Review

Insight into the Mechanisms and Clinical Relevance of Antifungal Heteroresistance

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Review

Insight into the Mechanisms and Clinical Relevance of Antifungal Heteroresistance

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Abstract: Antifungal resistance poses a critical global health threat, particularly in immuno-compromised patients. Beyond the traditional resistance mechanisms rooted in heritable and stable mutations, a distinct phenomenon known as heteroresistance has been concerned, wherein a minority of resistant fungal cells coexist within a predominantly susceptible population. It may be induced by pharmacological factors or non-pharmacological agents. The reversible nature of it presents significant clinical challenges, as it can lead to undetected resistance during standard susceptibility testing. As heteroresistance allows fungal pathogens to survive antifungal treatment, this adaptive strategy often leads to treatment failure and infection recurrence. Though extensively studied in bacteria, limited research has explored its occurrence in fungi. This review synthesizes the current findings on antifungal heteroresistance mechanisms, highlighting the clinical implications of fungal heteroresistance and the pressing need for deeper mechanism insights. We aim to combine the latest research advances in the field of antifungal heteroresistance, summarizing in detail the characteristics, inducing factors, molecular mechanisms, and clinical significance of it, and describing the similarities and differences between heteroresistance, tolerance and persistence. Further research is essential to elucidate this phenomenon and develop more effective antifungal therapies to combat fungal infections.

Keywords: antifungals; drug resistance; heteroresistance; aneuploidy; copy number variations (CNVs)

1. Introduction

Fungal infections pose a significant and growing threat to public health worldwide, particularly in immunocompromised patients [1,2]. The increasing prevalence of antifungal-resistant pathogens is complicating treatment, making fungal infection a major global health challenge [3]. Traditionally, antifungal resistance has been defined as the result of inherent and stable genetic mutations that

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diminish drug efficacy, with elevated MIC (Minimum Inhibitory Concentration). However, recent researches have uncovered a more complex and dynamic form of resistance: heteroresistance. Fungal heteroresistance, in which a small subpopulation of a fungal strain exhibits resistance to antifungal drugs while the majority remain susceptible, represents a significant global threat. This phenomenon complicates treatment as it can lead to treatment failure despite the apparent efficacy of the drug against most of the fungal cells, further exacerbating the public health crisis caused by fungal pathogens.

Heteroresistance was first discovered in 1947 in *Haemophilus influenzae* [4]. In bacteriology, it refers to the presence of a heterogeneous population with one or several subpopulations with increased levels of antibiotic resistance compared with the major population. This can be due to clonal heterogeneity during the co-infection of colonies with different levels of resistance, or microevolution of a single colony [5,6]. The heteroresistance phenotype can either be stable that do not rapidly revert to susceptible phenotype, or unstable that may affect the laboratory test results [7]. Under persistent selective pressure, it may even evolve into heritable ones, causing higher rate of treatment failure and create an antibiotic resistance reservoir [8]. As for mechanisms, they vary among different pathogens and various kinds of drugs. According to a research on Pseudomonas aeruginosa, heteroresistance to imipenem is due to biofilm formation and *OprD* gene mutation [9], while heteroresistance to levofloxacin is linked to elevated expression of genes involved in DNA replication and repair [10]. Another research on H. influenzae showed that imipenem heteroresistance was linked to alteration of *PBP3* (penicillin-binding protein 3), slowed drug influx and efflux alterations [11]. It can also be induced by antibiotic exposure, through upregulation of stress-related pathways, etc. [12,13].

While extensively studied in bacteria, research on fungal heteroresistance remains comparatively limited. From a clinical perspective, heteroresistance has been implicated in treatment failure in murine models [14], and relative mechanisms have not been studied in sufficient depth. However, the clinical impact of heteroresistance leading to drug resistance during treatment should not be underestimated, as it may lead to prophylaxis failure, recurrent infection or relapse [15–19]. In this review, we synthesize current research to outline the characteristics, mechanisms, and clinical prevalence of heteroresistance in fungal pathogens, with the goal of providing a foundation for future investigations.

