Acta Biochim Biophys Sin 2023, 55(4): 540–547
https://doi.org/10.3724/abbs.2023011
Advance Access Publication Date: 22 February 2023
Review

Review

Combating increased antifungal drug resistance in *Cryptococcus*, what should we do in the future?

Hengyu Deng[1,†], Jialin Song[1,†], Yemei Huang[2], Chen Yang[3], Xuelei Zang[2], Yangyu Zhou[2], Hongli Li[1], Bin Dai[2], and Xinying Xue[1,2,*]

[1]Affiliated Hospital of Weifang Medical University, School of Clinical Medicine, Weifang Medical University, Weifang 261053, China, [2]Department of Respiratory and Critical Care, Beijing Shijitan Hospital, Capital Medical University; Peking University Ninth School of Clinical Medicine, Beijing 100089, China, and [3]Department of Laboratory Medicine, the First Medical Centre, Chinese PLA General Hospital, Beijing 100853, China

[†]These authors contributed equally to this work.

*Correspondence address. Tel: +86-10-63926750; E-mail: xuexinying2988@bjsjth.cn

Received 6 September 2022 Accepted 8 December 2022

Abstract

Few therapeutic drugs and increased drug resistance have aggravated the current treatment difficulties of *Cryptococcus* in recent years. To better understand the antifungal drug resistance mechanism and treatment strategy of cryptococcosis. In this review, by combining the fundamental features of *Cryptococcus* reproduction leading to changes in its genome, we review recent research into the mechanism of four current anti-cryptococcal agents, coupled with new therapeutic strategies and the application of advanced technologies WGS and CRISPR-Cas9 in this field, hoping to provide a broad idea for the future clinical therapy of cryptococcosis.

Key words *Cryptococcus*, anti-cryptococcal agent, drug resistance mechanism, drug target

Introduction

Cryptococcus spp. are basidiomycete yeasts. Among them, *Cryptococcus neoformans* (*C. neoformans*) and *Cryptococcus gatti* (*C. gatti*) are the main pathogenic types that can infect humans and cause cryptococcal pneumonia and cryptococcal meningitis [1]. According to a 2017 survey, there are approximately 180,000 deaths from cryptococcal meningitis annually worldwide [2]. The high mortality may be caused by the evolution of strain virulence or drug resistance. Then, how does *Cryptococcus* develop drug resistance? This may be due to a combination of the following pathways: altered drug targets or overexpression resulting in decreased intracellular drug accumulation or increased drug efflux; altered cell membrane or cell wall components; and activation of cellular stress response pathways [3,4]. Most of the above resistance mechanisms are associated with mutations in genetically related genes, resulting in genotypic changes. This is especially true in *Cryptococcus*, which has a bipolar mating system. The mating type locus (MAT) and two selectable alleles constitute two compatible mating types: MATα and MATa. It reproduces bisexually after α-a mating, and MATα cells can also reproduce unisexually [5]. Genomic rearrangement and altered ploidy resulted from asexual reproduction can lead to increased drug resistance in strains. However, compared to offspring produced by asexual reproduction, the offspring produced by sexual reproduction exhibit genotypic and phenotypic diversity and achieve rapid adaptability to environmental evolution [6].

In this review, we discussed the mechanisms of resistance to four commonly used drugs (azoles, polyenes, flucytosine, and echinocandins) and the strategies to cope with drug resistance, hoping to provide feasible therapeutic targets for the research and development of new drugs.

Drug-resistance Mechanisms of *Cryptococcus*

Mutations in several main genes found to be associated with resistance to these four classes of drugs are as follows: (i) *ERG11*, *AFR1*, *MSH2*, and *YAP1* for fluconazole (FLC) resistance; (ii) *ERG11* and *HOB1* for amphotericin B (AMB) resistance; (iii) *FCY1*, *FCY2*, *FUR1*, *UXS1*, *URA6*, *UGD1* and *NRG1* for 5-fluorocytosine (5-FC) resistance; and (iv) *FKS1* and *CDC50* for caspofungin resistance (Figure 1).

