factors necessary for the proper cell response to amino acid starvation [28-30]. Arb1p and New1p are thought to play a role in the biogenesis of ribosomal subunits, and *ARB1* deletion is lethal [31, 32]. Rli1p is an essential iron-sulfur protein that plays a crucial role in the initiation and termination of translation [33].

PDR (ABCG) subfamily of yeast ABC proteins

The transporters belonging to the PDR (ABCG) subfamily of yeast ABC proteins are often associated with the extrusion of various growth inhibitors, but not all of them are multidrug efflux pumps. The best characterized are Pdr5p and Snq2p, which are localized to the plasma membrane and efflux a broad spectrum of drugs, including antibiotics, fungicides, detergents, ionophores, steroid hormones and anticancer drugs [34, 35]. Overexpression of *PDR5* confers resistance to many distinct growth inhibitors, such as antibiotics, azoles, chemotherapeutics and steroid hormones. Pdr5p functions as a homodimer and participates in cation export and lipid translocation as well as having a role in multidrug resistance. It is also suggested that Pdr5p has a role in quorum sensing as a pump exporting signal molecules, since $\Delta pdr5\Delta snq2$ cells arrest at a very high cell density [5, 36-39]. The absence of Pdr5p is not lethal but causes hypersusceptibility to drugs. *PDR5* expression is positively controlled by the transcription factors Pdr1p and Pdr3p, and negatively regulated by Rdr1p [5, 40]. Additionally, it is induced by the Yap1p and Yap2p transcription factors during heat shock [41].

Snq2p is a close homolog of Pdr5p and was identified as a protein conferring resistance to 4NQO (4-nitroquinoline 1-oxide). Later research showed that its overexpression caused resistance to many other compounds [1, 42]. Similarly to Pdr5p, Snq2p is involved in cation transport and quorum sensing. The expression of *SNQ2* is controlled by the transcription factors Pdr1p, Pdr3p and Stb5p [37, 38, 43]. Stb5p functions as a heterodimer with Pdr1p and is also involved in regulating the response to oxidative stress [44]. *SNQ2* expression is also induced by drugs (by means of the Yrr1p factor) and by heat shock (by means of the Yap1p factor) [41, 45]. Other homologs of Pdr5p are Pdr10p and Pdr15p [46]. Expression of both proteins is controlled by Pdr1p, Pdr3p and probably by Pdr8p [46, 47]. *PDR15* expression is also connected with the general stress response because the *PDR15* promoter comprises the STRE sequence recognized by the transcription factor Msn2p. The activity of Pdr15p is strongly induced by distinct stress signals: osmotic shock, heat shock, starvation and weak acids. Pdr15p contributes to cell detoxification during metabolic stress [48]. Pdr10p is responsible for the correct distribution and functioning of several proteins (e.g., chitin synthase Chs3p). It has also been shown that Pdr10p is involved in the distribution of another PDR transporter, Pdr12p. Strains that lack

PDR10 were strongly resistant to sorbate due to the increased amount of Pdr12p [49]. Pdr12p mediates resistance to weak organic acids. The substrate spectrum of this transporter includes common conservatives (benzoate, sorbate, propionic acid) and organic acids produced in the cell [50]. The expression of *PDR12* is negatively regulated by Pdr1p and Pdr3p and positively by War1p. War1p is constitutively bound to the WARE sequence in *PDR12* promoter. The appearance of weak organic acids induces War1p phosphorylation, which activates *PDR12* transcription. Pdr12p actively exports weak organic acids (C3-C7) from the cell [51, 52].

The remaining PDR proteins are only poorly characterized. Aus1p and Pdr11p have been suggested to participate in sterol transport, but the exact mechanism is not known. One hypothesis assumes sterol translocation directly from the plasma membrane to cytosolic sterol-binding proteins or to the endoplasmic reticulum membrane. These proteins may also indirectly facilitate sterol transport by catalyzing the transmembrane translocation of other lipids. Another model assumes the uptake of extracellular lipids and their incorporation into the plasma membrane [53]. Pdr18p is associated with resistance to some herbicides (2,4-D, MCPA), but its physiological role is in lipid homeostasis (probably direct incorporation of ergosterol into the plasma membrane). Its role in multidrug resistance might derive from the physiological functions of this protein [54]. Recently, it was shown that the expression of *PDR18* enhanced yeast tolerance to inhibitory concentrations of ethanol, possibly through decreasing plasma membrane permeabilization and lowering intracellular ethanol concentration [55].