2. Antifungal Drugs

Fungi are an important class of human pathogenic pathogens with immune escape, intra-host environmental adaptability and multiple virulence mechanisms, posing a great threat to human health [20–40]. Currently, there are four main classes of drugs approved for antifungal infection treatment: azoles, echinocandins, polyenes (Amphotericin B, AmB), and 5-fluorocytosine (5-FC). Azoles act on fungal 14-α-demethylases and inhibit ergosterol biosynthesis, leading to alterations in the permeability and metabolic state of fungal cells, which may result in growth inhibition or cell death [41,42]. Echinocandins target and inhibit the *FKS* gene which encodes β-1,3-glucan synthase, thereby inhibit β-1,3-glucan production and impairing fungal cells from maintaining their shape and resisting external stresses [43]. AmB targets membrane ergosterol, leading to pore formation, altered permeability, and reactive oxygen species (ROS) accumulation, all of which led to eventual fungal cell death [44–46]. 5-FC is a prodrug that is imported into cells by cytosine permease, which is encoded by the *FCY2* gene. Once inside the cell, 5-FC is converted to 5-fluorouracil (5-FU) through the action of cytosine deaminase and uracil phosphoribosyl transferase, both encoded by the *FCY1* gene, inhibiting DNA and RNA biosynthesis [47,48]. However, in recent years, there has been a concerning surge in the isolation of antifungal resistant strains, raising a critical alarm for public health.

Mechanisms associated with azole resistance mainly include mutations in targets, efflux pumps and biofilm formation etc. [49–51]. In *Candida* spp., up-regulation of the efflux transporter genes *CDR1, TAC1B* and *MDR1* and point mutations in the target coding genes *ERG11* and *ERG3*, such as Y132F and R398I in the *ERG11* gene, have been associated with fluconazole resistance [49,51–54]. In

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C. neoformans, a point mutation at the serine-substituted glycine 484S site of the 14-α-demethylase leads to single-drug resistance to fluconazole [55]. In *A. fumigatus*, mutations in the *cyp51A* gene encoding 14-α-demethylase and mutations in the ABC transporter gene *atrF* are common azole resistance mechanisms [56].

Currently, resistance to echinocandins is mainly associated with mutations in the *FKS1*/*FKS2* genes [49,57,58]. Other molecular mechanisms associated with echinocandin resistance have also been reported. Yu et al. found that the *ADA2* gene of *C. glabrata* is associated with resistance to three classes of antifungals, as evidenced by the Δ*ada2* knockout strains' significant downregulation of their MICs [59]. Singh et al. found that deletion of seven genes, including *MOH1, GPH1, CDC6, TCB1/2, DOT6, MRPL11* and *SUI2*, showed increased levels of resistance to echinocandins; but considering their small effect on caspofungin resistance or conjured occurrence with *FKS2* mutations, it is more likely that they create a genetic background in which the *FKS2* mutations are adaptive and less harmful, rather than a direct cause of resistance [60].

5-FC resistance is highly correlated with its pharmacological mechanism, i.e., mutations in any one or more of the key enzyme genes of the pyrimidine salvage pathway, which may involve mutations in the genes encoding the cytosine permease *FCY2*, purine *FCY1* encoding the cytosine deaminase, *FUR1* encoding the uracil phosphoribosyl transferase, and *ADE17* [49,61]. However, other mechanisms have also been reported: Kannan et al. found that V668G substitution of a putative transcriptional activator (*MRR1*) led to the up-regulation of *MFS7*, a multidrug transporter protein that mediated azole-5-FC cross-resistance in *C. lusitaniae* [62], and Billmyre et al. found that deletion of the mismatch repair gene, *MSH2*, in *C. deuterogattii* also led to an elevated rate of 5-FC mutations [63].

Fungal resistance to AmB is less common but has been reported in strains such as *C. auris* and the *C. haemulonii* complex [49,64]. Resistance to AmB in both species involves a variety of mechanisms, including altered cell membrane composition, altered cellular metabolic state, altered iron homeostasis, and altered ROS metabolism [44,45,64,65]. The molecular mechanisms of resistance to AmB in *C. haemulonii* have been systematically discussed in the author's previous review [66] .

3. Heteroresistance, Tolerance and Persistence of Antifungal Drugs

At the level of cell populations, not all survival under fungicidal concentrations of antifungals can be attributed to elevated MICs. This is when heteroresistance, tolerance and persistence come into play, while they are often confused conceptually. Here we describe the differences between the three from the perspective of the performance of cell populations under drug stress (Figure 1).