Azole resistance

Since the late 1960s, azole has become an important first-line drug

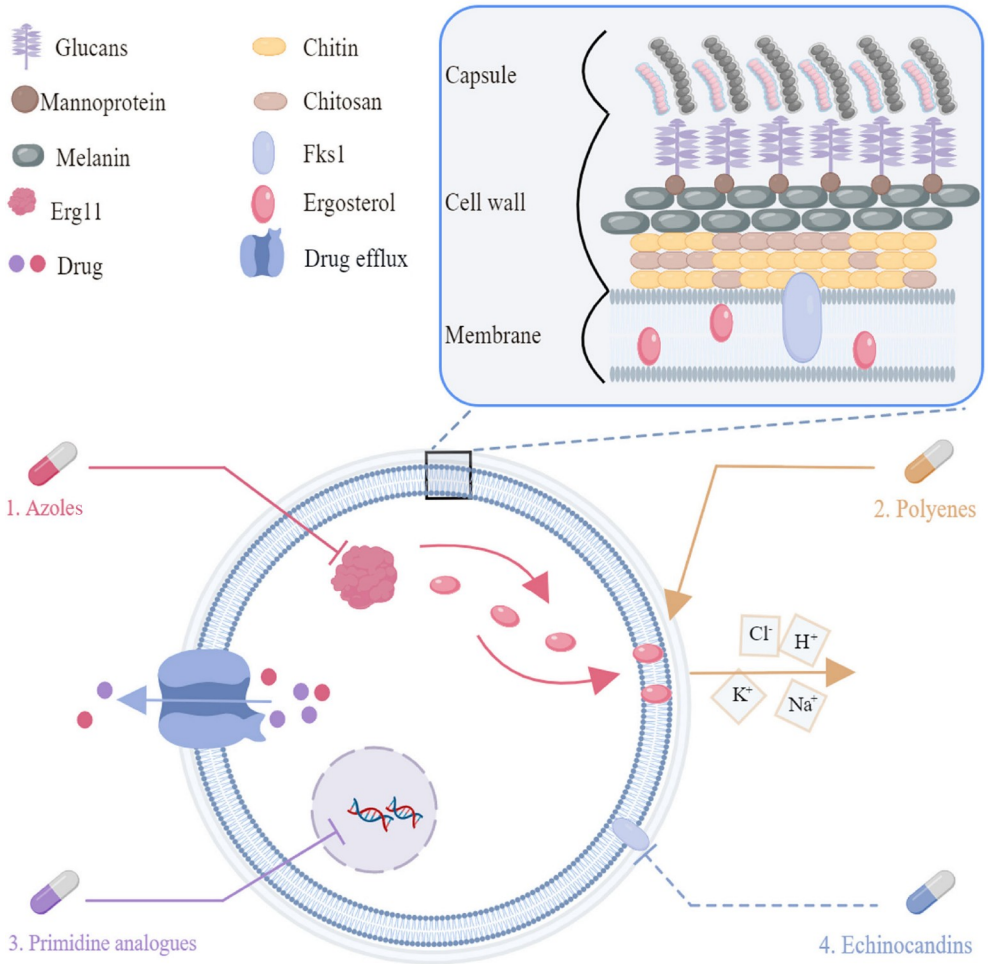

Figure 1. The mechanisms of drug resistance in Cryptococcus 1. Azoles inhibit lanosterol 14A-demethylase encoded by the *Erg11* gene, altering the normal sterol synthesis pathway. 2. Polyene drugs damage cell integrity by binding with ergosterol. 3. Pyrimidine analogs inhibit DNA and protein synthesis. 4. Echinocandins inhibit key enzymes for cell wall synthesis encoded by the *FKS1* gene and destroys cell integrity. The dotted line indicates the drug's inherent resistance to echinocandins.

in clinical antifungal therapy after miconazole became available. The representative drug fluconazole binds to the heme group at the active site and can inhibit lanosterol 14α-demethylase which is encoded by *ERG11*. Blocking the synthesis of a key component of fungal cell membrane ergosterol destroys the stability and permeability of the cell membrane and the function of the membrane-binding enzyme. Fluconazole has only fungistatic activity in *C. neoformans*; therefore, in the long-term treatment of *Cryptococcus* infection with fluconazole, *Cryptococcus* has the potential to acquire fluconazole resistance [7].

