Universidad Zaragoza
1542

VYTAUTAS MAGNUS UNIVERSITY
MCMXXII

Final Degree Project

Candida genus yeast resistance to antifungals

Author
Clara Navarro Villarreal

Director
Dr. Rimantas Daugelavičius

Biotechnology Degree – Natural Sciences Faculty

2020/2021

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1. INTRODUCTION

1.1. Abstract

Candida genus species are opportunistic fungi causing candidiasis**,** whose prevalence is on a growing trend. *C. albicans* is the commonest yeast causing opportunistic fungal diseases in humans, whose pleomorphism, highly adaptability and biofilm formation are essential for its virulence and pathogenicity. Other remarkable species are *C. glabrata* and the emergent multidrug resistant *C. auris*.

Main antifungals used for treatment of invasive candidiasis are azoles, whose target is 14α-lanosterol demethylase; polyenes, that target ergosterol; pyrimidine analogues, which inhibit DNA and RNA biosynthesis; and echinocandins, inhibiting β-(1,3)-glucan synthase. An inappropriate usage or prolonged exposure to these toxic compounds have developed the emergence of multidrug resistance phenomenon. One of the most prominent mechanisms of antifungal resistance in *Candida* genus is the rapid efflux of incoming drugs via active transport, due to the overexpression of two main classes of plasma membrane efflux pumps: ABC and MFS transporters. In addition, entry constraints, genomic alterations and cell plasticity are other mechanisms of the resistance.

Owing to the alarming life-threatening infections caused by *Candida* spp., several strategies have been found to reduce drug resistance. Some of them are the structure-based design of effective inhibitors of efflux, chemosensitization and design of new antifungal drugs. An effective antifungal stewardship programme and further investigation are urgently needed to circumvent the menace of multidrug resistance.

1.2. Antecedents

It is universally acknowledged that fungal diseases, related with substantial human morbidity and mortality, entail a major global impact. Even though they are gaining worldwide attention due to their progressive increase, they still remain unappreciated. According to a 2019 survey of 3,624 adults in the United States, there is a low public awareness of invasive fungal diseases. More than two thirds of the respondents had never heard about any of the fungal diseases that were listed and candidiasis showed the highest percentage of awareness, 24,6% (Benedict et al., 2020). By way of illustration, it has been estimated that over a quarter of the world's population have a fungal skin infection, three quarters of women suffer vulvovaginal candidiasis at least once during their lifetime and more than a million people yearly die due to invasive fungal infection (d'Enfert et al., 2021).

Our preparedness to tackle those pathogens is hindered by the development of antifungal resistance. Since the introduction in both agriculture and modern medicine and widespread use of triazoles in early 1990s, *Candida* resistant spp. have become a serious health threat. Clinical breakpoints of resistance are developed for *Candida* spp., mostly derived from episodes of invasive candidiasis in non-neutropenic patients and mucosal candidiasis. However, in patients with less frequent opportunistic yeast or mold infections, breakpoints only derive from epidemiological cut-off values and pharmacokinetic and pharmacodynamic data from animal models (Kontoyiannis, 2017).

In the pandemic world we are living nowadays, candidiasis is taking an active role in patients hospitalized for COVID-19. *Candida* infections resistant to antifungal treatment have been described in patients diagnosed with that disease (Posteraro et al., 2020). An increased incidence of antimicrobial resistance may be attributed to the excess use of antimicrobial drugs during this coronavirus pandemic.

1.3. Aim and objectives

This bibliographic review is aimed to provide a deeper insight into the current situation and findings of *Candida* genus resistance to antifungals. For that purpose, the objectives that have been achieved are:

1. Research, select and classify appropriate scientific reviews. Make citations and references using the reference management application Mendeley.
2. Increase the knowledge of *Candida* genus main representatives and antifungal drugs used to treat candidiasis.
3. Highlight the actual situation of resistance to antifungals focusing on *Candida* genus mechanisms.
4. Analysis of strategies to bypass antifungal resistance and tolerance developed by *Candida* genus, thereby contributing to public awareness of their urgent need.

2. LITERATURE

2.1. Yeasts

Yeasts are eukaryotic unicellular microfungi that along with molds belong to **Fungi Kingdom**. All members of this kingdom are comprised by not only a well-defined nucleus, mitochondria, Golgi bodies and endoplasmic reticulum, but also distinguishable features like a rigid cell wall containing chitin, glucan and unlike animals, **ergosterol**, its major sterol component of membranes. As opposed to molds that replicate either sexual or asexually, yeasts mostly replicate asexually by budding or fission. Curiously, for dimorphic fungi it is possible to assume both morphologies, yeast form in human tissues (except *Coccidioides*) and mold form in nature (Murray, 2018).

These microbes are widespread in the natural environment and have been studied as eukaryotic models and exploited biotechnologically. Nonetheless, they pose a threat in our health considering they are among the most prominent disease-causing fungi.

2.2. *Candida* genus

The largest group of pathogenic fungi are ascomycetous yeasts, most of which belong to the anamorphic genus *Candida*. *Candida* spp. are opportunistic fungi causing **candidiasis**,

whose prevalence is on a growing trend. In fact, it is the third-to-fourth most recurrent nosocomial infection in US and worldwide hospitals (Prasad, 2017).

There is a paucity of rapid diagnostic assays for invasive candidiasis and it is usually diagnosed by routine cultures of blood, urine tissue and other body fluids that have a low sensitivity. When mannan is found, a characteristic polysaccharide of *Candida* cell wall, an invasive candidiasis is suggested. Besides, viable or dead fungal elements can be detected in tissue sections by fluorescent antibody microscopy (Murray, 2018). Nevertheless, there is a scarcity of suitable diagnostic techniques to identify resistant species. Regarding treatment, it depends on the immunological status of patients, its location and the severity of the infection. *Candida* infection is also related with medical devices (Kojic and Darouiche, 2004).

On top of that, the situation is worsening by the emergence of **multidrug resistant species** of *Candida*. The US Center of Disease Control and Prevention (CDC) report published in 2019 estimates 34,800 cases and 1,700 deaths associated with drug-resistant *Candida* spp. (Centers for Disease Control and Prevention, 2019).

2.2.1. *Candida albicans*

Candida albicans (*C. albicans*), known as a **model system** of pathogenic fungi, is the commonest yeast causing opportunistic fungal diseases in humans and its economic impact and associated mortality challenge the intensive care units worldwide. It is considered both a **commensal** and **opportunistic** pathogen that produces infections whenever the immune system of the host is weakened or its microbiota becomes disturbed (Costa-de-oliveira and Rodrigues, 2020). It colonizes humans and other warm-blooded animals' mucosa producing endogenous infections in which it moves from normally colonized mucosal surfaces to the blood or other normally sterile locations. Exogenous infections can also occur in hospital (Murray, 2018). Once in the mucosa, it specially resides in the conjunctival flora and in the gastrointestinal and genitourinary tracts (Pristov and Ghannoum, 2019). The foremost virulence factors of this yeast are yeast-to-mycelia morphological transformation, biofilm formation and production of secretory hydrolytic enzymes, particularly aspartic proteases and phospholipases (Prasad, 2017).

Genome plasticity is a hallmark of *C. albicans* regarding fungal pathogenicity. Its successful infections are due to its **pleomorphism**, which is essential for its virulence and implies switching among different morphological phenotypes, from yeast to hyphal or pseudohyphal form (Costa-de-oliveira and Rodrigues, 2020). Hyphae formation, involved in adhesion to and invasion of host cells and tissues, is correlated with the upregulation of *ALS3* and *ECE1* genes, which are involved in pathogenicity processes like invasion, iron acquisition and host damage. These virulence-associated genes have been shown to be regulated by the transcription factor Ahr1 and the global repressor Tup1, which is a crucial antagonist of hyphal formation in *C. albicans* (Ruben et al., 2020). On top of that, this pathogen is highly **adaptable**, being able to develop resistance after a prolonged exposure to antifungals and thus, making the choice of medicines increasingly limited (Costa-de-oliveira and Rodrigues, 2020).

The medical impact of *C. albicans* depends on its ability to thrive as a **biofilm** on either biotic or abiotic surfaces. Biofilms, which are closely packed communities of cells, act as a physical barrier, preventing in some way drugs from reaching the underlying cells. Therefore, it leads to the enhancement of its virulence potential and to a higher resistance to antifungals and

the immune system than the planktonic forms, as it is able to withstand high antifungal concentrations (Prasad, 2017). The ability of *C. albicans* to form hyphae, the capacity of these hyphae to adhere to each other and to surfaces or host cells, and the dispersal of cells within a biofilm into the environment are critical for normal biofilm development and maintenance (Nobile and Johnson, 2015). So as to provide support and protection to the cells embedded within biofilms, thus contributing to biofilm drug resistance, biofilms are encased in an extracellular matrix (Prasad, 2017). Of note, once biofilms are formed on an implanted medical device, they can seed disseminated bloodstream infections and lead to invasive systemic infections (Nobile and Johnson, 2015).

2.2.2. Other members of *Candida* genus

As well as *C. albicans*, other *Candida* species categorised as non-*albicans Candida* (NAC) spp. can infect both healthy individuals and immunocompromised patients. Among them, some clinically important NAC spp. are *Candida glabrata* (*C. glabrata), Candida parapsilosis (C. parapsilosis), Candida tropicalis (C. tropicalis), Candida guilliermondii (C. guilliermondii), Candida krusei (C. krusei)* or *Candida auris (C. auris)*. Comparing with *C. albicans*, it has been demonstrated their higher resistance to some antifungals such as fluconazole and itraconazole and their tendency to cause more severe or fatal diseases, especially in bloodstream infection cases (Shigemura et al., 2014).

2.2.2.1 Candida glabrata

The second causative agent of candidiasis is *C. glabrata,* whose infections present a mortality rate about 40% (Fatahinia et al., 2020). It is a **nosocomial** pathogen that colonizes mucosal membranes and triggers challenging infections owing to their long hospitalization periods, high mortality rates and the emergence of resistance to azoles, echinocandins or multiple drugs (Khalifa et al., 2021).

The fact of being a **haploid** yeast, in contrast to the diploid genome of *C. albicans* or other NAC spp., has been ascribed to the development of resistance to antifungal agents, as a single DNA repair mutation is enough to cause a related mutator phenotype and the subsequent emergence of resistance-conferring mutations (Healey et al., 2016). Although there is some evidence of pseudohyphae formation in this microorganism, its pathogenicity seems to be independent of morphology, as well as lower compared to *C. albicans* and strongly related to **adhesion** and **flexibility** (Galocha et al., 2019). *C. glabrata* also forms biofilms, which increase mortality rates when placed on medical devices and show several differences comparing with *C. albicans,* for instance, *C. glabrata* **biofilms** are thinner, contain less biomass, have a different structure organization and are composed only by yeast cells. Nevertheless, they display a higher density of tightly packed cells. Regarding extracellular matrix, albeit being produced in a lesser extent than the one of *C. albicans,* they have the same main components: proteins and carbohydrates (Galocha et al., 2019).

2.2.2.2. Candida auris

Candidiasis infection deepens with the emergence of novel multidrug resistant (MDR) species of *Candida* genus such as *C. auris*, which was first described in 2009 when it was isolated from an ear discharge of a Japanese patient (Prasad, 2017). Since then, several reports of invasive infections and hospital outbreaks have appeared and it continues to spread unabated worldwide. Indeed, it is the only species having isolates **resistant to all classes of human antifungal drugs** (Pristov and Ghannoum, 2019). Unlike most *Candida* spp. which are mainly found in the gastrointestinal tract, *C. auris* is a colonizer of the skin (Lamoth et al., 2018).