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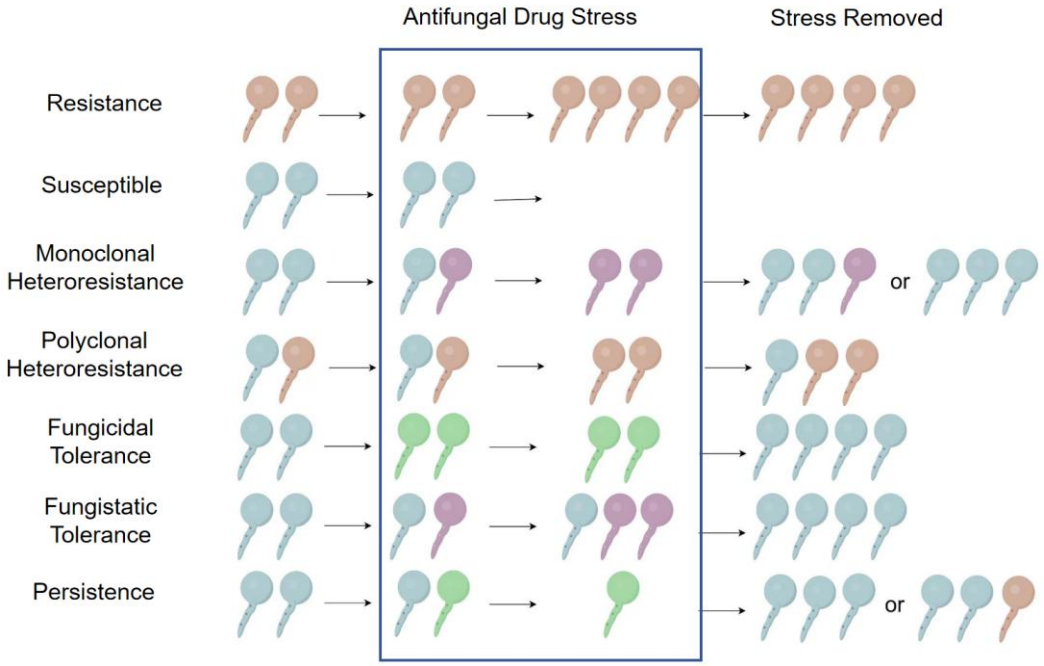

Figure 1. (By Figdraw). Explanation of resistance, susceptible, tolerance, persistence and heteroresistance from a cell population perspective. The state of cell proliferation is represented by the number of cells in the figure. Assorted colors indicate different genotypes and phenotypes, which are orange (genetically stable resistance), blue (susceptible), purple (genetically unstable or phenotypically resistance), green (phenotypically tolerant).

Heteroresistance , a concept first introduced in bacteria, is a form in which a minority subpopulation of resistant phenotype cells with higher MIC, sometimes as rare as one in one million cells, coexists with a majority population of susceptible cells with lower MIC [4]. It may be polyclonal or monoclonal. Polyclonal heteroresistance is related to heterogeneous consortia with genetically distinct subpopulations or to the appearance of rare resistant mutants whose frequency increases with antibiotic exposure. In turn, heteroresistance is monoclonal if present in pure clones [7]. As a variant, it is more of continuous than binary. A study on the nature of heteroresistance properties showed that incremental effects of multiple binary genetic switches, including *CDR1, PDH1, PDR1* and *SNQ1*, etc. produced a spectrum of heteroresistance states rather than binary, in *C. glabrata* [67]. From the perspective of population evolution, it can be seen as a strategy that helps the fungal population survive diverse environments [68]. In vitro, heteroresistance can be obtained through antifungal induction or non-antifungal agents and artificial selection, the latter of which may get the longer-lasting phenotype [69,70]. And repeated culture in stress-free environments can abolish the heteroresistance [71]. But interestingly, this majority subpopulation can never be purified, which means there's always a minority that is phenotypically resistant [72].

Tolerance is defined as an extension of the killing time, characterized by an increase in MDK_{99} (Minimum Duration of Killing 99%) time [73]. It refers to the whole population that can survive a transient exposure to antifungals at concentrations that would otherwise be lethal, without increasing antifungal MIC [74]. And some (5%-90%) can slowly proliferate [75]. This definition applies to fungicides such as echinocandins and polyenes. However, for antifungals rather than fungicidal drugs (e.g., azoles), tolerance has a different definition, i.e., the ability of a tolerant strain to overcome growth inhibition faster than a sensitive strain at drug concentrations above the MIC [76]. It can be measured through broth microdilution assay or disk diffusion assay [77]. And it is a reversible portrait, as confirmed in several fungi species or antifungals [78,79].

Persistence and persister cells have been well-studied in bacteriology [80]. For fungus, persistence is a subpopulation of genetically susceptible fungal cells that survives fungicidal concentrations of antifungals and may lead to the emergence of genetically resistant isolates [81–83]. This phenomenon has been observed in intra-macrophage *C. glabrata* cells, in which a subpopulation

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survives and gradually evolves heritable resistance mutations in *FKS* regions [81]. Sometimes "persistence" is used interchangeably with "tolerance," as the MIC values of the fungal cell population do not change in either phenotype. Most notably, persistence is characterized by a biphasic killing curve, whereas tolerance is not [80]. In other words, "tolerance" means that all survive and some may proliferate, whereas "persistence" means that some survive but no one proliferates.