Duplication of multiple chromosomes

FLC can penetrate well into cerebrospinal fluid with minimal toxic effects and can be used in the maintenance phase of treatment for cryptococcal meningitis in AIDS patients. However, drug resistance has increased due to long-term use [8]. The earliest discovery of drug resistance in azole was from a culture strain that was isolated from a patient with recurrent meningitis. This resistance turned out to be inherent [9]. The results of genomic hybridization and quantitative real-time PCR showed that *C. neoformans* adapted to high FLC concentrations by replicating multiple chromosomes. Replication of chromosome 1 is closely related to its two resident genes: *ERG11*, the target gene of FLC, and *AFR1*, the main gene encoding the drug transporter of azole in *C. neoformans* [10]. In addition to chromosome 1, chromosome 4 is the second most susceptible to diploid formation under high FLC concentrations [11]. *Cryptococcus* can improve its resistance to fluconazole drugs through changes in chromosome ploidy, enabling it to grow at a level higher than the minimal inhibitory concentration (MIC) of FLC, which is also the unique mechanism for *Cryptococcus* to develop drug resistance to azole.

Gene mutation and point mutation

Analysis of an FLC-resistant strain isolated from the cerebrospinal fluid of recurrent cryptococcal meningitis patients showed that there is a point mutation responsible for the amino acid substitution G484S in *ERG11* [12]. Another study identified that there is a single missense mutation of *ERG11* substituting tyrosine with phenylalanine at amino acid 145 (Y145F), which resulted in high FLC resistance of the *C. neoformans* strain [13]. The transcription factor Yap1 coexists with the *ERG11* and *AFR1* genes on chromosome 1, which are essential for FLC resistance in the wild-type strain. Yap1 plays a unique biological role in FLC resistance in the wild-type *C. neoformans* strain, and mutant strains of *C. neoformans* lacking

YAP1 are hypersensitive to a range of oxidative stress agents but importantly also to fluconazole [14]. In addition, the emergence of drug resistance is not limited to mutations in the above genes. By identification of deletion mutants and determination of ploidy changes through whole-genome sequencing (WGS), Albehaijani found that only *MSH2* gene mutations occurred in the genome of drug-resistant strains, and no mutations in *ERG11* or other genes of the ergosterol biosynthesis pathway were involved [15]. Boyce *et al.* [16] used WGS analysis to compare the mutation frequencies of eight mismatch repair pathway deletions and demonstrated that common deletions in three genes, *MSH2*, *MLH1*, and *PMS1*, resulted in increased mutation rates in *C. neoformans*. Due to the microevolution of mutations, phenotypic variation occurs in traits related to growth ability *in vivo*, which is a significant contributing factor to the development of antifungal resistance [16].

Upregulation of gene expression.

AFR1, *AFR2*, and *MDR1* are involved in encoding ATP binding cassette transporters (ABCTs) in *C. neoformans*. It reduces the intracellular accumulation of drugs by increasing drug efflux, leading to drug resistance [17,18]. Brunella *et al.* [19] used cDNA subtractive library technology to delete the *AFR1* gene in a resistant strain of (BPY22.17), and their data first demonstrated that overexpression of *AFR1* is a major factor causing increased resistance to azole antibiotics in *C. neoformans*. By identifying mutants with altered FLC sensitivity or an increased frequency of FLC heteroresistance, Chang *et al.* [20] demonstrated that *AFR1* was positively regulated by the transcription factors Crz1 and Yap1 in *C. neoformans*, and *AFR1* expression was upregulated under high FLC concentrations.