Functional analogs of PDR transporters are also found in pathogenic fungi. The *Candida albicans* genome possesses 28 ABC proteins, but only Cdr1p and Cdr2p play a role in multidrug extrusion [56]. Cdr1p has a topology highly similar to that of Pdr5p in *Saccharomyces cerevisiae*. However, they differ in some functional features. Cdr1p confers resistance to cycloheximide, chloramphenicol, and oligomycin, and while Pdr5p shares the specificity of Cdr1p to cycloheximide and chloramphenicol, neither amplification nor disruption of Pdr5p affects susceptibility to oligomycin. The substrate spectrum of Cdr1p includes many unrelated compounds such as azoles, lipids or steroids [57, 58]. Cdr2p exhibits about 84% identity with Cdr1p, but differs in its drug resistance profile. This protein mainly mediates resistance to azole compounds and terbinafine [59, 60].

ABC transporters that contribute to drug resistance are also found in other fungal pathogens. Posteraro *et al.* identified and characterized the antifungal resistance 1 gene (*AFR1*) in *Cryptococcus neoformans*. It encodes ABC protein involved in fluconazole resistance [61]. Several genes encoding ABC transporters have been found and partially characterized in *Aspergillus fumigatus*. Overexpression of *MDR1* conferred resistance to cilofungin (a lipopeptide related to echinocandins). Expression of another transporter *atrF* was induced by itraconazole, but only in itraconazole-resistant isolate [62, 63].

ABC transporters are common in human cells. Their expression, e.g., in the digestive system, is connected with the export of cAMP, lipids and hormones outside the cell [64]. Their overexpression in cancer cells is a major problem in chemotherapy, because they can effectively export most conventional drugs, thereby decreasing their intracellular concentrations, often to below the therapeutic threshold. The best characterized human ABC transporter is P-glycoprotein (Pgp). It is found in the plasma membrane of the cells of the liver, intestines, blood-brain barrier, placenta and other organs [65]. A characteristic feature of Pgp is a broad substrate spectrum, which includes anthracyclines, colchicines, methotrexate and mitoxantrone. Pgp is encoded by the *MDR1* gene, mutations of which not only increase the production of Pgp (causing tumor resistance), but also increase the risk of Parkinson's disease and ulcerative colitis [64]. Other ABC transporters involved in tumor resistance to anticancer drugs are BCRP (breast cancer resistance protein) and MRP1-3 and 5 (multidrug resistance proteins). BCRP is mainly present in the breast, placenta, bowel and liver, whereas the MRP proteins are found in almost every tissue. Both types of transporter have broad substrate spectra, and their overexpression in tumor tissues disrupts anticancer treatment [66].

REGULATION OF YEAST PDR (ABCG) PROTEINS

The expression of ABC transporters belonging to the PDR (ABCG) subfamily is controlled by several transcription factors, of which the best characterized are Pdr1p and Pdr3p. They belong to the GAL4 family of transcription factors containing the DNA-binding Zn₂Cys₆ zinc finger [67]. Both Pdr1p and Pdr3p bind the same sequence of PDRE (the Pdr1p/Pdr3p response element), which is present in different numbers of copies and orientation in the target gene promoters. The two transcription factors bind their cognate promoters via the zinc fingers recognizing CGG triplets in the PDRE motif. A single PDRE site is sufficient for Pdr1p or Pdr3p to bind, but full activation of the promoter requires at least three PDRE [68]. The PDRE consensus sequence is a perfect palindrome: 5'-TCCGCGGA-3'. However, Pdr1p and Pdr3p tolerate some single-base changes. It is believed that the binding of the transcription factors to PDRE is also dependent on other interactions, because the PDRE site in the *HXT11* promoter is not recognized by Pdr1/3p, whereas exactly the same sequence in the *PDR5*, *YORI* and *PDR10* promoters is bound by those transcription factors. The participation of other transcription factors in the binding has also been suggested [67].

The Pdr1p and Pdr3p structure comprises eight hydrophobic motifs (MI-MVIII) in the central part of the protein. They form a functional domain that regulates the activity of Pdr1/3p [69]. Pdr3p contains two activating regions (AR). ARI is localized near the N-terminus and has low binding efficiency, whereas ARII (C-terminal 180 residues) is more important in the activation of transcription. Pdr1p contains only one AR site near the C-terminus, and many spontaneous

mutations in this region increase the activity of Pdr1p (*pdr1-8*, *pdr1-10*, *pdr1-12*) [67]. An important role in the regulation of transcription activation is played by the co-activator/repressor protein Ngg1p. The C-terminal region of Pdr1p (aa 815-1063) and Pdr3p (aa 815-976) interacts directly or indirectly with Ngg1p, and adaptor proteins (Ada2p, Gcn5p) are involved in this process [70].