4. Mechanisms of Antifungal Tolerance

Fungal tolerance to antifungals arises from a variety of molecular mechanisms. For example, azole tolerance in *Candida* populations is often characterized by the ability to survive and proliferate slowly at concentrations above the minimum inhibitory concentration (MIC). This tolerance is typically temperature-independent and frequently associated with aneuploidy, which may be lost upon temperature changes or ploidy recovery [84,85]. Similarly, in *C. neoformans*, brain glucose has been shown to induce AmB tolerance without affecting the MIC. This process is mediated by the zinc-finger transcription factor Mig1 and involves more complex mechanisms, such as the inhibition of ergosterol synthesis and the promotion of competing compounds that contribute to antifungal tolerance [86]. And in *C. neoformans*, mitochondria metabolism alteration caused by cell aging drive increased ergosterol synthesis and ABC transporter upregulation and led to azole tolerance [87].

Alterations in cellular structure and metabolic levels also play a key role in antifungal tolerance. Cell wall remodeling contributes echinocandin tolerance, with changes in β-glucan synthesis or increased production of compensatory components, like chitin, strengthening the cell wall and reducing the drug's effectiveness [88,89]. Additionally, activation of cellular stress response pathways, such as the heat shock protein (Hsp) and calcineurin pathways, has been shown to contribute to echinocandin tolerance in C. albicans [90,91]. Moreover, a single-cell transcriptomics study demonstrated that the ribosome assembly stress response supports survival under fluconazole exposure above the MIC, further highlighting the importance of stress responses in antifungal tolerance [92]. Together, these mechanisms illustrate how fungi adapt to antifungal treatments through a combination of genetic, transcriptional, and physiological changes, making effective treatment more challenging.

5. Mechanisms of Antifungal Persistence

Persistence is always linked to cell dormancy, in a state which the cellular metabolism is temporarily ceased [82,93,94]. As the fungal pathogen enters the host organism, it is often phagocytosed by immune cells, like macrophages, etc. Studies on pathogen-phagocyte interactions have revealed the inducing effect of the phagocytic intracellular environment on the formation of persisters through comparing the metabolism of planktonic cells and persisters and exerting ROS or pH-related environmental stress, which also suggest a possible role for cellular stress response in persistence [81]. Ke et al. constructed a mouse model with pulmonary infection and demonstrated that AmB-tolerant persisters are enriched in *C. neoformans* cells with high levels of the stationary-phase protein Sps1 and the metabolic marker ergothioneine [83]. This also provides us with the critical role of the *EGT* gene, which encodes the ergothioneine, in regulating the AmB susceptibility [83].

There are also several key factors in the formation of persisters. The formation of persister cells does not require biofilm, but biofilm-containing persister populations are more tolerant to oxidative stress and better resist the fungicidal effects of AmB [95]. And inhibiting biofilm aids in the eradication of persister cells of *C. tropicalis* [96]. Also, as antifungals can cause an increased level of ROS, persister cells may possess stronger antioxidant capacity by upregulating enzymes like superoxide dismutases (SODs) or alkyl hydroperoxide reductase 1, etc. [97]. Such mechanisms can be can be confirmed inversely by SOD inhibition leading to down-regulation of persistence levels

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[98]. Formation of persister cells should be emphasized, as they can cause chronic or recurrent infections, complicating treatment.

6. Mechanisms of Antifungal Heteroresistance

The molecular mechanisms underlying fungal heteroresistance are diverse and involve a complex interplay of genetic, transcriptional, and physiological factors. However, they do not always include heritable changes. In many cases, heteroresistance arises through transient changes that allow the fungus to survive antifungal stress without permanent genetic changes. This adaptability complicates treatment strategies, as the resistant phenotype may not be stable and can fluctuate depending on environmental conditions.

5.1. Aneuploidy and Copy Number Variations (CNVs)

Aneuploidy is a common mechanism in heritable resistance and the most widely studied mechanism of heteroresistance [99,100]. It is the presence of an abnormal number of chromosomes, playing a crucial role in fungal survival and evolution by providing rapid adaptability in response to environmental stress, such as antifungal drug exposure [101,102]. Yang et al. found that exposure to fluconazole at subinhibitory concentrations for a short period of time (48 h) may cause *C. neoformans* to acquire different aneuploid chromosomes and confer heteroresistance to fluconazole and cross-resistance to 5-FC [103]. Thus, exposure to one class of antifungal can promote adaptation to similar or even more potent antifungal agents, highlighting the plasticity of the fungal genome and raising serious public health concerns. By increasing the copy number of chromosomes containing drug resistance genes, such as those encoding efflux pumps or involved in ergosterol biosynthesis, aneuploidy leads to resistance against multiple antifungals like azoles and echinocandins [67,104,105]. Also, non-antifungal agents can also induce heteroresistance. Zhang et al. found that the endoplasmic reticulum stress chemo inducer Brefeldin A (BFA) led to aneuploidy in *C. neoformans*: disomy in chromosome 1 led to cross-resistance to two classes of antifungal drugs, fluconazole and fluconazole and 5-FC and hypersensitivity to AmB [106]. By altering gene dosage or expression levels, aneuploidy allows fungi to quickly adjust to adverse conditions without relying on permanent genetic mutations, promoting survival in fluctuating environments [67,102,104,105,107]. Importantly, it is reversible, offering a dynamic mechanism for fungi to balance adaptation and stability, population and individuals [101,108]. As a result, aneuploidy plays a pivotal role in both fungal adaptability and the evolution of drug resistance, making it a key factor in heteroresistance and its clinical implications.