Polyene resistance

The gold standard antifungal regimen for the treatment of cryptococcosis is a combination of the polyene macrolide amphotericin B (AMB) with 5-fluorocytosine (5-FC) [21]. AMB has been used alone to treat cryptococcal meningitis for a long time, but polyene resistance is extremely rare [22]. This may be explained by the fact that compared with most of other anti-cryptococcus drugs, the targets of polyene drugs are not essential enzymes but major cell membranes [23]. AMB, by directly combining with the plasma membrane of ergosterol rather than destroying the ergosterol biosynthesis pathway [24], binds to ergosterol and participates in the formation of transmembrane channels, resulting in the leakage of cellular ions such as K^+, Na^+, Cl^- and H^+, ultimately leading to the disruption of *Cryptococcus* [25].

Genetic mutation may cause abnormal sterol synthesis

Changes in sterol composition in the fungal cell membrane are the most common mechanism of AMB resistance. Kelly *et al.* [26] first observed that sterolΔ8→7 isomerase was associated with resistance to AMB in the clinic, whose abnormal synthesis blocked the biosynthesis of ergosterol, and the failure of AMB treatment was associated with sterol alterations similar to *ERG2* mutations of *S. cerevisiae*. Moreover, long-term use of AMB can lead to changes in sterol biosynthesis and produce cross-resistance with azole drugs [26].

Transcription factors can regulate gene expression

Deletions of some transcription factors modulate azole and polyene sensitivity in opposite ways, probably because they may directly control the expression of *ERG11* and sterol biosynthesis, influencing the binding ability of polyene with sterol. Previously, the transcription factors (TFs) Sre1 and Mbs1 were shown to regulate the expression of *ERG11*. Sre1, a key sterol-regulated TF, plays a role in the oxygen-sensing pathway. It triggers membrane binding to Sre1 by cleavage of the activator protein Scp1 under hypoxic conditions as part of the sterol regulatory element-binding protein in *C. neoformans* [27,28]. *Mbs1*, a gene that negatively regulates basal *ERG11* expression, and the results showed that these two TF deletions can increase azole resistance but decrease polyene resistance in *C. neoformans* [29]. To prove whether other TFs participate in the regulation of *ERG11* expression levels, Jung *et al.* [30] demonstrated that the basal expression level of *ERG11* was significantly increased in *hob1* gene deletion mutants. Additionally, the TF HOB1 is also involved in the regulation of other *ERG* genes. The tight regulation of *ERG* expression appeared to be mostly absent in the *hob1Δ* mutant, suggesting that Hob1 is a key regulator of ergosterol gene expression [30].

Flucytosine resistance

Since 1968, 5-FC, the second class of drugs approved for antifungal therapy after amphotericin B, has been used for the treatment of cryptococcosis in humans [31]. 5-FC is one of the most commonly used antifungal drugs in the treatment of cryptococcosis. It acts as a prodrug to enter cells by the cytosine permeability of FCY2. 5-FC is not inherently toxic; however, it is converted to toxic 5-fluorouracil (5-FU) by cytosine deaminase after being taken up by fungal cells, which is an enzyme not present in human cells encoded by *FCY1* [32]. 5-FU is further processed by uracil phosphoribosyltransferase (URPT), a product encoded by the *FUR1* gene, and inhibits the synthesis of pathogen DNA and proteins [33]. Using 5-FC alone is highly susceptible to resistance. Therefore, it is usually used in combination with other antifungal agents. Combination with AMB is the basic principle of current guidelines for the treatment of cryptococcal meningoencephalitis [34].

