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Multidrug resistance ATP-binding cassette membrane transporters as targets for improving oropharyngeal candidiasis treatment

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Oropharyngeal candidiasis is caused by *Candida sp.*, opportunistic yeasts that infect immunocompromised patients. Chemotherapies are based on antifungal drugs against which yeast overexpress and address to the plasma membrane ATP-binding cassette (ABC) pumps for expelling these drugs out of the cell. More critical—because these pumps translocate structurally unrelated drugs—they confer to the yeast a broad resistance to antifungals when expressed, hampering the efficacy of these treatments whatever the drug used. We review here the disease, its treatment, and the role played by multidrug resistance ABC, and strategies to overcome this problem.

Keywords: *oropharyngeal candidiasis*; *pathogenic yeasts*; *fungal drug resistance*; *drug efflux*; *ABC transporters*; *P-glycoprotein*; *CDR1*

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Oropharyngeal candidiasis (OPC) is a fungal infection that affects oral and pharyngeal mucosa. In most cases, OPC is caused by an overgrowth of yeast from *Candida sp., albicans glabrata*, *tropicalis*, and *krusei* (1). These yeast are opportunistic and most of the time nonpathogenic; up to 60% stays in the buccal space of healthy people (1). They become aggressive in favorable conditions, typically those of immunocompromised patients after surgery or HIV-infected immunodeficient people, leading to superficial to life-threatening systemic infections with an elevated mortality level (2). OPC displays a large variety of clinical forms and classifications. The lesions can be acute or chronic and can involve other microorganisms such as bacteria (*candida*-associated injury). A brief description is summarized in Table 1.

Antifungal agents

Due to the similarities between fungal and mammalian cells, therapeutic options for fungal infections are limited compared to antibacterial treatments. Only four distinct fungal metabolic pathways are targeted (Fig. 1): (i) inhibition of ergosterol biosynthesis (azole derivatives and allylamines) and alteration of membrane function through ergosterol complex (polyenes); (ii) inhibition of glucan synthesis (echinocandins); (iii) inhibition of macromolecule synthesis (fluorinated pyrimidine analogs); and (iv) interaction with microtubules (griseofulvin).

- Azoles such as miconazole, fluconazole, and abafungi inhibit the sterol 14 α-demethylase, a protein encoded by ERG11, causing an ergosterol depletion and accumulation of 14-α-methyl-3,6-diol, a toxic sterol produced by the Δ-5,6-desaturase encoded by ERG3 (5).
- Allylamines such as terbinafin and naftifin inhibit the squalene epoxidase, encoded by ERG1, responsible for the first step of the biosynthesis of ergosterol. However, these inhibitors have a poor efficacy, being fungistatic for most of the *Candida sp.* (6). They are thus used mostly as topical agents (7).
- Polyenes such as nystatin and amphotericin B are cyclic amphiphilic molecules binding to the lipid bilayer and to ergosterol. They generate pores in the

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Table 1. Main clinical manifestation forms of OPC

	Type	Affected site	Appearance	Symptoms
Acute/ chronic	Pseudomembranous (also known as 'thrush')	Oral mucosa, tongue	Whitish-yellow creamy plaques that, when removed, leave an erythematous bleeding surface	Mild; patients complain of a slight tingling sensation or a foul taste
	Hyperplastic (also known as 'candida leukoplakia')	Most common in oral mucosa and less in the tongue and palate posterior to upper dentures	One adherent white plaque or multiple nodules that do not rub off	Soreness
	Erythematous	Oral mucosa, most commonly on the palate and tongue	Flat red patches	Burning sensation in mouth and altered taste
Associated lesions	Angular cheilitis	Corners of mouth	Cracking and inflammation	Pain, soreness, burning
	Denture related stomatitis	Oral mucosa	Erythema (redness) limited to the area beneath an upper denture	Asymptomatic, but patients complain of soreness and burning
	Median rhomboid glossitis	Center of the dorsal tongue	Elliptical or rhomboid reddened	Painless

OPC, oropharyngeal candidiasis.
According to ref. (3).

membrane promoting the leak of small cations such as K^+, Na^+, and Ca^{2+} (7).