Some spontaneous mutations in *PDR1* and *PDR3* increase transcription activation of the target genes, conferring multidrug resistance phenotype. Single point mutations in *PDR1* (*pdr1-2*, *pdr1-3*, *pdr1-6*, *pdr1-7*, *pdr1-8*) increase the mRNA level of target genes (*PDR5*, *SNQ2*, *YORI*, *PDR10*, *PDR15*) and mutations in *PDR3* (*pdr3-2* to *pdr3-10*) cause overexpression of *PDR5*, *SNQ2*, *PDR15*, *PDR10* and *PDR3* [46, 71, 72]. The *pdr1* and *pdr3* mutations result in multidrug resistance. Furthermore, the *pdr1-2* mutant is unable to grow under osmotic stress, heat shock or at increased pH, *pdr3-11* is not viable on lactate or glycerol/ethanol medium, and *pdr1-11* is temperature-sensitive [73, 74].

Numerous genes are regulated by the Pdr1/3p transcription factors. This group of genes includes the ABC transporters Pdr5p, Pdr10p, Pdr15p, Snq2p, and Yor1p, and the MFS transporters. Pdr1p also controls the expression of *PDR3*, *IPT1* (whose product is involved in sphingolipid biosynthesis) and *PDR16*, controlling sterol and phospholipid composition in the plasma membrane [72, 75]. Overexpression of *PDR5*, *SNQ2* or *YORI* confers resistance to anticancer drugs, antibiotics, fungicides, detergents, ionophores and many others. Pdr5p and Yor1p are also involved in phosphatidylethanolamine and steroid transport. *pdr1-11* and *pdr3-11* mutants exhibit decreased accumulation of phosphatidylethanolamine, which indicates the activation of proteins that promote its efflux or reduce influx [74, 76, 77]. Pdr1/3p also stimulates transcription of genes encoding MFS transporters, e.g., *TPO1* or *HXT11*, and genes, whose products are involved in lipid and cell wall metabolism (*PDR16*) [75, 78, 79].

The target genes have different affinities for the transcription factors. *PDR5* expression strongly depends on the activity of Pdr1p and Pdr3p. The double mutants $\Delta pdr1\Delta pdr3$ are hypersensitive to cycloheximide, which is a Pdr5p substrate [80].

SNQ2 is under the control of another transcription factor, Yrr1p, which also belongs to a zinc finger family. *SNQ2* transcription is activated in the *yrr1-1* gain-of-function mutant and similar changes are observed for *YORI*. Overexpression of *SNQ2* and *YORI* in the *yrr1-1* strain respectively results in resistance to 4-NQO and reveromycin A. The Pdr1p and Pdr3p factors are responsible for *SNQ2* expression at the basal level, but the overexpression is achieved by Yrr1p [45, 80, 81].

Another efflux pump, Pdr15p, is induced by Pdr1/Pdr3p, owing to the presence of the PDRE site in the *PDR15* promoter. It has been shown that *PDR15* is also under the control of Msn2p, which is a transcription factor that regulates the expression of about 200 genes in response to different stresses, including heat shock, osmotic and oxidative stress, and glucose starvation. Msn2p contains

a zinc finger domain that recognizes the STRE element in target gene promoters, and this element is also present in *PDR15* promoter [48, 82].

Pdr3p, besides regulating the expression of genes for the efflux pumps, can also positively autoregulate its own transcription due to the presence of two PDRE sites in its promoter. Those sites are also recognized and regulated by Pdr1p [83].

The regulation of proteins of the PDR (ABCG) subfamily also involves other factors. Pdr1p is post-translationally modified by Pdr13p, from the Hsp70 family, which affects its activity. No such effect of Pdr13p has been reported for Pdr3p [84].

War1p regulates *PDR12* transcription in response to weak organic acids. A model of War1p activity includes uncharged organic acids entering the cytoplasm, their dissociation, and lowering of the intracellular pH. The protons are then exported by Pma1p and the anionic acid residues by Pdr12p. War1p is directly or indirectly activated by anions. It is constitutively bound to the *PDR12* promoter at the WARE sequence. After induction by acids, it undergoes phosphorylation and activates the expression of *PDR12* [52].