Gene deletion mutations

The earliest studies showed that deletion mutations in either the *FCY1* or *FCY2* genes could lead to increased resistance of *C. neoformans* to 5-FC. Resistance in fcy1/fcy1 strains was associated with decreased UMP pyrophosphorylase activity, whereas resistance in *FCY2/FCY2* strains was related to decreased cytosine deaminase activity [35]. DNA mismatch repair defects lead to an increased mutation rate of isolates, especially *C. gattii*, which is more prone to develop drug resistance to 5-FC. Recently, a study identified mutations associated with 5-FC resistance *in vitro* by whole-genome sequencing of 16 independent isolates, including mutations in *UXS1*, in addition to the known resistance genes *FUR1* and *FCY2*. *UXS1* encodes an enzyme that converts UDP glucuronic acid to UDP xylose for capsule biosynthesis. Mutations in *UXS1* result in UDP glucuronic acid accumulation and altered nucleotide metabolism. As a result, they speculated that this may suppress the toxicity of both 5-FC and its toxic derivative 5-FU [36]. Ura6 catalyzes the seventh enzymatic step in the *de novo* biosynthesis of pyrimidines, which converts uridine monophosphate into uridine-5′-diphosphate in *S. cerevisiae* [37]. The resulting strain C2080 containing the $URA6^{V172L}$ variant by molecular manipulations through homologous integration displayed a markedly higher MIC than the wild type. This indicated that *URA6* contributes to 5-FC susceptibility, which has not been implicated in other fungi [38].

Formation of aneuploidy and transcriptional regulation

Cryptococci develop resistance to 5-FC at a high frequency when

exposed to concentrations several-fold above the minimal inhibitory concentration. Chang *et al.* [38] defined chr1 duplication as the major cause of increased copies of the *AFR1* gene, which also contributes to the 5-FC resistance of some strains. Unfortunately, the mechanism is still unclear. *uxs1Δ* may accumulate UDP-glucuronic acid, which appears to downregulate the expression of permease FCY2 and reduce cellular uptake of the drug. Mutations in the genes *UGD1* (UDP-glucuronic acid synthesis) or *NRG1*(TF) suppress UDP-glucuronic acid accumulation and 5-FC resistance in *uxs1Δ* [38].

Echinocandin resistance

Caspofungin, a representative drug of echinocandins, as the latest generation of antifungal drugs, targets the cell wall structure that is not found in mammalian cells. Such drugs disrupt cell wall integrity by blocking the production of β-1,3-glucan synthase, which is encoded by the *FKS1* gene, leading to osmotic pressure imbalance in fungal cells and rupture [39]. The *C. neoformans FKS1* sequence is closely related to the *FKS1* sequences from other fungal species and appears to be a single copy in *C. neoformans*. In addition, amino acid residues known to be critical for echinocandin susceptibility in *Saccharomyces* are conserved in the *C. neoformans FKS1* sequence, resulting in natural resistance to echinocandin antibiotics of *Cryptococcus* [40]. The mechanism of resistance of *Cryptococcus* to echinocandins is described in the following sections, which may provide a deeper understanding of how the intrinsic resistance of *Cryptococcus* is generated.

Gene mutation

Mutation of the *FKS1* gene alters the sensitivity of glucan synthase to echinin. Huang *et al.* [41] screened gene disruption and gene deletion libraries for mutants sensitive to caspofungin, identifying a mutation of *CDC50*, which encodes the β-subunit of membrane lipid flippase. Cdc50 protein is located in membranes, and its absence leads to plasma membrane defects and enhances caspofungin penetration into the cell, potentially explaining the increased caspofungin sensitivity. Loss of *CDC50* also results in hypersensitivity to FLC. In addition, genetic analysis showed that the *erg2 vph1* double mutant was more sensitive to micafungin than any single mutant, according to the analysis of a micafungin-resistant strain isolated from micafungin-sensitive *ERG* mutants [42]. This suggests that these two genes act differently in the resistance to micafungin.

The drug induces cell wall stress by inhibiting β-1,3-glucan synthase. The calcineurin pathway [43], cell wall integrity (CWI) pathway [44], high osmolarity glycerol (HOG) pathway [45], and Hsp90 protein [46] enable cells to adapt to cell wall stress through cell wall compensatory biosynthesis and remodelling. Cdc50 interacts with the calcium channel Crm1 to regulate intracellular calcium homeostasis and calcineurin signaling, which may drive resistance to caspofungin in *C. neoformans* [47].