Four or possibly five clades of this nosocomial fungus have been identified in circulation while sequencing its whole genome. There is a high inter-clade genetic diversity represented by 40000-200000 single nucleotide polymorphisms (SNPs) differing from each other. Meanwhile, their intra-clade diversity is 17-fold lower, with differences of a mere 2-600 SNPs between a given clade. Bearing this in mind, it is suggested that *C. auris* may have emerged independently in different parts of the world. By contrast, its local and international transmission is driven by some interconnected host-pathogen-environmental factors. Its **emergence** has been supported not only by the abuse of antimicrobials but also by several practices in agriculture, aquaculture, deforestation and land use, by the current warmer climate and by the changing human-population structure (Chakrabarti and Sood, 2021).

The antifungal resistance machinery of *C. auris,* mostly based on **mutations** and overexpression of **membrane transporters**, is a substantial impediment to successfully managing its infections. The 90% of *C. auris* strains are resistant to fluconazole, resistance rates up to 30 % correspond to amphotericin B and although remaining still limited, echinocandin resistance has been reported alongside other antifungal resistance phenotypes too (Kean and Ramage, 2019). Also, in the research carried by Kean et al. (2018), 41% of the strains showed multidrug resistance and 4% demonstrate pan-resistance (Kean et al., 2018). Another of its defence mechanisms is **biofilm** formation, which resist all classes of antifungals attack. Biofilms sequester up to 90% of the drug in the extracellular matrix, express efflux pumps and contain persister cells (Chakrabarti and Sood, 2021). Eventually, *C. auris* also forms **aggregative forms**, which are more resilient and less virulent than non-aggregative strains and exhibit high azole resistance (Chakrabarti and Sood, 2021). Although several of its resistance mechanisms are analogous to those in other *Candida* spp., it is a challenge to fully understand its high-level resistance to antifungals and disinfectants. Some of the newest azoles such as posaconazole and isavuconazole have shown favourable results *in vitro* against this formidable yeast (Pristov and Ghannoum, 2019).

2.3. Antifungal drugs

Unlike the large battery of clinical antibacterial agents, the array of available antifungal drugs is somewhat scarcer due to the fact that the generation of new ones has fallen behind when compared to the fast pace of emergence of fungal infections. The main antifungals used for treating invasive candidiasis are azoles, polyenes, pyrimidine analogues and echinocandins, usually considering the last the first-line therapy. Their spectrum of activity and their mechanisms of action and resistance are summarized in *Table 1* and detailed below.

Table 1. Spectrum of activity and mechanisms of action and resistance of the major antifungal drugs against candidiasis. Some examples of the most commonly used drugs of each class are also provided.

ANTIFUNGAL CLASS and representatives	**SPECTRUM OF ACTIVITY**	**MECHANISM OF ACTION**	**MECHANISM OF RESISTANCE**
AZOLES Fluconazole Voriconazole Posaconazole	Fungistatic	Inhibition of the fungal cytochrome P450 14α-lanosterol demethylase (Erg11p) and accumulation of a toxic sterol resulting in growth inhibition.	○ Overexpression of Cdr1, Cdr2 and Mdr1. ○ Altered sterol import. ○ Target alteration or overexpression (*ERG11, ERG3, ERG6, Upc2p).* ○ Genomic alterations and cell plasticity.
POLYENES Amphotericin B Nystatin Natamycin	Fungicidal	Targeting ergosterol and creation of pores in the lipid bilayers leading to membrane disruption and cell death.	○ Mutations in ERG3 and ERG6. ○ High catalase activity leading to susceptibility to oxidative damage. ○ Cell plasticity.
PYRIMIDINE ANALOGUES 5-Flucytosine	Fungicidal	Inhibition of DNA and RNA biosynthesis caused by the incorporation of fluorinated pyrimidine antimetabolites.	○ Mutations in FUR and FCY1/2 genes.
ECHINOCANDINS Caspofungin Anidulafungin Micafungin	Fungicidal	Inhibition of β-(1,3)-glucan synthase and consequent decrease in the production of the major cell wall biopolymer β-(1,3)-glucan and loss of cell wall integrity.	○ Point mutations in hot spot regions of FKS1/2 genes. ○ Genomic alterations.

2.3.1. Azoles

Azoles correspond to the largest class of antifungal compounds in clinical use, whose mechanism of action consists of binding and inhibiting the fungal cytochrome P450 enzyme **14α-lanosterol demethylase** (Erg11p), encoded by ***ERG11*** gene, also known as CYP51. This enzyme catalyses an important step of ergosterol biosynthesis so its inhibition results in decreased membrane ergosterol content and the synthesis of a fungistatic toxic sterol (14α methylergosta 8-24 (28) dienol). The accumulation of this intermediate accompanied by the

elevation of reactive oxygen species (ROS) characteristic of azoles, are responsible of membrane disruption and growth inhibition (Bhattacharya, Sae-Tia, and Fries, 2020). Their action is **fungistatic** against most yeasts as *Candida* spp., but **fungicidal** against molds. Their import is energy-independent and has been proved to be via facilitative diffusion in some pathogenic fungi including *C. albicans* or *C. krusei* (Mansfield et al., 2010).

Azoles are generally classified in two groups: **imidazoles** and **triazoles**. Imidazoles contain two nitrogen in the azole ring and include clotrimazole, econazole, ketoconazole, miconazole and tioconazole; while fluconazole, itraconazole, voriconazole, isavuconazole and posaconazole are triazoles with three nitrogen in the azole ring and comparing with imidazoles, display a broader antifungal spectrum with reduced toxicity (Prasad, 2017). **Second generation** of triazoles that comprises voriconazole, posaconazole and isavuconazole are more effective against resistant fungi (Bhattacharya et al., 2020). **Fluconazole** is the most commonly used and has a toxicity profile similar to that of isavuconazole, being the last more active (Wilson et al., 2016). Besides, that novel azole is highly water soluble and differently from voriconazole, does not require beta-cyclodextrin in its intravenous formulation, consequently offering lower side effects (Jenks et al., 2018).

2.3.2. Polyenes

Polyenes are a subgroup of macrolides that are poly-unsaturated and have at least one sequence of alternating double and single carbon-carbon bonds. Amphotericin B, nystatin and natamycin belong to this class of natural compounds with a heterocyclic amphipathic molecule. They are **fungicidal** and target **ergosterol** by inserting into the lipid bilayers and forming aqueous or nonaqueous (cation-selective) pores (Cohen, 2010). Thus, there is a leakage of monovalent ions (K^+, Na^+, H^+ and Cl^-) and subsequent fungal cell death.

The topical use of Nystatin is thought to be the most common route of administration, although its spectrum of activity is narrower than the one of **amphotericin B**, which is considered the gold standard in the treatment of fungal, especially severe, infections (Costa-de-oliveira and Rodrigues, 2020). However, it is unfortunately toxic to mammals. To reduce its toxicity, the conventional amphotericin B complexed with sodium deoxycholate has been modified as a cholesterol sulphate complex, as a lipid complex and as a liposomal formulation. Even if those reformulated versions have broad-spectrum activity against most fungi, their use is limited by their expensive cost. In addition, this drug is mainly preferred when tackling resistant *Candida* spp. to other antifungals (Bhattacharya et al., 2020). Regarding natamycin, it is specially chosen for the treatment of fungal keratitis and it is the only topical ophthalmic and potent drug with few side effects that has been approved by the Food and Drug Administration (Cui et al., 2021). Cui and co-workers have recently designed a promising drug delivery system with good biosafety and a significant anti-candidiasis effects consisting of nanoparticles for the co-delivery of natamycin and clotrimazole in chitosan and poly(lactic-co-glycolic acid) (Cui et al., 2021).

2.3.3. Pyrimidine analogues

The representative of this class of synthetic fluorinated analogues of cytosine is **5-Flucytosine**. It is orally administered and interferes with **nucleic acid biosynthesis**. Although it does not have intrinsic antifungal activity, once it is imported via the cytosine permease enzyme of susceptible fungal cells (Vermes et al., 2000), it is converted to 5-Fluorouracil by cytosine deaminase, which is then metabolized to 5-Fluorouridine triphosphate. This compound is incorporated in the fungal RNA, thereby inhibiting protein synthesis. Otherwise, 5-Flucytosine can also be converted to 5-Fluorodeoxyuridine monophosphate, that inhibits thymidylate synthase and consequently DNA synthesis (Vermes et al., 2000).

The spectrum of activity of pyrimidine analogues is restricted to pathogenic yeasts since most filamentous fungi are deprived of cytosine deaminase. It is preferable to use it in combination with other antifungal drugs rather than in monotherapy owing to its greatly frequent resistance development (Vermes et al., 2000).

2.3.4. Echinocandins

Echinocandins are a relatively recent class of cyclic non-ribosomal antifungal lipopeptides produced by filamentous fungi. As a result of their complex structure, **caspofungin**, **micafungin** and **anidulafungin** are manufactured semisynthetically (Hüttel, 2020). They target **β-(1,3)-glucan synthase** enzyme, encoded by ***FKS*** genes, located in the plasma membrane of fungal cells and responsible for the biosynthesis of the principal component of fungal cell walls: β-(1,3)-glucan. Thus, its inhibition results in loss of cell integrity. Moreover, the enzyme is composed of a minimum of two subunits: Fks1, the catalytic subunit, and Rho, a GTP-binding protein. However, some studies have revealed the presence of other membrane-associated components such as Pma1, which maintains transmembrane electrochemical proton gradients, and two other proteins of 40 and 18 kDa that may interact with the glucan synthase complex (Perlin, 2007). Against susceptible *Candida* spp., these antifungals showed *in vitro* fungicidal activity (Barchiesi et al., 2005). Simitsopoulou et al. (2013) proved that the three echinocandins are active against planktonic cells of all *C. albicans, C. parapsilosis, C. lusitaniae C. guilliermondii* and *C. krusei* strains as well as of biofilms of *C. albicans* and *C. krusei* bloodstream isolates. (Simitsopoulou et al., 2013).

Nowadays, echinocandins are used as front-line therapy against invasive mycosis and are considered equivalent or even superior to the amphotericin B and fluconazole therapies (Patil and Majumdar, 2017). The absence of cell wall in mammalian cells makes that structure an important antifungal agent and the low toxicity for humans allows echinocandins to be commonly the treatment of choice.

2.4. Mechanisms of resistance to antifungals

Eukaryotic cells count on several non-exclusive mechanisms to protect themselves from xenobiotic compounds. Their ability to survive at drug concentrations that exceed the minimum inhibitory concentration (MIC) is known as **antifungal tolerance**, which may promote the

acquisition of **antifungal resistance**. That resistance, on the contrary, show an increase of MIC values independent of the capacity to survive at drug concentrations beyond those MICs. It has been documented antifungal resistance to all currently available antifungal agents in several pathogens and in both laboratory and clinical settings (Delarze and Sanglard, 2015).

Antifungal resistance has been classified in different types (Figure 1): **primary or intrinsic**, when it is manifested before antifungal exposure; **secondary or acquired**, which is exhibited after exposure to an antifungal agent, and can be either **reversible** in case of a transient adaptation or **persistent** as a consequence of one or more genetic alterations; and **clinical resistance**, that refers to the persistence or progression of an infection even when an appropriate antifungal therapy is being applied with an *in vitro* susceptibility of the organism (Costa-de-oliveira and Rodrigues, 2020).

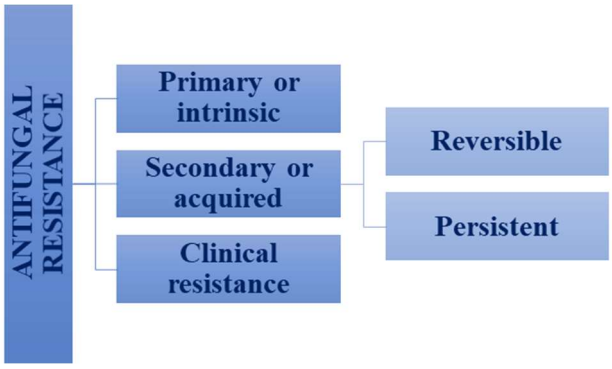

Figure 1. Classification of described antifungal resistance types.