- Echinocandins, such as caspofungin, micafungin, and anidulafungin, are noncompetitive inhibitors of (1, 3) β-D-glucan synthase, resulting in a cell wall unable to withstand osmotic stress.
- Fluoropyrimidines such as 5-fluorocytosin (5-FC) are first transported into the cell by cytosine permeases and pyrimidine transporters. Once in the cytoplasm, they are converted into 5-fluorouracil (5-FU) by cytosine deaminase, then phosphorylated to give the 5- fluorouracil monophosphate (5-FUMP), and

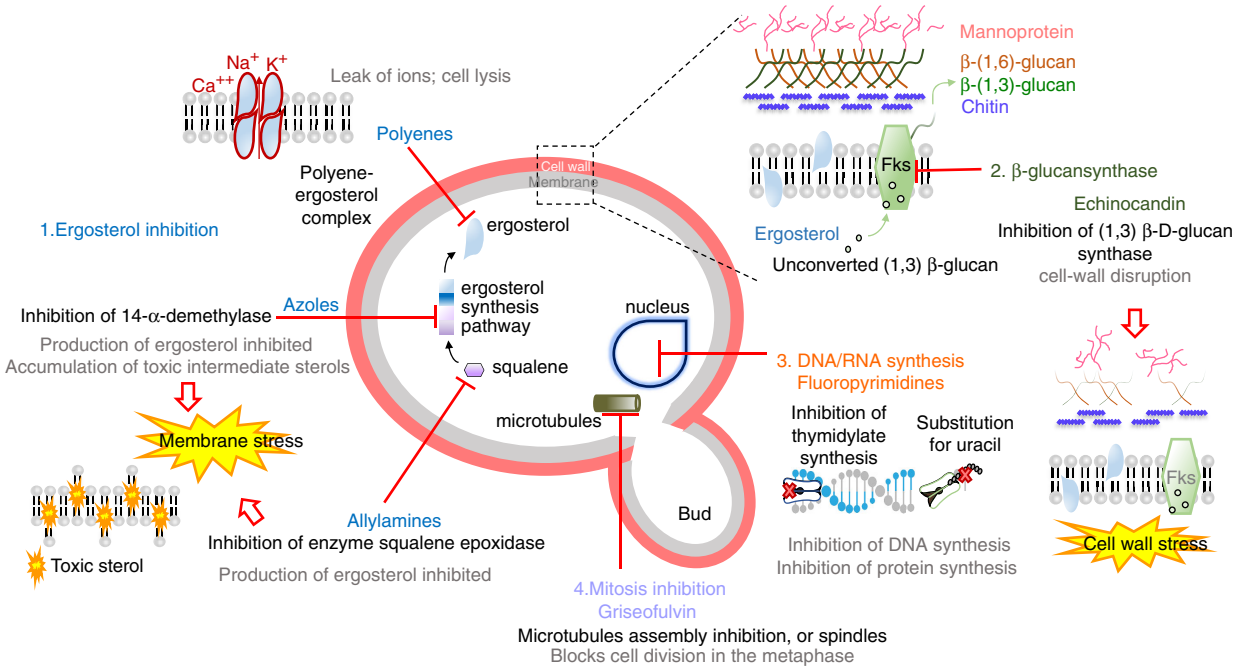

Fig. 1. Antifungal drugs and their mechanisms of action. Adapted from ref. (4) and http://www.doctorfungus.org/thedrugs/antif_pharm.php.

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converted into either the 5-fluorouracil triphosphate (5-FUTP) or the 5-fluorodeoxyuridine monophosphate (5-FdUMP); 5-FUTP can be incorporated into RNA while the 5-FdUMP interferes with DNA replication (8).

- Griseofulvin binds to tubulin, disrupting the mitotic spindle formation, and thus prevents the yeast division (7).

Oral candidiasis treatment

The first line of treatment for mild and localized candidiasis usually consists of topical antifungal drugs. A systemic therapy is recommended for patients with compromised immunology defenses. Table 2 summarizes the formulation of these antifungals.

Resistance mechanisms to antifungal chemotherapies

Despite the appropriate administration of antifungal drugs, the number of cases of persistence or infection progression is increasing. Factors that drive fungi resistance either take place prior to the drug treatment (natural resistance) or occur after the exposure to a drug (acquired resistance) (reviewed in (6–8, 11). Four to ten percent of *Candida* species are resistant to fluconazole and voriconazole (12). Each species can display a specific molecular mechanism of resistance to antifungal drugs (2, 5); however, four mechanisms are common and can function synergistically: (1) altered drug metabolism, (2) mutations in gene encoding target proteins, (3) prevented entry of the drug, and (4) removal of the drug from the cell through the upregulation of the expression of multidrug efflux pumps. Mutations in cytosine permease (uptake) or deficiency in enzymes implicated in the metabolism of fluoropyrimidines are a frequent cause of antifungal drug resistance. Mutations in ERG11, or in FKS1 (which encodes the β-1,3-glucan synthase), result in resistance to azoles and echinocandins, respectively. A recent study showed that upon fluconazole treatment, *Candida* species overexpress *Candida* drug resistance (CDR) membrane pumps to eliminate the drug (13). Overexpressed in *Saccharomyces cerevisiae*, they increase the resistance to fluconazole 600 times (14).