6.1.1. *C. albicans*

Mechanisms related to azole heteroresistance in *C. albicans* are diverse. Under fluconazole exposure, *C. albicans* rapidly evolve CNVs (Copy Number Variation) and aneuploidy in vitro [109]. Associated resistance mainly related to chr5, which has *ERG11* and *TAC1* gene [104,110]. *TAC1* encodes a transcription regulator of ABC transporter genes on chr3 [111]. Loss of chr5 can result in enhanced susceptibility to azoles, the mechanisms of which has been clarified [110]. However, loss of chr5 can also lead to the enhanced susceptibility to AmB and increased resistance to 5-FC, the latter of which is due to the location of negative regulator (s) of anti 5-fluorocytosin on chr5 and the former of which needs to be further clarified [110]. Harrison et al. reported the appearance of “trimeras,” three connected cells composed of a mother, daughter, and granddaughter bud, after exposure to fluconazole [112]. The same morphology cannot be found in genetically resistant strains, suggests the potential role of trimeras in heteroresistance. Also, these trimeras produce genetically progeny with different chromosomes, increasing chances of developing heteroresistance [112].

C. albicans’ heteroresistance to echinocandins is related to chr2, as chr2 trisomy can be induced after exposure to caspofungin and exhibits higher echinocandin resistance, according to Yang et al. [113]. It is also related to chr5, as chr5 aneuploidy after caspofungin exposure can obtain cross-

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resistance to caspofungin, micafungin and anidulafungin [114]. Yang et al. found three negative regulators of echinocandin susceptibility on chr5, including *CHT2* encoding a glycosylphosphatidylinositol (GPI)-dependent chitinase which is a covalently bound cell wall protein, *PGA4* encoding a GPI-anchored cell surface 1,3-β-d-glucanosyltransferase, and *CSU51* encoding another putative GPI-anchored protein, and two positive regulators, *CNB1* encoding a regulatory subunit of calcineurin B and *MID1* encoding a putative stretch-activated Ca^{2+} channel of the high-affinity calcium uptake system [88]. However, the exact gene copies and whether they are cumulative remain unclear, making it hard to simply summarize their specific roles in echinocandin resistance [88].

6.1.2. *C. glabrata*

C. glabrata has been considered a haploid and an asexual organism for decades, but recent years witnessed studies reporting the instability of clinical isolates genome, mainly due to the frequent change in ploidy forms [115]. Ploidy variation could not only promote the rapid adaptation of *C. glabrata* to the changing environment but also benefit the evolution of new traits, like heteroresistance. Ksiezopolska et al. found chrE aneuploidy contributes to heteroresistance after exposing clinical isolates to anidulafungin [116]. And heteroresistance obtained from environmental stress may remain even after the stress [116]. And when exposed to azoles, *C. glabrata* can also form "trimeras" that may be related to heteroresistance, but the exact mechanisms need further investigation [112].

6.1.3. *C. parapsilosis*

In *C. parapsilosis*, multi-center research proved that heteroresistance facilitates breakthrough infections in immunocompromised patients and may cause the prophylaxis failure [19]. However, unlike *C. neoformans* or itself when exposed to tunicamycin, this research couldn't find a significant relationship between aneuploidy and heteroresistance in *C. parapsilosis* [19,103]. Evidence of aneuploidy in the heteroresistance are put forward by Harrison et al., elaborating *C. parapsilosis* can also form "trimeras" when exposed to azoles, but the exact mechanisms need further investigation [112].

6.1.4. *C. auris*

For highly resistant pathogen *C. auris*, Zhai et al. reported heteroresistance of *C. auris* towards echinocandins, which is the first-line treatment for *C. auris* infection [19]. In vitro evolution under fluconazole exposure is relatively slow, compared to *C. albicans*, and mainly composed of SNP, with a minority of aneuploidy [109,117]. But due to its haploid genome, SNPs may have immediate phenotypic impact [118].