The emergence of **multidrug resistance (MDR)** phenomenon in yeasts has been developed by an inappropriate usage and prolonged exposure to those toxic compounds. It is characterized by simultaneous resistance to at least two different types of antifungals (Prasad et al. , 2019).

2.4.1. *Candida* efflux pumps

One of the most prominent mechanisms of antifungal resistance is the ability of *C. albicans* and NAC species to rapidly efflux incoming drugs via **active transport** (Prasad et al., 2015). This mechanism arises from the **overexpression** of the two main classes of plasma membrane efflux pumps that can be also found in vacuolar membranes (Khandelwal et al., 2019b) and mitochondrial membranes (Leighton and Schatz, 1995): the **ATP-Binding Cassette (ABC) proteins**, whose efflux is coupled with ATP hydrolysis, and the **Major Facilitator Superfamily (MFS) transporters** that utilize energy derived from proton motive force (Banerjee et al., 2021). Both classes are **promiscuous** transporters and they hamper the retention of detrimental concentrations of drugs inside the cells so that these can survive. In pathogenic fungi, including those of *Candida* genus, increased expression of these transporters correlates with azole resistance (Bhattacharya et al., 2020). In terms of the amount of proteins involved in clinical azole resistance and the frequency of overexpression in resistant isolates, ABC superfamily contributes to that phenomenon in a wider extent (Holmes et al., 2016).

2.4.1.1. ABC transporters

On the one hand, ABC proteins (Figure 2) are considered one of the largest superfamilies of proteins, specifically, in *C. albicans* a total of 28 putative ABC protein family members were identified (Gaur et al., 2005). In yeast species, aside from their role in drug transport, some of them also have physiological functions like vacuolar detoxification or metal ion, lipid and peptide transport (Kumari et al., 2021). They harbour at least one nucleotide binding domain (NBD) as energy source, each containing highly conserved motifs such as Walker A, Walker B and signature sequences. The hallmark of these transporters is their promiscuous nature that permit the efflux of many diverse substrates (Prasad et al., 2015).

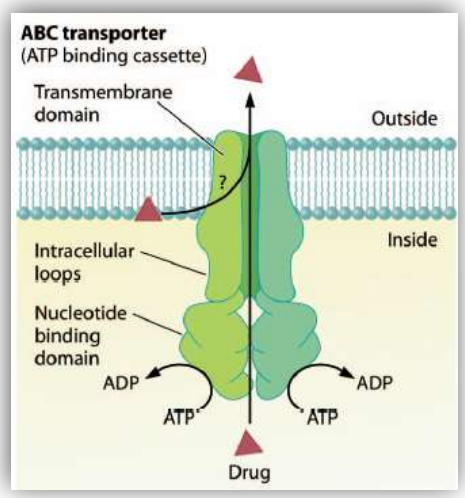

Figure 2. ABC protein representation (Cannon et al., 2009).

According to the Human Genome Organization, these proteins can be clustered into **six subfamilies** designated ABCB/MDR, ABCC/MRP, ABCD/ALDP, ABCF/YEF3, ABCE/RLI and ABCG/PDR (Prasad et al., 2015). Since the members of ABCB/MDR, ABCC/MRP, ABCD/ALDP and ABCG/PDR subfamilies possess transmembrane domains (TMDs) that act as substrate recognition entities, they are regarded as **ABC membrane-localized transporter proteins**. Among them, proteins with one NBD and one TMD are considered **half transporters**, while those containing NBD and TMD in duplicate are known as **full transporters** and can be present in forward $(TMD\text{-}NBD)_2$ or reverse $(NBD\text{-}TMD)_2$ topology. Quite the contrary, the ABCF/YEF3 and ABCE/RLI representatives do not contain TMDs, hence they are **nontransporter ABC proteins**. After a rearrangement of the open reading frames by *Candida* Genome Database (CGD) assembly, the number of ABC proteins was reduced to 26 and 19 of them are ABC transporter proteins (Prasad et al., 2015).

In all *Candida* species, ABCG/PDR subfamily is the largest, having 9 among 26 in *C. albicans* (Prasad et al., 2015), 7 among 25 in *C. glabrata* (Kumari et al., 2018), and 7 among 28 in *C. auris* (Wasi et al., 2019). In *C. albicans*, four members of the PDR subfamily have been characterized, showing that Cdr1p (*Candida* drug resistance 1 protein) and Cdr2p (*Candida* drug resistance 2 protein) are the only involved in drug and phospholipids transport, and Cdr3p (*Candida* drug resistance 3 protein) and Cdr4p (*Candida* drug resistance 4 protein) translocate

phosphoglycerides between the two lipid monolayers of plasma membrane (Smriti et al., 2002; Sanglard et al., 1999). The increased expression in *C. albicans* of Cdr1p and Cdr2p in different drug-resistant clinical isolates proved that these transporters are the only involved in *C. albicans* clinical drug resistance (D Sanglard et al., 1995), although Cdr1p has been proved to contribute to a greater extent than Cdr2p to fluconazole resistance in *C. albicans* (Holmes et al., 2008). Despite the fact that only CaCdr1p and CaCdr2p are the only clinically relevant, there are others significant ABC transporters in *C. albicans* that are linked to azole resistance/sensitivity. Indeed, Cdr6/Roa1p has demonstrated to be an exporter of some xenobiotics and its deletion leads to hyperactivation of Tor1 and consequently, increased azole resistance (Khandelwal et al., 2018). Afterwards, the same group revealed the role of the vacuolar-membrane-localized ABC transporter Mlt1p in *C. albicans* importing azoles into vacuoles, that is to say, vacuolar sequestration (Khandelwal et al., 2019a). *C. albicans* homolog *CDR1* in *C. auris* is related to azole resistance, as well as the overexpression of *CgCDR1*, *CgSNQ2*, *CgPDH1* and *CgPDR16* in C. *glabrata* (Bhattacharya et al., 2020).

Cdr1p is a 169.9-kDa protein and full transporter with reverse topology. Each TMD is made up of six transmembrane helices (TMHs), which are interlinked by six extracellular loops (ECL1-6) and four intracellular loops (ICL1-4). In turn, the NBDs have the hallmark β-sheet sub-domain containing the Walker A and Walker B motifs and an α-helical sub-domain that consists of the conserved ABC signature sequence (Prasad et al., 2015). Regarding polyspecificity, Cdr1p has a wide range of structurally unrelated molecules as substrates, whose features are high hydrophobicity, molecular branching, high aromatic and the presence of an atom-centered fragment (R-CH-R) (Puri et al., 2010). Some of these substrates are antifungals, fluorescent dyes, plant products, herbicides, anticancer drugs, steroids and phosphoglycerides (Prasad et al., 2015). In order to understand the molecular basis of polyspecificity in *Ca*Cdr1p and unveil the location of its drug binding site, Rawal and co-workers (2013) employed alanine scanning mutagenesis. That study revealed the presence of multiple overlapping minibinding sites within a polyspecific substrate binding site and some critical residues in the TMDs that, likewise the substrates of this efflux pump, were mainly hydrophobic in nature. Of note, the importance of a residue in substrate binding or transport and its degree of conservation did not directly correlate (Rawal et al., 2013). The main reason of this **polyspecificity** seems to be the primary sequence variation of TMDs (Banerjee et al., 2021).

Concerning regulation, the transcription of *CDR1* is controlled by several well-characterized *trans* factors such as *TAC1*, which is often associated with gain-of-function mutations that lead to hyperresistance in clinical *Candida* isolates (Coste et al., 2006). There are other factors like *UPC2*, that is involved in sterol biosynthesis, acts as an activator or repressor depending on its own activation and the upc2∇/∇ cause azole hypersusceptibility; or the bZIP transcription factor coded by *CAP1* gene (Prasad et al., 2015). Zinc-cluster transcription factors were analysed using artificial activation by fusion of the C terminus of the protein with the heterologous Gal4 activation domain. This method helped in the characterization of the multidrug resistance regulator *MRR2* as mediator of fluconazole resistance through overexpression of *CDR1* (Schillig and Morschhäuser, 2013). Additionally, *CDR1* promoter has some regulatory elements including AP-1, yeast AP-1 (YAP-1), heat shock elements (HSEs) and MDR nuclear factor-1 (MDR-NF1), also known as drug response element (DRE) (Puri et al., 1999).

2.4.1.2. MFS transporters

On the other hand, the ubiquitous MFS transporters (Figure 3), abundant in *Candida* spp., can act as **uniporters**, **antiporters** and **symporters** (Banerjee et al., 2021). In *C. albicans*, 95 members of MFS group have been identified, divided in 17 families among which **Drug: H^+ Antiporter-1 (DHA1)** and **Drug: H^+ Antiporter-2 (DHA2)** are the major families with 22 and 9 representatives respectively (Manisha Gaur et al., 2008).

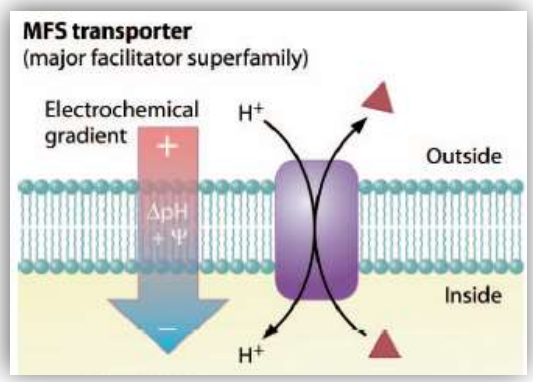

Figure 3. MFS protein representation (Cannon et al., 2009).

Conjointly with *Ca*Cdr1p and *Ca*Cdr2p, ***Ca*Mdr1p** is the only multidrug transporter of this family showing clinical significance (Prasad et al., 2017). However, *Ca*Flu1p has experimentally demonstrated to confer azole resistance albeit not contributing to clinical drug resistance (Calabrese et al., 2000). Mdr1p, formerly known as BEN^r for benomyl resistance (Banerjee et al., 2021), is a typical DHA1 subfamily antiporter that contains 12 TMHs, five ICLs, six ECLs, an "antiporter motif" within TMH5, a large ICL3 considered as the central cytoplasmic loop or CCL and a long hydrophilic N-terminal extension whose importance is yet to be clarified (Redhu et al., 2016; Pasrija et al., 2007; Banerjee et al., 2021). In *C. glabrata,* the transporters clinically significant are *CgFLR1* and *CgQDR2*, whose increased expression is associated with azole-resistant clinical isolates (Bhattacharya et al., 2020). Similar to the study of Rawal et al. (2013) on *Ca*Cdr1p, Redhu et al. also performed alanine scanning mutagenesis for all the residues present within the TMDs of *Ca*Mdr1p (Redhu et al., 2018). Around 33% (84 out of 252) of the variants showed total or selective loss of drug resistance, and of those, 53 were sensitive to all of the tested drugs and the rest were sensitive to at least one (Redhu et al., 2018). In addition, apart from *Ca*Mdr1p TMH4, which resulted to be maximally conserved and have the maximal number of critical residues, no direct relationship was detected between the TMH conservation and their functional importance (Redhu et al., 2018). This study also provided some insight about Mdr1p broad substrate promiscuity, revealing that the structural basis for **polyspecificity** is conferred by certain residues situated at the periphery of the central core that can accommodate compounds of different size or type (Redhu et al., 2018).

The multidrug resistance regulator, *MRR1*, is the transcription factor responsible for *MDR1* expression and the gain of function in Mrr1p, followed by loss of heterozygosity, represents the principal cause of *MDR1* overexpression in *C. albicans* strains resistant to fluconazole (Dunkel et al., 2008).