Posttranscriptional regulation

Puf4 is a member of the pumilio/FBF RNA-binding protein family involved in the regulation of endoplasmic reticulum stress in *C. neoformans* [48]. *Puf4* participates in promoting inherent resistance to caspofungin through posttranscriptional regulation of *FKS1* mRNA in *C. neoformans*. Puf4Δ mutant strains showed increased resistance to caspofungin; in contrast, overexpression of *Puf4* resulted in caspofungin sensitivity. In addition to *FKS1*, the abundance of other cell wall biosynthesis genes, including chitin synthase (*CHS3*, *CHS4*, and *CHS6*) and deacetylase (*CDA1*, *CDA2*, and *CDA3*) and β-1,6-glucan synthase gene (*SKN1*), are regulated by Puf4 [49].

In summary, the emergence of drug resistance is the result of the long-term microevolution of *Cryptococcus*, which includes the accumulation of multiple gene mutations that can change stress signal transduction, membrane transport, epigenetic modification, and chromosomal formation.

Strategies for the Future Treatment of *Cryptococcosis*

New therapies and the development of new drugs are urgently needed to improve the current treatment of *Cryptococcus*, especially as resistance evolves. Therefore, to improve efficacy and avoid drug resistance, new drug targets must be explored in the process of developing new drugs (Figure 2).

Combination therapy

AR-12 is an antifungal derivative of celecoxib that competitively inhibits essential acetyl-CoA synthase in fungi. Therefore, acetyl-CoA synthase has become a potential therapeutic target [50]. AR-12 can reduce the fungal burden in the brain of the mouse cryptococcosis model as a sensitizer in combination with FLC [51].

The biofilms formed by *Cryptococcus* are tolerant to the host immune system and antifungal agents [52,53]. The antifolate combination of sulfamethoxazole/trimethoprim (SMX/TMP) and sulfamethoxazole/pyrimidine (SDZ/PYR) has an inhibitory effect on the biofilms of *C. neoformans* and *C. gattii*. They can also enhance the susceptibility of *Cryptococcus* to amphotericin B by reducing ergosterol content in *C. neoformans* and *C. gattii* [54]. Kim *et al.* [55] indicated that mutants lacking iron oxidase (encoded by the *Cfo1* gene) showed reduced intracellular iron levels and decreased ergosterol biosynthesis in *C. neoformans*, leading to increased sensitivity to several azole drugs. Therefore, iron plays a key role in antifungal susceptibility, and combining iron-chelating agents with fluconazole can synergistically inhibit the growth of *C. neoformans* [55]. In summary, combination therapy can not only enhance the efficacy of drugs but also slow the development of drug resistance. Therefore, it is a good treatment strategy.

Coping with stress response

The stress response is a key strategy for *C. neoformans* survival under antifungal treatment, and inhibition of *Hrk1* increases susceptibility to azole drugs. The HOG pathway controls the expression of ergosterol biosynthesis genes, such as *ERG11*, affecting sensitivity to polyenes and azole drugs. Recently, a transcriptomic analysis of the HOG pathway identified the Hog1 regulatory gene *Hrk1*, which encodes a putative protein kinase. Inhibition of Hrk1 significantly increases sensitivity to azole drugs and has the potential to be a novel strategy for combined antifungal therapy [56].

The FK506 analog compensates for the immunosuppression of traditional calcineurin inhibitors, thus allowing the development of new antifungal agents [57]. Further analysis demonstrated that tryptanthrin exerted fungistatic and potent antifungal activity at elevated temperatures. It exerts antifungal activity by interfering with the cell cycle and signaling pathways through various transporters and signaling pathways. In addition, tryptanthrin exhibited a synergistic effect with FK506 and cyclosporine A against *C. neoformans*, which could be a potential drug or adjuvant to treat