6.1.5. *C. neoformans* and *C. gattii*

For *Cryptococcus* spp., heteroresistance means a subpopulation appears under antifungal exposure and retain the potential to grow under continuous drug stress [77]. Because of the broad definition, many studies have reported "tolerance" as cryptococcal azole heteroresistance and vice versa [119]. It is a crucial factor in the treatment failure of cryptococcosis. For *C. neoformans* and *C. gattii*, the heteroresistance to fluconazole is intrinsic and can be induced to increase [72,120]. According to a comparative study, *C. gattii* showed a higher heteroresistance level to fluconazole than *C. neoformans* [121]. For *C. neoformans*, heteroresistance can be reduced or inhibited by several environmental factors, including temperature, media type, growth phase, and the age of cells [122]. One of its mechanisms is associated with the multiple types of aneuploid daughter cells produced by titan cells [123–125]. Stone et al. carried out a genomic analysis of clinical *C. neoformans* strains and found a high rate of aneuploidy in heteroresistant colonies and recurrent isolates, with a predominance of chr1 disomy [126]. Strains with chr1 disomy can also be isolated from mice brain during treatment with fluconazole [127]. Ngamskulrungroj et al. demonstrated the high incidence of

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chr4 disomy, which may be related to *SEY1* (GTPase with a role in Endoplasmic Reticulum morphology), *GCS2* (ADP-ribosylation factor GTPase activating proteins) or *GLO3* (ADP-ribosylation factor GTPase activating proteins) genes on it [128]. And if any of the *ERG11, SEY1, GCS2* or *GLO3* genes are relocated to chr3, then the frequency of chr3 disomy alternatively increases [128,129]. This is due to the presence on chr1 of *ERG11*, which is the drug target of fluconazole, and AFR1, which encodes the drug efflux pump. But *AFR1* gene does not directly lead to heteroresistance, though [72]. Another research on *C. neoformans*' ploidy found that exposure to inhibitory concentrations of fluconazole leads to diminished budding and subsequent growth while permitting nuclear events, resulting in populations with an increase in DNA content grow better in the presence of fluconazole, through which aneuploidy rates increase and the fitter survive [130]. Specific mechanisms of *C. neoformans* heteroresistance need to be explored, and their development may help to optimize clinical therapies for the effective elimination of drug-resistant *Cryptococcus* subpopulations.

6.2. Alterations in Gene Expression

Efflux pumps, such as those belonging to the ABC transporter and major facilitator superfamily (*MFS*), play a crucial role in antifungal resistance by actively expelling drugs from the fungal cell. In heteroresistant subpopulations, the overexpression of efflux pumps can confer a temporary survival advantage. Stone et al. carried out a genomic analysis of clinical *C. neoformans* strains and found a high rate of aneuploidy in heteroresistant colonies and recurrent isolates, with a predominance of chr1 disomy and upregulated activity of efflux pumps [126]. Marr et al. carried out molecular researches on one *C. albicans* strain and demonstrated the induction effect of fluconazole on heteroresistance, marked with elevated mRNA level of ABC superfamily *CDR* genes [107]. As mRNA level can represent the expression of one or more genes, this coincides with the continuous characteristic of *C. glabrata* [67], suggesting that heteroresistance in *C. albicans* may also be a continuous variable. However, further research is needed.

6.3. Environmental Stress Induction

In addition to specific antifungals that can induce aneuploidy, non-pharmacological stressors can also have an impact on antifungal heteroresistance and even cross-heteroresistance. Although resistance phenotype is not stable due to the intrinsic instability of aneuploidy, such studies still suggest the vital role of external inducing factors and genomic instability in drug resistance in *C. neoformans*. "Titanization" in *C. neoformans* also plays a role in formation of aneuploidy. Gerstein et al. found that during cryptococcosis treatment, newly emerged fungal cells -- polyploid titan cells -- produced daughter cells that were more resistant to fluconazole and thus adapted to the host environment, and that a single titan mother cell was able to produce multiple types of aneuploid daughter cells, which contributes to the survival rate of the progeny under different environmental stresses [124]. This process is associated with intracellular ROS accumulation and mitochondrial responses [125]. For *C. neoformans*, heteroresistance can be reduced or inhibited by several environmental factors, including temperature, media type, growth phase, and the age of cells [122]. And from these can we speculate that stress response pathways may play a role in the generation of antifungal heteroresistance. Bosch et al. demonstrated that environmental stress, such as nitrogen limitation commonly encountered in the natural habitat of the fungus, increases the resistance of *C. neoformans* to AmB and fluconazole, and increases the frequency of heterogeneous resistance to fluconazole [70]. For *A. fumigatus*, clinically, long-term itraconazole treatment may cause decreased susceptibility. And progressive itraconazole exposure in labs can reproduce such phenomenon, independent of the mutation in *cyp51A* gene [131]. This is called secondary resistance, suggesting the existence of heteroresistance. However, exact mechanisms need to be further explored.