2.4.1.3. Impact of efflux pumps on cell functioning

The potentially high operational cost of these efflux pumps brings up to the question of whether they interfere with cell functioning. Indeed, while these transporters prevent cells from being attacked by toxic compounds, on the contrary, their excessive activity can compromise the overall cellular fitness under normal conditions (Knorre et al., 2020). Firstly, activity of MDR transporters is linked to an **energy expenditure** due to basal ATP-hydrolysing, which is not stimulated by the addition of substrates (Ernst et al., 2008). Secondly, it can **compromise plasma membrane properties** such as ergosterol content under stress conditions or sterols distribution, since MDR transporters contribute to plasma membrane lipid asymmetry, consequently being involved in the maintenance of lipid homeostasis (Knorre et al., 2020). Also, MDR transporters can promote **unintentional efflux** of some natural metabolic intermediates, thus decreasing metabolic efficiency; and **cell-to-cell communication disturbance** because of an excessive efflux of quorum-sensing factors. Zhu et al. (2011) showed for the first time the involvement in *C. albicans* of Cdr1p-mediated glutathione efflux as a mechanism preceding the farnesol-induced apoptotic process. Farnesol, a quorum-sensing molecule in *C. albicans*, conjugates with glutathione, a crucial antioxidant for cellular detoxification, and the resulting conjugates act as substrates of ABC transporters, finally leading to oxidative stress and fungal cell death (Zhu et al., 2011).

2.4.2. Other mechanisms of resistance

2.4.2.1. Entry constraints

There are several hypotheses of defective **azole entry** that could contribute to drug resistance reducing intracellular azoles. However, there is still no evidence of identified azole importers and their roles in azole resistance (Bhattacharya et al., 2020). Where we have clear evidence is in the mechanism of **sterol import** that reduces the need for sterol biosynthesis and thus compensates the effect of azoles decreasing the content of ergosterol. This mechanism has been well characterized in the human fungal pathogens *C. albicans* and *C. glabrata*, showing some differences between them (Zavrel et al., 2013). *C. glabrata* can uptake sterols either in aerobic or anaerobic conditions and the limited aerobic import can be stimulated by the presence of serum with fluconazole; whereas in *C. albicans* the mechanism only occurs in aerobic conditions, increasing azole resistance in the presence of both serum and cholesterol (Zavrel et al., 2013). Bearing this in mind, it can be stated that *C. albicans* could develop azole resistance by importing cholesterol and serum from the blood (Bhattacharya et al., 2020).

2.4.2.2. Genomic alterations

Given that azoles target ergosterol, altered biosynthesis of that component is an essential mechanism of resistance. ***ERG11*** overexpression has been linked to **azole resistance** in many fungi, including *C. albicans, C. glabrata* and *C. auris* (Bhattacharya et al., 2020). Besides, azole resistance can be acquired by mutated versions of *ERG11* mutations like A61V, A114S, Y132F, Y132H, K143Q, K143R, Y257H, S405F, G448E, F449S, G464S, R467K and

I471T in *C. albicans* (Xiang et al., 2013). Point mutations in that gene have also been identified in other azole-resistant *Candida* spp. like *C. glabrata* mutations C108G, C423T and A1581G; and *C. krusei* mutations A497 and G1570A (dos Santos Silva et al., 2016). When azoles inhibit Erg11p, Erg3p and Erg6p synthesize the toxic sterol. Disruption of ***ERG3*** increases azole resistance in *C. albicans* (Sanglard et al., 2003), just as heterozygous ***ERG6*** deletion (Yoo et al. , 2010). **Upc2p** regulates the majority of ergosterol biosynthetic genes, among which we find *ERG11*. Mutations G648D, G648S, A643T, A643V, Y642F, A646V and W478C in CaUpc2p exhibited increased expression of ERG11 and increased resistance to fluconazole (Flowers et al., 2012). In *C. glabrata*, deletion of *CgUPC2A*, one of the two homologues of *CaUPC2*, has been linked to azole susceptibility (Nagi et al., 2011).

In addition to azoles, there are target alterations causing resistance to other drugs. **Polyene resistance**, despite not being well characterized, is associated with changes in ***ERG3*** and ***ERG6*** genes (Bhattacharya et al., 2020). Whilst still quite unusual, resistance to amphotericin B can be mediated by a higher catalase activity and a consequent decrease in oxidative damage susceptibility (Costa-de-oliveira and Rodrigues, 2020).

Point mutations in **FKS** genes, which encode echinocandins' target β-(1,3)-glucan synthase, hold relevance in **echinocandin resistance**. Mutations in the catalytic subunit of that enzyme, ***FKS1*** gene, contribute to a higher extent than mutations in ***FKS2*** gene, both leading to amino acid substitutions in two different regions of these genes (Hot spot 1 and 2, or HS1 and HS2) (Costa-de-oliveira and Rodrigues, 2020). In *C. albicans*, where therapeutic failure still occurs rarely, amino acid substitutions at Ser645 and Phe641 are the most pronounced phenotypes, accounting for more than 75% of resistance in that species; while in *C. glabrata,* where resistance is rising, are Ser629, Ser663 and Fks2 position F659S (Perlin, 2015). Eventually, *C. auris* isolates initially susceptible to echinocandins can develop de novo FKS1-mediated resistance after echinocandin exposure (Prasad et al., 2019).

With regard to resistance to **5-Flucytosine** among *Candida*, it is associated with mutations in the enzyme uracil phosphoribosyl transferase (**Fur1p**) that unable the conversion of 5-fluorouracil to 5-fluorouridine monophosphate (Costa-de-oliveira and Rodrigues, 2020). Furthermore, mutations in ***FCY1*** and ***FCY2*** genes have been related to 5-Flucytosine resistance in some *Candida* spp. like *C. auris, C. lusitaniae or C. glabrata* (Frías-De-León et al., 2020; Florent et al., 2009; Edlind and Katiyar, 2010).

Genomic plasticity seems to be a conserved and central adaptative mechanism of resistance (Revie et al., 2018). In fungi, **azole resistance** is correlated with aneuploidy and loss of heterozygosity (LOH), in which the mutation of one allele can be copied to the second (Bhattacharya et al., 2020). LOH in *CaTAC1*, *CaERG11* and *CaMRR1* have been correlated with increased levels of resistance (Ford et al., 2015). Also related with major azole resistance, clinical isolates of *C. albicans* have shown segmental aneuploidy, in which two copies of the left arm on chromosome 5 containing *CaERG11* and *CaTAC1* form an isochromosome (Selmecki et al., 2006); trisomy in chromosome 4 (Anderson et al., 2017), whose mechanism of resistance is still unknown; loss of one homologue of chromosome 4, aneuploidy in chromosome 6 and trisomy in chromosome 3 (Bhattacharya et al., 2020). Further, trisomy in chromosome R contributes to azole resistance too (Li et al., 2015). In *C. glabrata*, aneuploidy and alteration in gene copy numbers have been associated with high levels of azole resistance (Bhattacharya et al., 2020). Recently, transient duplication in *CDR1* and *ERG11* genes in generationally aging *C. auris* cells cause increased tolerance to fluconazole (Bhattacharya et al.,

2019). Even though its mechanism has not been studied deeply, chromosome 2 trisomy in *C. albicans* causes **caspofungin resistance** (Bhattacharya et al., 2020).

In some cases, genomic alterations comprising the **generation of an isochromosome**, are an alternative way to enhance antifungal efflux and thus resistant traits in *Candida* species. It has been observed a duplication of the left arm of chromosome 5 (**i(5L)**) as a common aneuploidy in *C. albicans* **azole-resistant** isolates, leading into an increased dosage of both *ERG11* and *TAC1*, hence enabling a dual mechanism of azole resistance (Revie et al., 2018).

2.4.2.3. Cell plasticity

Cell plasticity is another contributor to antifungal resistance, mainly the extracellular matrix of biofilms that promotes adherence and protection from hostile environmental conditions. In fact, biofilm production by *C. albicans* confer up to a 1000-fold greater drug resistance compared with non-biofilm cells *in vitro* (Pristov and Ghannoum, 2019). *C. albicans* **biofilms** are resistant to **azoles** and conventional **amphotericin B**, while liposomal amphotericin B and echinocandins are effective upon them (Costa-de-oliveira and Rodrigues, 2020).

Besides, in some *Candida* species, in particular *C. albicans*, there is an alternative respiratory pathway corresponding to an **alternative oxidase (AOX)**. It has been implicated in reduced susceptibility to **azoles** through a mechanism in which exposure to antifungal agents decrease intracellular reactive oxygen species (ROS) production (Yan et al., 2009). Inhibition of this alternative pathway leads to enhanced recognition by macrophage cells but when that inhibition is removed, a stress response takes place increasing the levels of virulence traits (Duvenage et al., 2019).

2.5. Cellular stress responses mediating resistance

Antifungal exposure is one of the stressing conditions under which *Candida* cells activate several cellular stress response pathways. Firstly, the **cyclic AMP (cAMP)-protein kinase A (PKA) signalling pathway** is often involved in the stress responses mediating **triazole resistance**. In *C. albicans*, it contributes to the recovery process facilitating the resume of growth after stress conditions or fluconazole exposure (Costa-de-oliveira and Rodrigues, 2020). Secondly, another cellular response is the **Ca^{2+}-calmodulin-calcineurin pathway**. Calcium acts as a secondary messenger molecule in both mammals and fungi, regulating cellular processes and playing crucial roles in cell survival. In conjunction with mainly calcineurin and other components of the fungal calcium signalling pathway, they can mediate antifungal resistance of invasive fungal strains (Liu et al., 2015). Therefore, they could be seen as targets for new therapies. Particularly against candidemia, Jia et al. (2012) found that calcium-activated-calcineurin can severely reduce the efficacy of **fluconazole** treatment both *in vitro* and *in vivo* through its target Rta2p and the transcription factor *CRZ1* (Jia et al., 2012). Calcineurin is stabilized by the molecular chaperone heat shock protein 90 (Hsp90), giving way to cellular stress responses that are required for **echinocandin** resistance. Actually, calcineurin may be defined as the key mediator of Hsp90-dependent echinocandin resistance (Singh et al., 2009). A previous work of the same group stated the genetic reduction of Hsp90 levels in a

murine model of disseminated *C. albicans* infection enhances the efficacy of fluconazole activity (Singh et al., 2009).

Furthermore, most *Candida* species have shown the potential of **increasing chitin synthesis** as a mechanism of tolerance to echinocandins, as cells with high chitin content are less susceptible to caspofungin (Walker et al., 2013). **Echinocandin tolerance** has also been related to the *C. albicans* transcription factor ***CAS5***, which makes echinocandin resistance possible in a FKS1-mutated strain (Xie et al., 2017).

2.6. Strategies to overcome antifungal resistance and tolerance

Owing to the alarming life-threatening infections caused by *Candida* spp., it is vital to find new strategies so that we could reduce drug resistance.

2.6.1. Inhibition of efflux

An approach that has attracted significant attention after the growing incidence of resistant *Candida* isolates is the **structure-based design of effective inhibitors of efflux** (Figure 4). These can affect pumps directly, either by binding as a pseudosubstrate and blocking access to the binding site, or by locking the drug in a conformation so that efflux reaction cycle is impeded (Cannon et al., 2009). Unfortunately, the lack of high-resolution structures for yeast PDR pumps and the incomplete understanding of the mechanisms required for efflux pump inhibition and modulation hinder the implementation of these inhibitors into the clinic (Banerjee et al., 2021).

Some inhibitors with potential that have been identified are the immunosuppressant **FK520**, **clorgyline**, **jatrophanes**, **curcumin**, **disulfiram** inhibiting the oligomycin-sensitive ATPase activity of Cdr1p, **farnesol** acting as modulator of the same transporter and some peptide and peptide-derivatives including the D-octapeptide derivative **RC21v3** (Prasad et al., 2015; Prasad et al., 2019). Unlike *Ca*Cdr1p, only few molecules are able to inhibit *Ca*Mdr1p so far. For example, **clorgyline**, certain **chalcones**, **jatrophanes** or **lathyrane diterpenes** (Prasad et al., 2019).