Multidrug efflux pumps

The most ubiquitous mechanism resistant to xenobiotic toxicity is the elimination of drugs out of the cell mediated by ATP-binding cassette (ABC) membrane transporters. These act as molecular pumps that actively translocate drugs through the plasma membrane by using the energy gained from ATP hydrolysis. These pumps are

Table 2. Antifungal medications of OPC

Antifungal	Topical antifungal formulation (indication)	Systemic antifungal formulation
Polyenes		
Amphotericin B	100 mg/ml (Intraoral candidiasis)	100 mg/ml OS
Nystatin	Ointment 100,000 u/g (Angular cheilitis)	100,000 u/ml OS
	Topical powder 100,000 u/g (Denture stomatitis)	200,000 u/ml pastille
	OS 100,000 u/g (Intraoral candidiasis)	500,000 u/ml Tablet
Azoles		
Clotrimazole	Cream 1% (Angular cheilitis)	10 mg troche
	Troches 10 mg (Intraoral candidiasis)	
Miconazole	Cream 2% (Angular cheilitis)	
Ketoconazole	Cream 2% (Angular cheilitis)	200 mg tablet
Fluconazole		100 mg tablet
		10 mg/ml OS
		40 mg/ml OS
Itraconazole		100 mg capsule
		10 mg/ml OS
Fluoropyrimidines		
5-Flucytosine		Often in combined therapy with amphotericin
Echinocandins		Intravenous
Caspofungin		50–75 mg/day
Micafungin		100–200 mg/day
Anidulafungin		50–150 mg/day

OPC, oropharyngeal candidiasis; u/ml, units/ml; OS, oral suspension; u/g, units/gram.
According to refs. (9) and (10).

polyspecific—able to translocate structurally unrelated drugs. This property has a deep impact on chemotherapies because once the resistance is acquired for one drug, it is also acquired for most of them, from common to distinct chemical classes. These proteins thus cover a critical field in drug disposition and drug resistance to chemotherapeutic treatments, for which solutions remain to be found. The pleiotropic drug resistance (PDR) in fungi displays several similarities to the multidrug resistance (MDR) phenotype in humans (15, 16) and bacteria (17–19). Thirty-one ABC transporters have been identified in *S. cerevisiae* (20), compared with the 48 ABC transporters found in humans (21). They are classified in five subfamilies according to their phylogenetic relationships: PDR, MDR, multidrug-resistance protein (MRP)/cystic fibrosis transmembrane (TM) conductance regulator (CFTR), adrenoleukodystrophy protein (ALDP), and yeast elongation factor 3 (YEF3)/RNase L inhibitor 1 (RLI) (22). Among them, Pdr5p is the most studied, with Snq2p (20) and Yor1p (23). These pumps constitute the main shield against xenobiotics, including antifungal drugs. Most ABC transporters in *Candida* species (http://www.candidagenome.org) are orthologs of *S. cerevisiae* Pdr5 and are equally implicated in MDR (22). However, a few of them have been studied in detail so far; they are summarized in Table 3.

ABC transporter topology

The basic structure that defines the members of the ABC transport family is a combination of a cytosolic nucleotide-binding domain (NBD) and a transmembrane domain (TMD) organized either in 1 to 4 polypeptides (Fig. 2). These domains are arranged in any possible combination in a protein that includes two NBDs and two TMDs. Some members have additional TM segments or cytosolic domains. The human P-glycoprotein (P-gp) has a TMD1-NBD1-TMD2-NBD2 topology in a single polypeptide while PDR or CDR pumps, which are functionally close in terms of polyspecificity, display a reverse topology: NBD1-TMD1-NBD2-TMD2. The reason for such specificities is not known. The TMD has six hydrophobic α-helices, poorly conserved in length or amino acid sequence. Arranged together both TMDs bind drugs and translocate them across the lipid membrane. Note that in that frame, strictly speaking, drugs are not substrates because they do not undergo a molecular transformation by the pump; but because the term is widely used, we will keep it here. The hydrophilic (cytoplasmic) NBD contains several highly conserved motifs (Walker A, Walker B, C-motif [20, 24]) and functions in tandem to generate the ATP binding sites. ATP is thought to be hydrolyzed to reset the protein to its initial state after drug translocation.