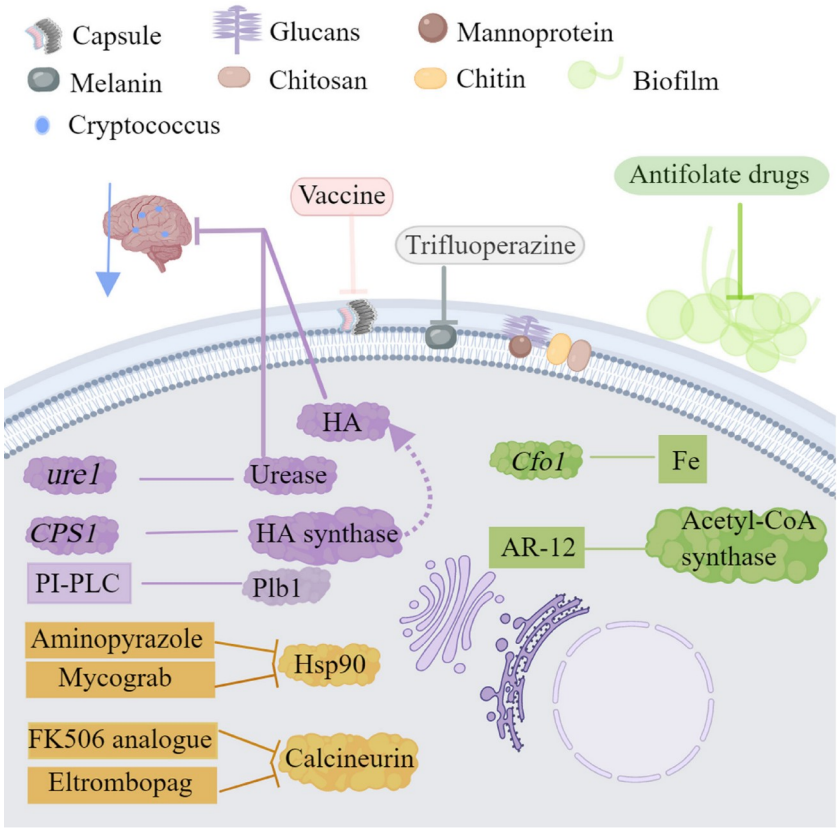

Figure 2. New targets for the treatment of *Cryptococcus* infection Combination therapy (green). AR-12 inhibits acetyl-CoA synthase. Antifolate drugs inhibit biofilm production. Iron oxidase encoded by the *Cof1* gene can bind to intracellular iron. Coping with stress response (yellow). The FK506 analog and eltrombopag inhibit the calcineurin pathway. Aminopyrazole and mycograb inhibit the Hsp90 protein pathway. Addressing virulence factors (pink, gray, and purple). Immunomodulation with monoclonal antibodies against the capsule. Trifluoperazine is effective in melanized cells. PI-PLC regulates the release of Plb1. *CPS1* encodes hyaluronic acid synthase, and its product HA prevents *Cryptococcus* from entering the CNS. Urease-negative transformant caused by deletion of *ure1* reduces the fungal burden on the brain and mortality in mice.

cryptococcosis [58]. Eltrombopag exhibits excellent antifungal activity against cryptococcal species, probably through its effects on virulence factors and calcineurin pathways, suggesting that eltrombopag can be effectively repurposed as an antifungal agent for the treatment of cryptococcosis [59]. Huang *et al.* [60] developed a series of synthetic aminopyrazole-substituted resorcylate amides with broad, potent, and fungal-selective Hsp90 inhibitory activity, replacing the concomitant host toxicity issues of traditional Hsp90 inhibitors. A previous study showed that Hsp90 inhibitors combined with azole drugs can enhance its antifungal activity [61]. Mycograb, a genetically recombinant human antibody against Hsp90, exhibits significant synergistic effects when combined with AMB, caspofungin, and FLC [62].

Addressing virulence factors

Some studies have shown that capsule, melanin, survival at physiological temperature, and secretion of various extracellular proteins are closely related to the pathogenicity of *Cryptococcus* [63].

Capsule

The *Cryptococcus* polysaccharide capsule is a major virulence factor due to its resistance to innate immunity. Clarithromycin, a macrolide drug, may contribute to inhibiting capsule formation through the MAPK signaling pathway and inhibiting toxic genes. Therefore, it may be a useful adjunct drug for the treatment of refractory *C. gattii* infection [64]. In addition, immunomodulation with monoclonal antibodies against the capsular polysaccharides glucuronoxylomannan and melanin has also yielded promising results in animal models [65]. A recent study used glucan particles (GPs) as a combined delivery system and adjuvant for *Cryptococcus* vaccines. Four vaccine candidates (GP-CDA1, GP-CDA2, GP-CDA3, and GP-SOD1) all showed significant survival advantages in at least one mouse model [66].