Some data indicate a correlation between the genes encoding ABC efflux pumps and the YAP network, which is regulated mainly by Yap1p. Yap1p is a zinc-finger transcription factor and a major determinant of the oxidative stress response system. Yap1p directly activates the transcription of genes under stress conditions by binding to the YRE (Yap responsive element) site in their promoters. YRE elements are present in the *PDR5* and *SNQ2* promoters and Yap1p can induce those transporters in response to external stress (e.g., heat shock). The Yap1p-mediated diazaborine resistance is dependent on Pdr1p and Pdr3p, which suggests interactions among these transcription factors [41, 85, 86].

MODULATION OF MULTIDRUG ABC TRANSPORTERS

Active efflux of drugs is one of the main mechanisms of resistance among microorganisms and cancer cells. Inhibition of efflux pumps by chemosensitizers is a promising approach to overcoming drug resistance. These modulators can inhibit the ABC transporters by specific interactions; by lowering the intracellular ATP level, crucial for the functioning of the pump; or by affecting the plasma membrane composition [87].

The research on efflux pump inhibitors mainly concerns human ABC transporters, such as glycoprotein P (PgP), the overexpression of which in cancer cells disturbs chemotherapy. To date, many modulators of multidrug resistance have been identified: calcium channel blockers (verapamil), calmodulin antagonists, steroids, protein kinase C inhibitors, immunosuppressants (cyclosporine A), phenothiazines, indole alkaloids (reserpine), steroid hormones, antisteroids (tamoxifen), detergents, and surfactants [88].

The first generation of the pump modulators includes compounds that are already pharmacologically used for other purposes. These compounds have a low affinity to the ABC transporters and usually compete with the drugs being

transported for the binding site, which requires the use of highly toxic doses of the modulators. This group of efflux inhibitors includes verapamil, quinine and cyclosporine A [89-91].

Second generation modulators (e.g., cyclosporine D and valspodar) were designed to decrease the side effects of the first generation ones. However, it emerged that those modulators disrupted the clearance of anticancer drugs, thereby increasing their plasma level and leading to toxic effects. Moreover, many chemotherapeutics, similarly to the second generation inhibitors, are substrates of the cytochrome P450 3A4 isoenzyme. The competition of the modulators and the drugs for this enzyme can cause unpredictable pharmacokinetic interactions that limit their use in overcoming drug resistance. Finally, the affinity of the second generation modulators to ABC transporters is still too low [88].

The synthesis of third generation inhibitors was undertaken to eliminate their interactions with cytochrome P450. These compounds do not change the pharmacokinetics of anticancer drugs and they are specific for Pgp (rarely other transporters). The most promising inhibitor is the anthranilamide derivative tariquidar (XR9576), which inhibits the ATPase activity of Pgp and is not a substrate for the pump. It is more effective and its inhibitory effects last longer in comparison to the second generation modulators [92-94]. Another third generation compound, VX-710 (biricodar), exhibits high affinity to Pgp and MRP and does not interact with doxorubicin [88, 95].

Numerous MDR pump modulators have been isolated from natural sources. It was shown that the carotenoids capsanthin, capsorubin and lysophyl (isolated from *Capsicum* spp.) are particularly active against Pgp in murine lymphoma cells [96]. Other carotenoids like (5R, 8R)-capsochrome, monoepoxy- β -caroten, luteoxantin or violaxantin have a high fluorescence activity ratio (FAR), which points to Pgp inhibition. Carotenoids interact with the lipid bilayer and in this manner affect the biophysical properties of the plasma membrane [97].

Another group of plant-derived compounds that inhibit efflux pumps are flavonoids. The most active flavonoids are amorphenine, pharmonetine, rotenone, chrysin and epigallocatechin, which caused increased accumulation of rhodamine 123 in murine lymphoma cells overexpressing Pgp. Rotenone was active also against human colon cancer cells. The mechanism of action of these compounds is probably inhibition of the ATPase activity of the transporters [98]. The reversal of multidrug resistance in cancer cells by flavonoids could be also achieved by lowering the expression of drug transporters. It was shown that wogonin and oroxylin A, isolated from *Scutellaria baicalensis* Georgi, did downregulate the expression of Pgp [99, 100].

Chalcones are bioprecursors of flavonoids and are also associated with the inhibition of Pgp. Their lipophilicity is an important feature for Pgp inhibitory activity. These compounds are generally specific for Pgp modulation, because the activity of functionally related protein BCRP was unaffected by the studied

chalcones. This might suggest different structural requirements for BCRP and Pgp inhibitory activities [101, 102].