Table 3. ABC proteins in pathogenic candida species

Candida sp.	Function	Localization	Length (amino acids)	Family	Topology
Albicans					
*Ca*Cdr1	Drug efflux, transport of phospholipid	PM	1,501	PDR	NBD1–TMD1- NBD2–TMD2
*Ca*Cdr2	Drug efflux, transport of phospholipid	id	1,499	id	id
*Ca*Cdr3	Transport of phospholipid	id	1,501	id	id
*Ca*Cdr4			1,490	id	id
*Ca*Hst6	Transport of a-factor		1,323	MDR	TMD1–NBD1-TMD2–NBD2
*Ca*Yor1	Drug efflux	PM	1,488	id	id
*Ca*Ycf1	Drug efflux	VA?	1,580	id	id
*Ca*Mlt1	Involved in virulence	VA?	1,606	id	id
Glabrata					
*Cg*Cdr1	Drug efflux	PM	1,499	PDR	NBD1–TMD1- NBD2–TMD2
*Cg*Cdr2	Drug efflux		1,542	id	id
*Cg*Snq2	Drug efflux		1,507	id	id
*Cg*Aus1	Involved in sterol uptake		1,398	id	id
Dubliniensis					
*Cd*Cdr1	Drug efflux		1,501	PDR	NBD1–TMD1- NBD2–TMD2
*Cd*Cdr2	Drug efflux		1,500	id	id
Krusei					
*Ck*Abc1	Drug efflux				
*Ck*Abc2	Drug efflux				
Tropicalis					
*Ct*Cdr1	Drug efflux?				

ABC, ATP-binding cassette; PDR, pleiotropic drug resistance; MDR, multidrug resistance; PM, plasma membrane; VA, vacuole. According to ref. (22).

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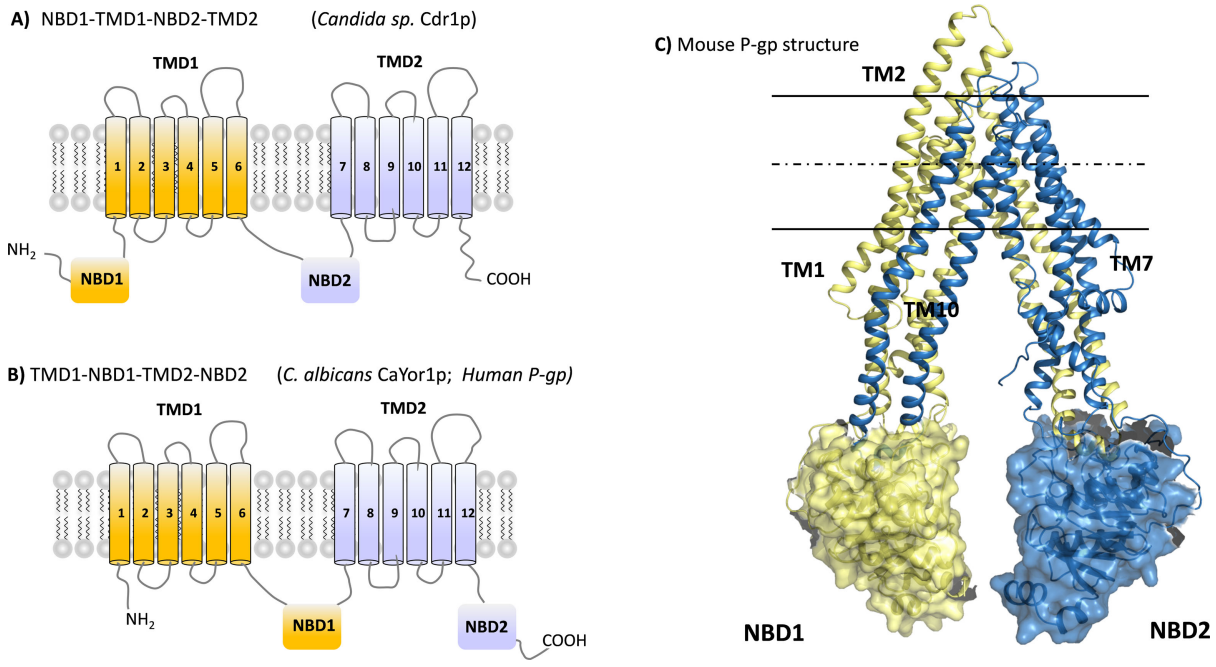

Fig. 2. A–B. Topology of ATP-binding cassette (ABC) transporters belonging to pleiotropic drug resistance (PDR) and multidrug resistance (MDR) subfamily. C. 3D-structure of mouse P-gp in cartoon and colored in yellow and blue illustrating each moiety of the protein.