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Table 1. Heteroresistance Mechanisms in Common Fungal Pathogens.

Types	Species	Antifungals	Mechanisms	Related Components	References
Aneuploidy and CNVs	*C. albicans*	Fluconazole	Chr5 disomy	*ERG11* and *TAC1*	[104,110]
	C. albicans	5-Flucytosine	Loss of chr5, due to the location of negative regulator (s) of anti 5-FC	None.	[110]
	C. albicans	Fluconazole	"Trimeras," three connected cells composed of a mother, daughter, and granddaughter bud	None	[112]
	C. albicans	Echinocandins	Chr2 trisomy	*RNR1, RNR21*	[113]
	C. albicans	Echinocandins (caspofungin, micafungin and anidulafungin)	Chr5 aneuploidy after caspofungin exposure can obtain cross-resistance	Three negative regulators *CHT2, PGA4* and *CSU51*, and two positive regulators, *CNB1* and *MID1*.	[88,114]
	C. glabrata	Echinocandins (anidulafungin)	ChrE aneuploidy contributes to heteroresistance after exposing clinical isolates to anidulafungin.	None	[116]
	C. glabrata	Azoles	Incremental effects of these multiple binary genetic switches	*CDR1, PDH1, PDR1* and *SNQ1*	[67]
	C. glabrata	Azoles	Formation of "trimeras"	None	[112]
	C. parapsilosis	Azoles	Formation of "trimeras"	None	[112]
	C. auris	Azoles (fluconazole)	Genome changes mainly composed of SNP, with a minority of aneuploidy. But due to its haploid genome, SNPs may have immediate phenotypic impact.	SNPs	[109,117,118]
	C. neoformans	Cross-resistance to 5-FC and Fluconazole	Chr1 disomy	*ERG11, AFR1*	[106]
	C. neoformans	Fluconazole	Overexpression of *AFR1* on chr1 and *GEA2* on chr3	*AFR1, GEA2*	[106]
	C. neoformans	Azoles (fluconazole)	Titan cells that produce multiple types of aneuploid daughter cells	None	[123–125]
	C. neoformans	Azoles (fluconazole)	Chr1 disomy	*ERG11, AFR1*	[126]
	C. neoformans	Azoles	Chr4 disomy	*SEY1, GCS2, GLO3*	[128]

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	C. neoformans	(fluconazole) Azoles (fluconazole)	Chr3 disomy caused by gene relocation	*ERG11, SEY1, GCS2, GLO3*	[128,129]
Alterations in Gene Expression	*C. albicans*	Azoles	Elevation of mRNA	ATP Binding Cassette superfamily *CDR* genes	[107]
	C. neoformans	Azoles (fluconazole)	Up-regulated activity of efflux pumps	*AFR1*	[126]
Environmental Stress	*C. neoformans*	Polyene (AMB) and Azoles (fluconazole)	Nitrogen limitation	None	[70]
	C. neoformans	Azoles	Temperature, media type, growth phase, and the age of cells	None	[122]

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7. Clinical Relevance of Antifungal Heteroresistance

7.1. Outcomes of Antifungal Heteroresistance

Heteroresistance poses significant challenges in the clinical management of fungal infections. In bacteria, heteroresistance may proceed during antibiotic therapy, leading to changes in clinical tests and treatment failures and even create an antimicrobial resistance reservoir [8,132]. Similarly, the transient nature of antifungal heteroresistance allows pathogenic fungus to survive conventional antifungal treatment, even when most of the population is inhibited. Prior to the onset of infection, this can facilitate breakthrough infection or result in prophylaxis failure [19]; for diagnosed fungal infections, it may lead to persistent infections that require longer or more aggressive treatment regimens [15–18]. Despite initial susceptibility to the drug, heteroresistant subpopulations can expand during therapy, partially caused by antifungal exposure, characterized molecularly as emergence of resistance-related genetic mutations and clinically as relapse or progression of the infection [81,101–103].