Another important application of efflux pump modulators is overcoming the mycosis caused by drug-resistant fungi. This aspect of research on multidrug transporter inhibitors often concerns *Candida albicans*, a common opportunistic pathogen. The mechanisms of its resistance to fungicides include overexpression of Cdr1p and Cdr2p pumps that, similarly to the *S. cerevisiae* PDR proteins, belong to the ABC family and actively export drugs [60]. The resistance of *C. albicans* to azoles can be reversed by immunosuppressive drugs, e.g., tacrolimus (FK506) and cyclosporine, but the high cardio- and nephrotoxicity of these compounds severely limits their clinical use [103-106]. Another compound, this time of low toxicity, is tetrandrine (TET), which is isolated from *Stephania tetrandra* S. Moore roots. It acts synergistically with azoles and increases the sensitivity of *C. albicans* to those drugs. The likely mechanism of TET action is the inhibition of *CDR1* and *CDR2* expression [107]. The azole resistance of *C. albicans* can also be reduced by ibuprofen [108]. Other compounds that inhibit efflux pumps in *C. albicans* are terbinafine and propafenones [109]. Also disulfiram, clinically used in treating alcoholism, has been shown to modulate ABC transporters (human Pgp and MRP and Cdr1p in *C. albicans*) by inhibiting ATP hydrolysis. When used with antifungal drugs, disulfiram increases their effectiveness [110].

Due to the homology of human Pgp and Pdr5p, the research on efflux pump modulators often exploits *S. cerevisiae*. It was shown that Pdr5p was inhibited by steroids such as progesterone, estradiol and deoxycorticosterone and some of their synthetic derivatives [111-113]. Inhibition of Pdr5p was also observed for some flavonoids like 6-(3,3-dimethylallyl)galangin, but the strongest effect was exhibited by phenothiazines [114, 115]. Phenothiazine-derived drugs are mainly antipsychotics, which block dopamine receptors and interact with many ion channels. Phenothiazines influence the biophysical properties of lipid bilayers, e.g., the asymmetry of aminophospholipids of the erythrocyte plasma membrane [116, 117]. These compounds most likely inhibit ABC transporters by direct binding to the protein's drug-binding site, but the inhibition profile might be different for various transporters, e.g., phenothiazine, which modulated the activity of Snq2p did not inhibit Pdr5p or Cdr1p [118].

The direct interactions of phenothiazines with efflux pumps were also indicated by research on bacterial transporters. In *S. aureus*, NorA-mediated efflux was inhibited by phenothiazine differently for various substrates, which suggests that phenothiazines may interact with the drug-recognition site of the transporter [119]. In the Pgp binding pocket, drug-binding sites are most likely formed by various regions, so the efflux inhibition by phenothiazine would depend on the substrate bound by the transporter, e.g., phenothiazines interacted with Pgp at the same site as verapamil or cyclosporine A, but not prazosin [116, 120]. Some phenothiazines (e.g., fluphenazine) increased the sensitivity of *C. albicans* to fluconazole. A strong inhibition of efflux pumps was observed for an aminoacyl

derivative of phenothiazine, M961, which inhibited the drug efflux from yeast cells in micromolar concentrations and decreased their resistance to ketoconazole [118].

Another group of effective and specific Pdr5p inhibitors are enniatins B, B1 and D, isolated from *Fusarium* 4-53. They are neutral ionophores that increase plasma membrane permeability [121]. A similar mechanism was observed for KN20 (a D-octapeptide derivative), which increased the plasma membrane permeability and inhibited the ATPase activity of Pma1p and ABC transporters, which reversed the resistance of *S. cerevisiae* to azoles [122]. Also, CTBT (7-chlorotetrazole-[5,1-c]-benzo-[1,2,4]-triazine) could be a potential pump inhibitor. This compound has antifungal activity and enhances the efficacy of other antifungals, such as cycloheximide, fluconazole or 5-fluorocytosine. Its exact mode of action is not well understood, but it was shown that it causes oxidative stress in the cell with the damage of mitochondria and DNA [123]. Some oligopeptides also reverse multidrug resistance, for example FKRRWQWRM (Pep2), which enhances itraconazole efficacy by increasing ATP leakage from the cell [124].

Numerous new efflux pump modulators are still in the trial phase. Developing effective compounds that would inhibit pathogenic yeast resistance and their usage in combined therapy should enhance the efficacy of antifungal drugs and greatly improve the odds of overcoming fungal infections.

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