Transport cycle

In a typical drug efflux cycle, the drug binds first to the TMDs. This binding triggers a conformational change by which each NBD comes closer to the other, generating the ATP binding sites. The binding of two ATPs blocks the protein in a close state that leads it to undergo a second large conformational change from the inward-facing to the outward-facing conformation (Fig. 3). In this new conformation, both NBDs remain bound together with their ATP while the outer leaflet part of each TMD becomes distant, open to the extracellular face of the drug-binding sites. Drugs are released by

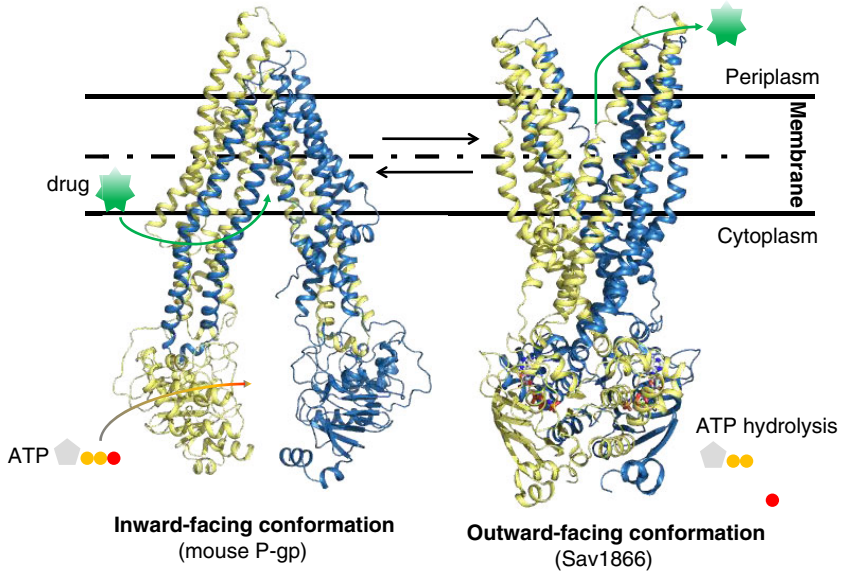

Fig. 3. Conformational changes of ATP-binding cassette (ABC) exporters. The 3D-structure of mouse P-gp in the inward-facing conformation (34, 35) is shown on the left and the homodimer Sav1866 in the outward-facing conformation (32) is displayed on the right. Nonhydrolysable ATP-analog AMPPNP (adenosine-5′(βγ-imido)triphosphate) bound to Sav1866 is shown in CPK-colored stick molecules (red, O atom; gray, C atom). The drug is symbolized in green.

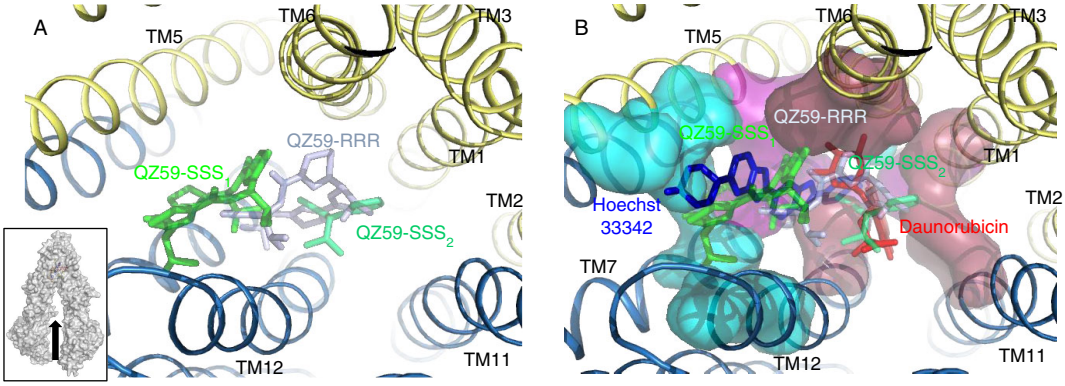

Fig. 4. Drug binding site of the mouse P-gp. A. The 3D-structure of mouse P-gp in the inward-facing conformation with the hexapeptides inhibitors QZ59SSS and QZ59RRR bound in separate P-gp (34, 35). The inset shows the direction of observation. B. This time, the same view is displayed showing the location of Hoechst 33,342 (H site) and daunorubicin (R site) as determined in (42). Blue, red, and magenta areas correspond to residues belonging to the H site, the R site, and to both sites.