Up to now, there are no approved first-line therapies for antifungal heteroresistance. However, a combination of drugs or dosage escalation may aid in alleviating treatment failure. One research on *C. neoformans* showed that combining 5-FC with fluconazole may effectively reduce heteroresistance and treatment failure, suggesting the importance of combined therapy [126]; another research suggested the feasibility of fluconazole dose escalation and combination therapy in the treatment of cryptococcal meningitis but also revealed limitations in combination options that only 5-FC was comprehensively available [133]. To make things worse, antifungal heteroresistance can also contribute to cross-resistance between different classes of antifungal drugs. For example, in *C. albicans* chr5 aneuploidy caused simply by caspofungin can result in cross-resistance to caspofungin, micafungin and anidulafungin [114], and in *C. neoformans*, chr1 disomy may confer cross-resistance to both azoles and 5-FC [106]. This cross-resistance makes treatment options even more restrictive. Even if there is a suitable combination therapy or escalation dose therapy, both can increase the risk of toxicity and adverse effects. And in-depth study of the mechanisms of heteroresistance will help to develop low-toxicity combination therapies or new drugs.

7.2. Diagnosis of Antifungal Heteroresistance

Conventional antifungal susceptibility testing methods, such as broth microdilution and disk diffusion assays, may not detect heteroresistant subpopulations [134]. These tests typically measure the MIC for the bulk population, potentially overlooking small subpopulations that can survive higher drug concentrations. As a result, infections caused by heteroresistant strains may be mistakenly classified as susceptible, leading to inappropriate therapy.

For heteroresistance, the population analysis profile (PAP) assay is recommended as the golden standard [135]. PAP has been proved to efficiently test the heteroresistance to azoles and echinocandins in *C. albicans, C. haemulonii, C. tropicalis, C. glabrata, C. parapsilosis, C. neoformans* and *S. cerevisiae*, etc. [18]. Single-cell assays can also be used in heteroresistance rest. These include techniques such as flow cytometry, single-cell RNA sequencing (scRNA-seq), and microfluidics-based assays, allowing for the analysis of gene expression, cell viability, and drug tolerance in individual fungal cells [136,137]. Though the protocol is lengthier, it may reveal a wide spectrum of adaptation mechanisms [18]. And Time-kill assays, conventionally used to monitor the survival of fungal cells over time in the presence of an antifungal drug, can be used to detect heteroresistance, too. By measuring the rate of cell death at various time points, this method can identify delayed growth or survival of heteroresistant subpopulations that might not be detected by static MIC assays, and even evaluate the kinetics of heteroresistance and assess whether heteroresistant cells eventually adapt to drug pressure, which makes it widely applicable clinically [138–140]. In *C. neoformans*, it has been used to study azole and AmB heteroresistance, revealing that subpopulations of cells can

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survive for prolonged periods despite the presence of high drug concentrations [70]. These make time-kill assays a valuable tool in understanding heteroresistance dynamics.

8. Conclusions

Heteroresistance is a reversible form of antifungal resistance that fluctuates under varying conditions, primarily driven by genome aneuploidy and changes in gene expression. Unlike bacterial resistance, fungal heteroresistance remains largely underexplored, with most research focusing on azole resistance. A unique characteristic of heteroresistance is its inducibility—resistant phenotypes can emerge in response to antifungal exposure or other factors, leading to reduced drug efficacy over time.

Molecularly, aneuploidy and CNVs play a crucial role, influencing mechanisms such as gene loss, gene amplification, modifications of antifungal-binding sites, and upregulation of efflux pumps. These genetic changes enhance fungal adaptability to antifungal pressure. One point worth exploring is that CNVs can be stably inherited and may occur in both resistance and heteroresistance, which means it may contribute greatly to the evolution of antifungal resistance. Understanding the evolutionary and molecular mechanisms of heteroresistance is crucial for predicting and controlling clinical resistance.

Antifungal heteroresistance poses a significant clinical challenge, as it can develop during treatment, leading to therapeutic failure and recurrent infections. While escalating drug dosages or using combination therapies may sometimes be effective, these approaches come with limited options and increased toxicity risks. Therefore, the development of novel antifungal agents is critical, offering more therapeutic choices and combination possibilities. Additionally, gaining deeper insights into the specific mechanisms of heteroresistance could inform the development of innovative treatment strategies targeting multiple pathways, ultimately improving patient outcomes and reducing recurrence.

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Abbreviations

The following abbreviations are used in this manuscript:

MIC	Minimum Inhibitory Concentration
CNVs	Copy Number Variations
AmB	Amphotericin B
5-FC	5-fluorocytosine
5-FU	5-fluorouracil
ROS	Reactive Oxygen Species
MDK_{99}	Minimum Duration of Killing 99%
SOD	Superoxide Dismutases
chr	Chromosome
GPI	Glycosylphosphatidylinositol

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PAP	Population Analysis Profile
scRNA-seq	Single-Cell RNA Sequencing
SNP	Single Nucleotide Polyphorism
ABC	ATP-Binding Cassette

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