The development of **indirect inhibitors** of efflux is also considered in that context. They would act depriving cells of the energy required for drug efflux by lowering the cytoplasmic ATP concentration or by depleting the electrochemical potential of the plasma membrane (Cannon et al., 2009). **Proton pump inhibitors**, combined with fluconazole, enhance the efficacy of that azole both *in vitro* and *in vivo*. Moreover, the synergistic antifungal effects lead to efflux pump activity suppression and some *Candida* virulence factors inhibition, specifically, morphology switching and phospholipase activity (Lu et al., 2020).

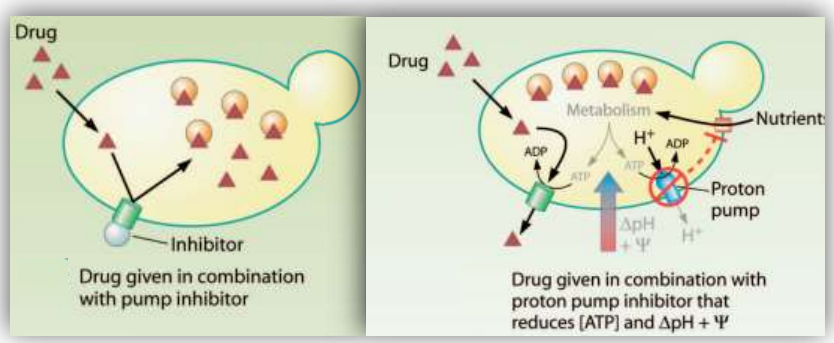

Figure 4. Direct and indirect inhibition of efflux pumps (Cannon et al., 2009).

2.6.2. Chemosensitization

In the study of chemosensitizers, it has been reported that **FK506** and **cyclosporine A** (**CsA**) chemosensitize *C. albicans* cells to azoles, inhibiting calcineurin and turning the spectrum of activity of those drugs to fungicidal (Costa-de-oliveira and Rodrigues, 2020). Though being both immunosuppressive is a disadvantage when it comes to treat candidiasis patients, who are already immunocompromised. Furthermore, **calcium channel blockers** such as amlodipine, nifedipine, benidipine and flunarizine can increase the sensitivity of fluconazole to resistant *C. albicans* strains (Liu et al., 2016); and **transmembrane peptide mimics (TMPMs)** of efflux pumps have the ability to chemosensitize CaCdr1 overexpressing clinical isolates toward fluconazole (Prasad et al., 2019; Maurya et al., 2013). The synergistic relationship between **sulfa antibacterial drugs** and fluconazole has been studied in *C. albicans,* as well as it has been confirmed the ability of these drugs to inhibit *Candida* biofilms by 40% (Eldesouky et al., 2018). However, it has been reported that antibacterial agents administration can unbalance the fungal microbiome and consequently increase colonization and proliferation of yeasts (Costa-de-oliveira and Rodrigues, 2020).

Therapies based on the **combination of drugs** can also play a key role potentiating antifungal action and leading to shorter treatment periods, lower doses of drugs and higher efficiency of single agents. Nim et al. (2018) found that **chalcone derivatives** diphenylpropenones combined with fluconazole act as antifungal sensitizers by antagonizing the drug efflux pump activity of *Ca*Cdr1p and *Ca*Mdr1p (Nim et al., 2018). Among the different derivatives assessed by Nim and co-workers, four compounds were able to sensitize yeasts overexpressing both transporters, were not considered cytotoxic to yeast cells and were easily synthesized. Everything together underlines their potential to undergo further development for preclinical trials to make azoles active again (Nim et al., 2018).

2.6.3. Design of new antifungal drugs

Currently, one of the routes for designing new drugs is on a styrylquinoline scaffold. In 2017, it came to light the first evidence that **styrylquinolines**, heterocyclic and lipophilic compounds, decrease the activity of ABC multidrug transporters and act synergistically with fluconazole (Szczepaniak et al., 2017). More recently, it has been revealed that metal

complexation through hydroxyl groups could be the mechanisms of action of styrylquinolines and that synergistic interactions are reliant on the substitution pattern (Cieslik et al., 2020).

Styrylpyridinium compounds, initially synthesized as fluorescent probes, are promising drug candidates too. Vaitkienė and her group lately demostrated the antifungal activity of a set of new and earlier described styrylpyrydinium compounds against *C. albicans* cells and evaluated their possible synergism with fluconazole (Vaitkienė et al., 2020a). Eight out of thirteen reduced *C. albicans* growth and some of them were considered substrates of *C. albicans* MDR pumps. The compound with a NEt_2 group as substituent showed the strongest fungicidal properties, effectively inhibiting respiration but possessing the highest toxicity to mammalian cells. Nonetheless, the most active synthesized styrylpyridinium compound was 4-(4-cyanostyryl)-1-dodecylpyridin-1-ium ($CSDP^+$) bromide with a CN-group as substituent. It presented at fungicidal concentrations the least toxicity to mammalian cells, the most effective synergism with fluconazole and a strong inhibition of the growth and respiration of *C. albicans* (Vaitkienė et al., 2020a).

Later, it was evaluated the interaction of low concentrations of **$CSDP^+$ bromide** with yeast and mammalian cells, resulting in an absence of toxicity to HEK-293 cells and a reduction of *C. albicans* adhesion, both alone and in combination with fluconazole (Vaitkienė et al., 2020c). Preventing adhesion was such an encouraging result, as it is a crucial factor for colonization and persistence of the pathogen. This study also suggests the possible association of the expression of *MDR1* and *MRR1* genes *in C. albicans* with fluconazole resistance related efflux, as they are overexpressed after the exposure to that antifungal in combination with $CSDP^+$ (Vaitkienė et al., 2020c).

On the basis of the highest efficiency against *C. albicans*, Vaitkienė and colleagues selected and evaluated eight styrylpyridinium compounds from their previous study (Vaitkienė et al., 2020a) on *C. glabrata* strains containing or not drug efflux pumps (Vaitkienė et al., 2020b). Their mechanism of action, based on permeabilization of the plasma membrane, resulted to be dependent on their structure, exposure time and concentration. All compounds were exported from *C. glabrata* cells as substrates of their MDR pumps, mainly Cdr1p, so they could be properly applied in combined therapy by increasing the concentration of another antifungal drug above the effective threshold due to binding site competition. Their potential was also emphasised with their large Stokes shifts and small overlaps between the absorption and fluorescence spectra. Ultimately, styrylpyridinium compounds may be applied not only as new antifungal drugs, showing significant synergism with FK506 and terbinafine, but also as fluorescence dyes with low toxicity in yeast research (Vaitkienė et al., 2020b).

2.6.4. Other strategies

The first approach that come to mind in order to bypass drug resistance due to efflux pumps would be the research on **antifungals that are not substrates of them** (Figure 5), like echinocandins or amphotericin B (Costa-de-oliveira and Rodrigues, 2020). That could be possible taking into account size and hydrophobicity constraints, at the same level as complicated since efflux pumps have developed flexible and large drug-binding sites (Cannon et al., 2009). Otherwise, focusing on a **promotion of antifungal uptake** (Figure 6) may promote the maintenance of high intracellular concentrations of antifungal in spite of any

upregulation of efflux. Inclusion of multiple arginine residues in antifungals would enhance drug delivery to their intracellular target (Cannon et al., 2009).

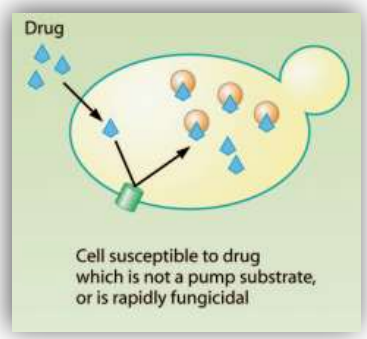

Figure 5. Use of antifungals that are not substrates of efflux pumps (Cannon et al., 2009).

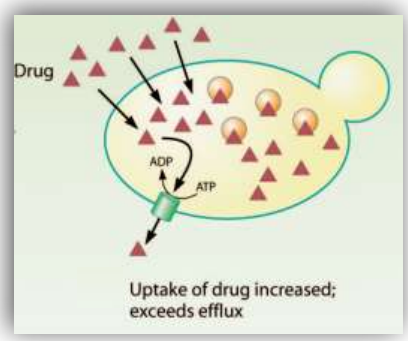

Figure 6. Promotion of antifungal uptake (Cannon et al., 2009).

More combination therapies have shown promising results. In a murine model of systemic infection caused by *C. albicans*, the very well-known anti-inflammatory drug **ibuprofen** exhibited a synergistic effect with fluconazole against fluconazole-resistant strain (Costa-de-Oliveira et al., 2015); the combination of the peptidyl nucleoside **Nikkomycin Z** with caspofungin and micafungin demonstrated to be synergic in *C. albicans* biofilms (Kovács et al., 2019); and a synergistic effect in *C. albicans* fluconazole resistant strains was exhibited by the flavonoid **kaempferol** (Shao et al., 2016). More flavonoids have been studied in the field of novel therapeutic approaches. For instance, selected **flavones** (luteolin, apigenin), **flavonols** (quercetin), and their **glycosylated derivatives** (quercitrin, isoquercitrin, rutin and apigetrin), have shown antifungal capacity not only reducing biofilm and hyphal formation but also, lowering the expression of the resistance linked genes *CDR1* and *ERG11* (Ivanov et al., 2020). This year, a study analysing a wide range of antifungal combinations against echinocandin-resistant and echinocandin-susceptible *C. glabrata* has confirmed that most of the synergistic action takes place among the echinocandin-resistant isolates (Khalifa et al., 2021). For the first time, it has been discovered a sustained fungicidal activity against an echinocandin-resistant isolate by the synergistic action of **caspofungin-fluconazole**, **caspofungin-voriconazole**, **caspofungin-posaconazole**, and **5-flucytosine-amphotericin B** combinations, with **caspofungin-voriconazole** and **5-flucytosine-amphotericin B** combinations (Khalifa et al., 2021).

Further, other prospects for resistance prevention would be the **identification of the molecular mechanisms behind biofilm state or adherence** that could pave the way for new biofilm-specific therapeutics (Nobile and Johnson, 2015) and **targeting the stress-response components** that permit tolerance and development of resistance. Compromising Hsp90 function *in vitro* enhances the efficacy of echinocandins against isolates that evolved resistance in a human host and isolates not previously exposed to these drugs (Singh et al., 2009). Additionally, an overview of the components of the fungal **calcium-calcineurin signalling pathway** and their potential roles as antifungal targets would be convenient for the development of new antifungal drugs. Interestingly, on account of the deleterious effects of excessive MDR transporters activity, cells keep MDR pumps genes repressed under normal conditions, activate them only upon exposure to chemical stress and their basal expression can vary with changes in

the metabolic state of the cells. Notwithstanding, this can make cells vulnerable to hypothetical toxins that could avoid detection inside the cells and **do not activate MDR transporters**. Therefore, studying this approach may be a promising strategy in the research of new efficient antifungals (Knorre et al., 2020).

3. CONCLUSIONS AND RECOMMENDATIONS

1. Scientific information from different databases has been obtained, including PubMed, ScienceDirect, Google Scholar and Scopus. From science journals including Nature and Science; and from web pages and books available on Internet. There is a clear **agreement** in the information provided and, especially, in the relationship between *Candida* infections and colonization of those pathogens in the medical setting. To prevent outbreaks and collapse of the health system, **surface disinfection** in hospital rooms and finding ways to **decolonize** serious pathogens such as *C. auris* should be emphasised.

2. Cryo-electron microscopy, pharmacophore-based screening and other bioinformatics technologies are worth of further application in studies of the most clinically important *Candida* efflux pumps. Their functioning could be completely understood with the availability of **more data on 3D structures**.