changes in the affinity of the sites. Finally, the protein comes back to the initial inward-facing conformation by hydrolyzing ATP, which gives the energy to the protein to allow separation of each moiety (25). Several x-ray structures of bacterial and eukaryotic ABC transporters have now been resolved at high to medium resolution, 2.2–5.5 Å, BtuCD (26), HI1470/1 (27), HmuUV (28), ModBC (29), $MalFGK_2$ (30), MetNI (31), Sav1866 (32), MsbA (33), ABCB1 (34, 35), ABCB10 (36), and TM287/288 heterodimer (37). They give some hints about

Table 4. Substrates and inhibitors of ABC transporters from *Candida* sp. and human P-glycoprotein

		C. albicans		*C. glabrata*		*C. dubliniensis*		*C. krusei*		
	Compound	*Ca*Cdr1	*Ca*Cdr2	*Cg*Cdr1	*Cg*Cdr2	*Cd*Cdr1	*Cd*Cdr2	*Ck*Abc1	*Ck*Abc2	P-gp
Antifungals	Azoles	X	X	X	X	X	X	X		
	Fluconazole								X	
	5-flucytosine			X	X					
	Cycloheximide	X	X	X	X			X		
	Cerulenin	X	X	X	X			X		
Dyes	Rhodamine 6G	X	X	X						X
	Rhodamine 123	X		X				X		X
Anticancer	Tamoxifine	X								
	Doxorubicin	X		X						X
	Daunorubicin	X								X
	Etoposide	X								X
	Vinblastine	X								X
	Topotecan	X								X
Others	Trifluoperazine	X								
	Verapamil	X								X
	Nigericin	X								
Inhibitors	Milbemycins	X	X		X			X		
	Enniatin	X						X		
	FK506	X		X	X			X		
	FK520	X								
	Unnarmicins	X		X	X					
	Curcumin	X								X
	Disulfiram	X								
	Verapamil			X						X

ABC, ATP-binding cassette.
According to refs. (6), (24), and (44).

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the putative mechanism; however, the structure of intermediate conformational states is required to elucidate on a molecular level the mechanism by which drugs are translocated. In this way, three new conformations in the inward-facing conformation of P-gp could be solved (38).

The human P-gp contains at least two well-identified drug-binding sites, one binding Hoechst 33342 (the H site) and the other rhodamine 123 (the R site) (39, 40). Both dyes are also transported by PDR/CDR transporters because the structural organization of their drug-binding sites is probably close to that of the P-gp. In that frame, 3D models could be proposed (41) that may be helpful in studying such proteins for which no structural information has yet been released. More importantly, no structural information is available for CDR pumps, while the present 3D structures cannot be transposed to them due to their reversed topology. Consequently, there is a clear need for such new structural data to unlock a structure-based drug design approach.

Regardless, these structures open the way to molecular enzymology to elucidate how the drug-binding sites are formed, organized, and how they bind drugs. The cocrystallization of two cyclic hexapeptides with the mouse P-gp (34, 35, 38) allowed us to precisely locate the H and R drug-binding sites by characterizing the mode of inhibition of these compounds on the transport of drugs binding to each site (Fig. 4) (42).

Modulation of MDR ABC transporters for restoring antifungal drug sensitivity

Altering the capacity of pathogenic yeast that overexpress MDR ABC pumps to expel antifungal drugs can be achieved with inhibitors. Ideally such compounds display the following characteristics: effective at low concentrations, specific to the targets, no pharmacokinetic interactions with the coadministrated antifungal drug, and nontoxic (43). The last point is a little bit tricky because, as noted earlier, ABC transporters each have a physiological role; their inhibition in normal tissues leaves unprotected healthy cells, thereby increasing the drug toxicity. Using specific inhibitors for a single transporter can overcome this obstacle. Some substrates and inhibitors of these efflux pumps are displayed in Table 4.

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