3. The actual COVID-19 pandemic has promoted an unforeseen and unavoidable increase in antifungal resistance. It should be pointed out the high rate of antimicrobial agent use in COVID-19 patients with relatively low rates of co-secondary infections. That brings up to the urgent need of **effective antifungal stewardship programme**, comprising quicker fungal diagnostics, appropriate prescription of antifungals, therapeutic drug monitoring and clinical intervention teams to control resistance.

4. In spite of the fact that many strategies are being devised to avoid resistance, identification of **innovative fungal targets** and exploration of **multiple targeting therapies** are being underrated in the antifungal drug discovery. Exploiting these challenges with the help of *in silico* investigations may provide a substantial impact on that field.

4. REFERENCES

1. Anderson, M. Z., Saha, A., Haseeb, A., and Bennett, R. J. (2017). A chromosome 4 trisomy contributes to increased fluconazole resistance in a clinical isolate of Candida albicans. *Microbiology (Reading, England)*, *163*(6), 856–865. https://doi.org/10.1099/mic.0.000478
2. Banerjee, A., Pata, J., Sharma, S., Monk, B. C., Falson, P., and Prasad, R. (2021). Directed mutational strategies reveal drug binding and transport by the MDR transporters of Candida albicans. *Journal of Fungi*, *7*(2), 1–16. https://doi.org/10.3390/jof7020068
3. Barchiesi, F., Spreghini, E., Tomassetti, S., Arzeni, D., Giannini, D., and Scalise, G. (2005). Comparison of the fungicidal activities of caspofungin and amphotericin B against Candida glabrata. *Antimicrobial Agents and Chemotherapy*, *49*(12), 4989–4992. https://doi.org/10.1128/AAC.49.12.4989-4992.2005
4. Benedict, K., Molinari, N. A. M., and Jackson, B. R. (2020). Public Awareness of Invasive Fungal Diseases — United States, 2019. *MMWR. Morbidity and Mortality Weekly Report*, *69*(38), 1343–1346. https://doi.org/10.15585/mmwr.mm6938a2
5. Bhattacharya, S., Holowka, T., Orner, E. P., and Fries, B. C. (2019). Gene Duplication Associated with Increased Fluconazole Tolerance in Candida auris cells of Advanced Generational Age. *Scientific Reports*, *9*(1), 5052. https://doi.org/10.1038/s41598-019-41513-6
6. Bhattacharya, S., Sae-Tia, S., and Fries, B. C. (2020). Candidiasis and mechanisms of antifungal resistance. *Antibiotics*, *9*(6), 1–19. https://doi.org/10.3390/antibiotics9060312
7. Calabrese, D., Bille, J., and Sanglard, D. (2000). A novel multidrug efflux transporter gene of the major facilitator superfamily from Candida albicans (FLU1) conferring resistance to fluconazole. *Microbiology*, *146*(11), 2743–2754. https://doi.org/10.1099/00221287-146-11-2743
8. Cannon, R. D., Lamping, E., Holmes, A. R., Niimi, K., Baret, P. V., Keniya, M. V., … Monk, B. C. (2009). Efflux-mediated antifungal drug resistance. *Clinical Microbiology Reviews*, *22*(2), 291–321. https://doi.org/10.1128/CMR.00051-08
9. Centers for Disease Control and Prevention. (2019). Antibiotic resistance threats in the United States. *Centers for Disease Control and Prevention*, 1–113. Retrieved from https://www.cdc.gov/drugresistance/biggest_threats.html
10. Chakrabarti, A., and Sood, P. (2021). *On the emergence , spread and resistance of Candida auris : host , pathogen and environmental tipping points*. https://doi.org/10.1099/jmm.0.001318
11. Cieslik, W., Szczepaniak, J., Krasowska, A., and Musiol, R. (2020). Antifungal styryloquinolines as candida albicans efflux pump inhibitors: Styryloquinolines are ABC transporter inhibitors. *Molecules*, *25*(2). https://doi.org/10.3390/molecules25020345
12. Cohen, B. E. (2010). Amphotericin B membrane action: role for two types of ion channels in eliciting cell survival and lethal effects. *The Journal of Membrane Biology*, *238*(1–3), 1–20. https://doi.org/10.1007/s00232-010-9313-y
13. Costa-de-Oliveira, S., Miranda, I. M., Silva-Dias, A., Silva, A. P., Rodrigues, A. G., and Pina-Vaz, C. (2015). Ibuprofen potentiates the in vivo antifungal activity of fluconazole against Candida albicans murine infection. *Antimicrobial Agents and Chemotherapy*, *59*(7), 4289–4292. https://doi.org/10.1128/AAC.05056-14

14. Costa-de-oliveira, S., and Rodrigues, A. G. (2020). Candida albicans antifungal resistance and tolerance in bloodstream infections: The triad yeast-host-antifungal. *Microorganisms*, *8*(2). https://doi.org/10.3390/microorganisms8020154
15. Coste, A., Turner, V., Ischer, F., Morschhäuser, J., Forche, A., Selmecki, A., … Sanglard, D. (2006). A Mutation in Tac1p, a Transcription Factor Regulating CDR1 and CDR2, Is Coupled With Loss of Heterozygosity at Chromosome 5 to Mediate Antifungal Resistance in Candida albicans. *Genetics*, *172*(4), 2139–2156. https://doi.org/10.1534/genetics.105.054767
16. Cui, X., Li, X., Xu, Z., Guan, X., Ma, J., Ding, D., and Zhang, W. (2021). Fabrication and Characterization of Chitosan/Poly(Lactic-Co-glycolic Acid) Core-Shell Nanoparticles by Coaxial Electrospray Technology for Dual Delivery of Natamycin and Clotrimazole. *Frontiers in Bioengineering and Biotechnology*, *9*(March), 1–13. https://doi.org/10.3389/fbioe.2021.635485
17. d'Enfert, C., Kaune, A. K., Alaban, L. R., Chakraborty, S., Cole, N., Delavy, M., … Brown, A. J. P. (2021). The impact of the Fungus-Host-Microbiota interplay upon Candida albicans infections: current knowledge and new perspectives. In *FEMS microbiology reviews* (Vol. 45). https://doi.org/10.1093/femsre/fuaa060
18. Delarze, E., and Sanglard, D. (2015). Defining the frontiers between antifungal resistance, tolerance and the concept of persistence. *Drug Resistance Updates*, *23*, 12–19. https://doi.org/https://doi.org/10.1016/j.drup.2015.10.001
19. dos Santos Silva, D. B., Carbonera Rodrigues, L. M., De Almeida, A. A., de Oliveira, K. M. P., and Grisolia, A. B. (2016). Novel point mutations in the ERG11 gene in clinical isolates of azole resistant Candida species. *Memorias Do Instituto Oswaldo Cruz*, *111*(3), 192–199. https://doi.org/10.1590/0074-02760150400
20. Dunkel, N., Blass, J., Rogers, P. D., and Morschhäuser, J. (2008). Mutations in the multi-drug resistance regulator MRR1, followed by loss of heterozygosity, are the main cause of MDR1 overexpression in fluconazole-resistant Candida albicans strains. *Molecular Microbiology*, *69*(4), 827–840. https://doi.org/10.1111/j.1365-2958.2008.06309.x
21. Duvenage, L., Walker, L. A., Bojarczuk, A., Johnston, S. A., MacCallum, D. M., Munro, C. A., and Gourlay, C. W. (2019). Inhibition of Classical and Alternative Modes of Respiration in Candida albicans Leads to Cell Wall Remodeling and Increased Macrophage Recognition. *MBio*, *10*(1), e02535-18. https://doi.org/10.1128/mBio.02535-18
22. Edlind, T. D., and Katiyar, S. K. (2010). Mutational analysis of flucytosine resistance in Candida glabrata. *Antimicrobial Agents and Chemotherapy*, *54*(11), 4733–4738. https://doi.org/10.1128/AAC.00605-10
23. Eldesouky, H. E., Mayhoub, A., Hazbun, T. R., and Seleem, M. N. (2018). Reversal of Azole Resistance in Candida albicans by Sulfa Antibacterial Drugs. *Antimicrobial Agents and Chemotherapy*, *62*(3). https://doi.org/10.1128/AAC.00701-17
24. Ernst, R., Kueppers, P., Klein, C. M., Schwarzmueller, T., Kuchler, K., and Schmitt, L. (2008). A mutation of the H-loop selectively affects rhodamine transport by the yeast multidrug ABC transporter Pdr5. *Proceedings of the National Academy of Sciences*, *105*(13), 5069 LP – 5074. https://doi.org/10.1073/pnas.0800191105
25. Fatahinia, M., Halvaeizadeh, M., Mahmoudabadi, A. Z., AboualiGalehdari, E., and Kiasat, N. (2020). In vitro antifungal susceptibilities of six antifungal drugs against clinical Candida glabrata isolates according to EUCAST. *Current Medical Mycology*, *6*(2), 1–6. https://doi.org/10.18502/CMM.6.2.2692

26. Florent, M., Noël, T., Ruprich-Robert, G., Da Silva, B., Fitton-Ouhabi, V., Chastin, C., … Chapeland-Leclerc, F. (2009). Nonsense and missense mutations in FCY2 and FCY1 genes are responsible for flucytosine resistance and flucytosine-fluconazole cross-resistance in clinical isolates of Candida lusitaniae. *Antimicrobial Agents and Chemotherapy*, *53*(7), 2982–2990. https://doi.org/10.1128/AAC.00880-08
27. Flowers, S. A., Barker, K. S., Berkow, E. L., Toner, G., Chadwick, S. G., Gygax, S. E., … Rogers, P. D. (2012). Gain-of-function mutations in UPC2 are a frequent cause of ERG11 upregulation in azole-resistant clinical isolates of Candida albicans. *Eukaryotic Cell*, *11*(10), 1289–1299. https://doi.org/10.1128/EC.00215-12
28. Ford, C. B., Funt, J. M., Abbey, D., Issi, L., Guiducci, C., Martinez, D. A., … Regev, A. (2015). The evolution of drug resistance in clinical isolates of Candida albicans. *ELife*, *4*, e00662. https://doi.org/10.7554/eLife.00662
29. Frías-De-León, M. G., Hernández-Castro, R., Vite-Garín, T., Arenas, R., Bonifaz, A., Castañón-Olivares, L., … Martínez-Herrera, E. (2020). Antifungal Resistance in Candida auris: Molecular Determinants. *Antibiotics (Basel, Switzerland)*, *9*(9), 568. https://doi.org/10.3390/antibiotics9090568
30. Galocha, M., Pais, P., Cavalheiro, M., Pereira, D., Viana, R., and Teixeira, M. C. (2019). Divergent approaches to virulence in C. Albicans and C. Glabrata: Two sides of the same coin. *International Journal of Molecular Sciences*, *20*(9). https://doi.org/10.3390/ijms20092345
31. Gaur, M, Choudhury, D., and Prasad, R. (2005). Complete Inventory of ABC Proteins in Human Pathogenic Yeast, *Candida albicans*. *Microbial Physiology*, *9*(1), 3–15. https://doi.org/10.1159/000088141
32. Gaur, Manisha, Puri, N., Manoharlal, R., Rai, V., Mukhopadhayay, G., Choudhury, D., and Prasad, R. (2008). MFS transportome of the human pathogenic yeast Candida albicans. *BMC Genomics*, *9*(1), 579. https://doi.org/10.1186/1471-2164-9-579
33. Healey, K. R., Zhao, Y., Perez, W. B., Lockhart, S. R., Sobel, J. D., Farmakiotis, D., … Perlin, D. S. (2016). Prevalent mutator genotype identified in fungal pathogen Candida glabrata promotes multi-drug resistance. *Nature Communications*, *7*, 1–10. https://doi.org/10.1038/ncomms11128
34. Holmes, A. R., Cardno, T. S., Strouse, J. J., Ivnitski-Steele, I., Keniya, M. V, Lackovic, K., … Cannon, R. D. (2016). Targeting efflux pumps to overcome antifungal drug resistance. *Future Medicinal Chemistry*, *8*(12), 1485–1501. https://doi.org/10.4155/fmc-2016-0050
35. Holmes, A. R., Lin, Y.-H., Niimi, K., Lamping, E., Keniya, M., Niimi, M., … Cannon, R. D. (2008). ABC Transporter Cdr1p Contributes More than Cdr2p Does to Fluconazole Efflux in Fluconazole-Resistant Candida albicans Clinical Isolates. *Antimicrobial Agents and Chemotherapy*, *52*(11), 3851 LP – 3862. https://doi.org/10.1128/AAC.00463-08
36. Hüttel, W. (2021). Echinocandins: structural diversity, biosynthesis, and development of antimycotics. *Applied Microbiology and Biotechnology*, *105*(1), 55–66. https://doi.org/10.1007/s00253-020-11022-y
37. Ivanov, M., Kannan, A., Stojković, D. S., Glamočlija, J., Calhelha, R. C., Ferreira, I. C. F. R., … Soković, M. (2020). Flavones, flavonols, and glycosylated derivatives—impact on candida albicans growth and virulence, expression of cdr1 and erg11, cytotoxicity. *Pharmaceuticals*, *14*(1), 1–12. https://doi.org/10.3390/ph14010027
38. Jenks, J. D., Salzer, H. J. F., Prattes, J., Krause, R., Buchheidt, D., and Hoenigl, M. (2018). Spotlight on isavuconazole in the treatment of invasive aspergillosis and

mucormycosis: Design, development, and place in therapy. *Drug Design, Development and Therapy*, *12*, 1033–1044. https://doi.org/10.2147/DDDT.S145545

39. Jia, Y., Tang, R.-J., Wang, L., Zhang, X., Wang, Y., Jia, X.-M., and Jiang, Y.-Y. (2012). Calcium-Activated-Calcineurin Reduces the In Vitro and In Vivo Sensitivity of Fluconazole to Candida albicans via Rta2p. *PLOS ONE*, *7*(10), e48369. Retrieved from https://doi.org/10.1371/journal.pone.0048369
40. K. Redhu, A., Shah, A. H., and Prasad, R. (2016). MFS transporters of Candida species and their role in clinical drug resistance. *FEMS Yeast Research*, *16*(4). https://doi.org/10.1093/femsyr/fow043
41. Kean, R., Delaney, C., Sherry, L., Borman, A., Johnson, E. M., Richardson, M. D., … Ramage, G. (2018). Transcriptome Assembly and Profiling of Candida auris Reveals Novel Insights into Biofilm-Mediated Resistance. *MSphere*, *3*(4). https://doi.org/10.1128/mSphere.00334-18
42. Kean, R., and Ramage, G. (2019). Combined Antifungal Resistance and Biofilm Tolerance: the Global Threat of Candida auris *MSphere*, *4*(4), e00458-19. https://doi.org/10.1128/mSphere.00458-19
43. Khalifa, H. O., Majima, H., Watanabe, A., and Kamei, K. (2021). In vitro characterization of twenty-one antifungal combinations against echinocandin-resistant and-susceptible candida glabrata. *Journal of Fungi*, *7*(2), 1–15. https://doi.org/10.3390/jof7020108
44. Khandelwal, N. K., Chauhan, N., Sarkar, P., Esquivel, B. D., Coccetti, P., Singha, A., … Prasad, R. (2018). Azole resistance in a Candida albicans mutant lacking the ABC transporter CDR6/ROA1 depends on TOR signaling. *Journal of Biological Chemistry*, *293*(2), 412–432. https://doi.org/10.1074/jbc.M117.807032
45. Khandelwal, N. K., Wasi, M., Nair, R., Gupta, M., Kumar, M., Mondal, A. K., … Prasad, R. (2019a). Vacuolar sequestration of azoles, a novel strategy of azole antifungal resistance conserved across pathogenic and nonpathogenic yeast. *Antimicrobial Agents and Chemotherapy*, *63*(3), 1–15. https://doi.org/10.1128/AAC.01347-18
46. Khandelwal, N. K., Wasi, M., Nair, R., Gupta, M., Kumar, M., Mondal, A. K., … Prasad, R. (2019b). Vacuolar Sequestration of Azoles, a Novel Strategy of Azole Antifungal Resistance Conserved across Pathogenic and Nonpathogenic Yeast. *Antimicrobial Agents and Chemotherapy*, *63*(3), e01347-18. https://doi.org/10.1128/AAC.01347-18
47. Knorre, D. A., Galkina, K. V., Shirokovskikh, T., Banerjee, A., and Prasad, R. (2020). Do Multiple Drug Resistance Transporters Interfere with Cell Functioning under Normal Conditions? *Biochemistry (Moscow)*, *85*(12–13), 1560–1569. https://doi.org/10.1134/S0006297920120081
48. Kojic, E. M., and Darouiche, R. O. (2004). Candida Infections of Medical Devices. *Clinical Microbiology Reviews*, *17*(2), 255–267. https://doi.org/10.1128/CMR.17.2.255-267.2004
49. Kontoyiannis, D. P. (2017). Antifungal resistance: An emerging reality and a global challenge. *Journal of Infectious Diseases*, *216*(Suppl 3), S431–S435. https://doi.org/10.1093/infdis/jix179
50. Kovács, R., Nagy, F., Tóth, Z., Bozó, A., Balázs, B., and Majoros, L. (2019). Synergistic effect of nikkomycin Z with caspofungin and micafungin against Candida albicans and Candida parapsilosis biofilms. *Letters in Applied Microbiology*, *69*(4),

271–278. https://doi.org/https://doi.org/10.1111/lam.13204
51. Kumari, S., Kumar, M., Gaur, N. A., and Prasad, R. (2021). Multiple roles of ABC transporters in yeast. *Fungal Genetics and Biology*, *150*(February), 103550. https://doi.org/10.1016/j.fgb.2021.103550
52. Kumari, S., Kumar, M., Khandelwal, N. K., Kumari, P., Varma, M., Vishwakarma, P., … Gaur, N. A. (2018). ABC transportome inventory of human pathogenic yeast Candida glabrata: Phylogenetic and expression analysis. *PLOS ONE*, *13*(8), e0202993. Retrieved from https://doi.org/10.1371/journal.pone.0202993
53. Lamoth, F., Lockhart, S. R., Berkow, E. L., and Calandra, T. (2018). Changes in the epidemiological landscape of invasive candidiasis. *Journal of Antimicrobial Chemotherapy*, *73*, i4–i13. https://doi.org/10.1093/jac/dkx444
54. Leighton, J., and Schatz, G. (1995). An ABC transporter in the mitochondrial inner membrane is required for normal growth of yeast. *The EMBO Journal*, *14*(1), 188–195. https://doi.org/https://doi.org/10.1002/j.1460-2075.1995.tb06989.x
55. Li, X., Yang, F., Li, D., Zhou, M., Wang, X., Xu, Q., … Jiang, Y. (2015). Trisomy of chromosome R confers resistance to triazoles in Candida albicans. *Medical Mycology*, *53*(3), 302–309. https://doi.org/10.1093/mmy/myv002
56. Liu, S., Hou, Y., Liu, W., Lu, C., Wang, W., and Sun, S. (2015). Components of the Calcium-Calcineurin Signaling Pathway in Fungal Cells and Their Potential as Antifungal Targets. *Eukaryotic Cell*, *14*(4), 324–334. https://doi.org/10.1128/EC.00271-14
57. Liu, S., Yue, L., Gu, W., Li, X., Zhang, L., and Sun, S. (2016). Synergistic Effect of Fluconazole and Calcium Channel Blockers against Resistant Candida albicans. *PLOS ONE*, *11*(3), e0150859. Retrieved from https://doi.org/10.1371/journal.pone.0150859
58. Lu, M., Yan, H., Yu, C., Yuan, L., and Sun, S. (2020). Proton pump inhibitors act synergistically with fluconazole against resistant Candida albicans. *Scientific Reports*, *10*(1), 1–10. https://doi.org/10.1038/s41598-019-57174-4
59. Mansfield, B. E., Oltean, H. N., Oliver, B. G., Hoot, S. J., Leyde, S. E., Hedstrom, L., and White, T. C. (2010). Azole Drugs Are Imported By Facilitated Diffusion in Candida albicans and Other Pathogenic Fungi. *PLOS Pathogens*, *6*(9), e1001126. Retrieved from https://doi.org/10.1371/journal.ppat.1001126
60. Maurya, I. K., Thota, C. K., Verma, S. D., Sharma, J., Rawal, M. K., Ravikumar, B., … Prasad, R. (2013). Rationally Designed Transmembrane Peptide Mimics of the Multidrug Transporter Protein Cdr1 Act as Antagonists to Selectively Block Drug Efflux and Chemosensitize Azole-resistant Clinical Isolates of Candida albicans* [♦]. *Journal of Biological Chemistry*, *288*(23), 16775–16787. https://doi.org/10.1074/jbc.M113.467159
61. Murray author, P. R. (2018). *Basic medical microbiology*. Retrieved from http://www.vlebooks.com/vleweb/product/openreader?id=none&isbn=9780323478526
62. Nagi, M., Nakayama, H., Tanabe, K., Bard, M., Aoyama, T., Okano, M., … Miyazaki, Y. (2011). Transcription factors CgUPC2A and CgUPC2B regulate ergosterol biosynthetic genes in Candida glabrata. *Genes to Cells : Devoted to Molecular & Cellular Mechanisms*, *16*(1), 80–89. https://doi.org/10.1111/j.1365-2443.2010.01470.x
63. Nim, S., Baghel, P., Tran-Nguyen, V. K., Peres, B., Nguyen, K. A., Pietro, A. Di, … Boumendjel, A. (2018). Make azoles active again: Chalcones as potent reversal agents of transporters-mediated resistance in Candida albicans. *Future Medicinal Chemistry*, *10*(18), 2177–2186. https://doi.org/10.4155/fmc-2018-0081
64. Nobile, C. J., and Johnson, A. D. (2015). Candida albicans Biofilms and Human

Disease. *Annual Review of Microbiology*, *69*, 71–92. https://doi.org/10.1146/annurev-micro-091014-104330
65. Pasrija, R., Banerjee, D., and Prasad, R. (2007). Structure and Function Analysis of CaMdr1p, a Major Facilitator Superfamily Antifungal Efflux Transporter Protein of Candida albicans: Identification of Amino Acid Residues Critical for Drug/H⁺ Transport. *Eukaryotic Cell*, *6*(3), 443 LP – 453. https://doi.org/10.1128/EC.00315-06
66. Patil, A., and Majumdar, S. (2017). Echinocandins in antifungal pharmacotherapy. *Journal of Pharmacy and Pharmacology*, *69*(12), 1635–1660. https://doi.org/10.1111/jphp.12780
67. Perlin, D. S. (2007). Resistance to echinocandin-class antifungal drugs. *Drug Resistance Updates : Reviews and Commentaries in Antimicrobial and Anticancer Chemotherapy*, *10*(3), 121–130. https://doi.org/10.1016/j.drup.2007.04.002
68. Perlin, D. S. (2015). Mechanisms of echinocandin antifungal drug resistance. *Annals of the New York Academy of Sciences*, *1354*(1), 1–11. https://doi.org/10.1111/nyas.12831
69. Posteraro, B., Torelli, R., Vella, A., Leone, P. M., De Angelis, G., De Carolis, E., … Fantoni, M. (2020, September). Pan-Echinocandin-Resistant Candida glabrata Bloodstream Infection Complicating COVID-19: A Fatal Case Report. *Journal of Fungi (Basel, Switzerland)*, Vol. 6. https://doi.org/10.3390/jof6030163
70. Prasad, R. (2017). Candida albicans: Cellular and Molecular Biology. In *Candida albicans: Cellular and Molecular Biology: Second Edition*. https://doi.org/10.1007/978-3-319-50409-4
71. Prasad, R., Banerjee, A., Khandelwa, N. K., and Dhamgaye, S. (2015). The ABCs of Candida albicans multidrug transporter Cdr1. *Eukaryotic Cell*, *14*(12), 1154–1164. https://doi.org/10.1128/EC.00137-15
72. Prasad, R., Banerjee, A., and Shah, A. H. (2017). Resistance to antifungal therapies. *Essays in Biochemistry*, *61*(1), 157–166. https://doi.org/10.1042/EBC20160067
73. Prasad, R., Nair, R., and Banerjee, A. (2019). Multidrug transporters of Candida species in clinical azole resistance. *Fungal Genetics and Biology*, *132*(June), 103252. https://doi.org/10.1016/j.fgb.2019.103252
74. Pristov, K. E., and Ghannoum, M. A. (2019). Resistance of Candida to azoles and echinocandins worldwide. *Clinical Microbiology and Infection*, *25*(7), 792–798. https://doi.org/10.1016/j.cmi.2019.03.028
75. Puri, Neeti, Krishnamurthy, S., Habib, S., Hasnain, S. E., Goswami, S. K., and Prasad, R. (1999). CDR1, a multidrug resistance gene from Candida albicans, contains multiple regulatory domains in its promoter and the distal AP-1 element mediates its induction by miconazole. *FEMS Microbiology Letters*, *180*(2), 213–219. https://doi.org/10.1111/j.1574-6968.1999.tb08798.x
76. Puri, Nidhi, Prakash, O., Manoharlal, R., Sharma, M., Ghosh, I., and Prasad, R. (2010). Analysis of physico-chemical properties of substrates of ABC and MFS multidrug transporters of pathogenic Candida albicans. *European Journal of Medicinal Chemistry*, *45*(11), 4813–4826. https://doi.org/https://doi.org/10.1016/j.ejmech.2010.07.050
77. Rawal, M. K., Khan, M. F., Kapoor, K., Goyal, N., Sen, S., Saxena, A. K., … Prasad, R. (2013). Insight into Pleiotropic Drug Resistance ATP-binding Cassette Pump Drug Transport through Mutagenesis of Cdr1p Transmembrane Domains * . *Journal of Biological Chemistry*, *288*(34), 24480–24493. https://doi.org/10.1074/jbc.M113.488353
78. Redhu, A. K., Banerjee, A., Shah, A. H., Moreno, A., Rawal, M. K., Nair, R., … Prasad, R. (2018). Molecular Basis of Substrate Polyspecificity of the Candida albicans

Mdr1p Multidrug/H+ Antiporter. *Journal of Molecular Biology*, *430*(5), 682–694. https://doi.org/https://doi.org/10.1016/j.jmb.2018.01.005
79. Revie, N. M., Iyer, K. R., Robbins, N., and Cowen, L. E. (2018). Antifungal drug resistance: evolution, mechanisms and impact. *Current Opinion in Microbiology*, *45*(Figure 2), 70–76. https://doi.org/10.1016/j.mib.2018.02.005
80. Ruben, S., Garbe, E., Mogavero, S., Albrecht-Eckardt, D., Hellwig, D., Häder, A., … Martin, R. (2020). AHR1 and TUP1 contribute to the transcriptional control of virulence-associated genes in candida albicans. *MBio*, *11*(2), 1–15. https://doi.org/10.1128/mBio.00206-20
81. Sanglard, D, Ischer, F., Monod, M., Dogra, S., Prasad, R., and Bille, J. (1999). Analysis of the ATP-binding cassette (ABC)-transporter gene CDR4 from Candida albicans. *ASM Conference on Candida and Candidiasis, Charleston, SC, USA, March*, 1–4.
82. Sanglard, D, Kuchler, K., Ischer, F., Pagani, J. L., Monod, M., and Bille, J. (1995). Mechanisms of resistance to azole antifungal agents in Candida albicans isolates from AIDS patients involve specific multidrug transporters. *Antimicrobial Agents and Chemotherapy*, *39*(11), 2378 LP – 2386. https://doi.org/10.1128/AAC.39.11.2378
83. Sanglard, Dominique, Ischer, F., Parkinson, T., Falconer, D., and Bille, J. (2003). Candida albicans mutations in the ergosterol biosynthetic pathway and resistance to several antifungal agents. *Antimicrobial Agents and Chemotherapy*, *47*(8), 2404–2412. https://doi.org/10.1128/aac.47.8.2404-2412.2003
84. Schillig, R., and Morschhäuser, J. (2013). Analysis of a fungus-specific transcription factor family, the Candida albicans zinc cluster proteins, by artificial activation. *Molecular Microbiology*, *89*(5), 1003–1017. https://doi.org/https://doi.org/10.1111/mmi.12327
85. Selmecki, A., Forche, A., and Berman, J. (2006). Aneuploidy and isochromosome formation in drug-resistant Candida albicans. *Science (New York, N.Y.)*, *313*(5785), 367–370. https://doi.org/10.1126/science.1128242
86. Shao, J., Zhang, M., Wang, T., Li, Y., and Wang, C. (2016). The roles of CDR1, CDR2, and MDR1 in kaempferol-induced suppression with fluconazole-resistant Candida albicans. *Pharmaceutical Biology*, *54*(6), 984–992. https://doi.org/10.3109/13880209.2015.1091483
87. Shigemura, K., Osawa, K., Jikimoto, T., Yoshida, H., Hayama, B., Ohji, G., … Arakawa, S. (2014). Comparison of the clinical risk factors between Candida albicans and Candida non-albicans species for bloodstream infection. *Journal of Antibiotics*, *67*(4), 311–314. https://doi.org/10.1038/ja.2013.141
88. Simitsopoulou, M., Peshkova, P., Tasina, E., Katragkou, A., Kyrpitzi, D., Velegraki, A., … Roilides, E. (2013). Species-specific and drug-specific differences in susceptibility of candida biofilms to echinocandins: Characterization of less common bloodstream isolates. *Antimicrobial Agents and Chemotherapy*, *57*(6), 2562–2570. https://doi.org/10.1128/AAC.02541-12
89. Singh, S. D., Robbins, N., Zaas, A. K., Schell, W. A., Perfect, J. R., and Cowen, L. E. (2009). Hsp90 Governs Echinocandin Resistance in the Pathogenic Yeast Candida albicans via Calcineurin. *PLOS Pathogens*, *5*(7), e1000532. Retrieved from https://doi.org/10.1371/journal.ppat.1000532
90. Smriti, Krishnamurthy, S., Dixit, B. L., Gupta, C. M., Milewski, S., and Prasad, R. (2002). ABC transporters Cdr1p, Cdr2p and Cdr3p of a human pathogen Candida albicans are general phospholipid translocators. *Yeast*, *19*(4), 303–318. https://doi.org/https://doi.org/10.1002/yea.818

91. Szczepaniak, J., Cieślik, W., Romanowicz, A., Musioł, R., and Krasowska, A. (2017). Blocking and dislocation of Candida albicans Cdr1p transporter by styrylquinolines. *International Journal of Antimicrobial Agents*, *50*(2), 171–176. https://doi.org/10.1016/j.ijantimicag.2017.01.044
92. Vaitkienė, S., Bekere, L., Duburs, G., and Daugelavičius, R. (2021). Interaction of styrylpyridinium compound with pathogenic candida albicans yeasts and human embryonic kidney hek-293 cells. *Microorganisms*, *9*(1), 1–11. https://doi.org/10.3390/microorganisms9010048
93. Vaitkienė, S., Daugelavičius, R., Sychrová, H., and Kodedová, M. (2020). Styrylpyridinium Derivatives as New Potent Antifungal Drugs and Fluorescence Probes. *Frontiers in Microbiology*, *11*(August), 1–14. https://doi.org/10.3389/fmicb.2020.02077
94. Vaitkienė, S., Kuliešienė, N., Sakalauskaitė, S., Bekere, L., Krasnova, L., Vigante, B., … Daugelavičius, R. (2020). Antifungal activity of styrylpyridinium compounds against Candida albicans. *Chemical Biology and Drug Design*, (June), 1–13. https://doi.org/10.1111/cbdd.13777
95. Vermes, A., Guchelaar, H. J., and Dankert, J. (2000). Flucytosine: A review of its pharmacology, clinical indications, pharmacokinetics, toxicity and drug interactions. *Journal of Antimicrobial Chemotherapy*, *46*(2), 171–179. https://doi.org/10.1093/jac/46.2.171
96. Walker, L. A., Gow, N. A. R., and Munro, C. A. (2013). Elevated Chitin Content Reduces the Susceptibility of Candida Species to Caspofungin. *Antimicrobial Agents and Chemotherapy*, *57*(1), 146 LP – 154. https://doi.org/10.1128/AAC.01486-12
97. Wasi, M., Khandelwal, N. K., Moorhouse, A. J., Nair, R., Vishwakarma, P., Bravo Ruiz, G., … Prasad, R. (2019). ABC Transporter Genes Show Upregulated Expression in Drug-Resistant Clinical Isolates of Candida auris: A Genome-Wide Characterization of ATP-Binding Cassette (ABC) Transporter Genes. *Frontiers in Microbiology*, *10*, 1445. https://doi.org/10.3389/fmicb.2019.01445
98. Wilson, D. T., Dimondi, V. P., Johnson, S. W., Jones, T. M., and Drew, R. H. (2016). Role of isavuconazole in the treatment of invasive fungal infections. *Therapeutics and Clinical Risk Management*, *12*, 1197–1206. https://doi.org/10.2147/TCRM.S90335
99. Xiang, M.-J., Liu, J.-Y., Ni, P.-H., Wang, S., Shi, C., Wei, B., … Ge, H.-L. (2013). Erg11 mutations associated with azole resistance in clinical isolates of Candida albicans. *FEMS Yeast Research*, *13*(4), 386–393. https://doi.org/10.1111/1567-1364.12042
100. Xie, J. L., Qin, L., Miao, Z., Grys, B. T., Diaz, J. D. L. C., Ting, K., … Cowen, L. E. (2017). The Candida albicans transcription factor Cas5 couples stress responses, drug resistance and cell cycle regulation. *Nature Communications*, *8*(1), 499. https://doi.org/10.1038/s41467-017-00547-y
101. Yan, L., Li, M., Cao, Y., Gao, P., Cao, Y., Wang, Y., and Jiang, Y. (2009). The alternative oxidase of Candida albicans causes reduced fluconazole susceptibility. *Journal of Antimicrobial Chemotherapy*, *64*(4), 764–773. https://doi.org/10.1093/jac/dkp273
102. Yoo, J. Il, Choi, C. W., Lee, K. M., and Lee, Y. S. (2010). Gene Expression and Identification Related to Fluconazole Resistance of Candida glabrata Strains. *Osong Public Health and Research Perspectives*, *1*(1), 36–41.

https://doi.org/10.1016/j.phrp.2010.12.009
103. Zavrel, M., Hoot, S. J., and White, T. C. (2013). Comparison of Sterol Import under Aerobic and Anaerobic Conditions in Three Fungal Species, Candida albicans, <span class="named-content genus-sp. *Eukaryotic Cell*, *12*(5), 725 LP – 738. https://doi.org/10.1128/EC.00345-12
104. Zhu, J., Krom, B. P., Sanglard, D., Intapa, C., Dawson, C. C., Peters, B. M., … Jabra-Rizk, M. A. (2011). Farnesol-Induced Apoptosis in Candida albicans Is Mediated by Cdr1-p Extrusion and Depletion of Intracellular Glutathione. *PLOS ONE*, *6*(12), e28830. Retrieved from https://doi.org/10.1371/journal.pone.0028830