Melanin

Melanized *Cryptococcus* cells showed low sensitivity to AMB and caspofungin [67]. The antipsychotic drug trifluoperazine is effective in killing melanized cells, and this drug has been in the stage of clinical validation [68].

Extracellular enzymes

Phosphatidylinositol-specific phospholipase C (PI-PLC) is a central regulator of cryptococcal virulence, acting through the protein kinase C/MAPK pathway. It regulates the release of Plb1 from the plasma membrane, which is a candidate antifungal drug target [69]. Experiments showed that treatment with hyaluronidase resulted in a reduction in the level of *C. neoformans* binding to human brain microvascular endothelial cells (HBMECs). As a result, Jong *et al.* [70] demonstrated that *C. neoformans CPS1* encodes hyaluronic acid synthase and that its product, hyaluronic acid (HA), plays a

role as an adhesion molecule during the association of endothelial cells with yeast. This study also demonstrated that hyaluronidase treatment of yeast reduced the binding level of *C. neoformans* to human microvascular endothelial cells, thereby preventing *Cryptococcus* from entering the central nervous system [70]. Urease (encoded by *ure1*) contributes to the spread of *Cryptococcus* from the lungs to the central nervous system. Deletion of *ure1* reduces the fungal burden on the brain and mortality in mice [71]. Therefore, it can be used as a new target for the development of therapeutic drugs.

WGS for Detecting *Cryptococcus* Drug Resistance

At present, WGS is a very reliable technology to study the mechanism of *Cryptococcus* drug resistance and can quickly sequence the complete *Cryptococcus* genomes. By sequencing the entire genomes of representative strains of both *C. neoformans* and *C. gatti*, hundreds of individual strains were identified. We have gained a deeper understanding of the evolution and diversity of *Cryptococcus* [72]. Sequencing was also used to study the microevolution of isolates during prolonged infections, highlighting the potential role of hypermutations in evolution over a short period. To determine the cause of recurrence of cryptococcal meningitis infection, WGS was used in some studies to evaluate the genetic basis of infected patients. It was found that nonsense mutations can cause hypermutation status, and synonym and nonsynonym replacement rates are significantly increased, which may be used for rapid adaptation in the host and evolution of resistance to first-line antifungal agents in the future [73]. Moreover, this technique has been applied to reveal the genetics of drug resistance, contributing to the molecular epidemiology and phylogenetic analysis of *Cryptococcus*. In the study of Wongsuk *et al.* [74], WGS and multilocus sequence typing (MLST) were used to determine the genetic characterization of *C. neoformans*, and they first reported the *in vitro* antifungal susceptibility profiles of *C. neoformans* isolated from Thailand.

CRISPR-Cas9 Editing for Antifungal Resistance Genes

Deciphering the mechanisms underlying virulence signatures and drug resistance relies strongly on genetic manipulation techniques, such as producing mutant strains that carry specific mutations or gene deletions. Davidson *et al.* [75] used both biolistic and electroporation transformation systems for gene disruption in *C. neoformans*; however, the low frequency of homologous recombination obtained by these methods resulted in low efficiency of gene disruption, which to some extent hindered the development of genomic studies in *Cryptococcus*. In contrast, the CRISPR-Cas9 system has highly efficient gene editing capabilities, and this technology is constantly being refined and improved, showing great promise for gene editing using it [76,77]. Methods utilizing CRISPR-Cas9 have become the preferred technique for gene editing in many organisms due to their simplicity, efficiency, and versatility [78]. Wang *et al.* [79] investigated a "suicide" CRISPR-Cas9 system that effectively generates an indel mutation and efficiently performs targeted gene disruption via homology-directed repair by electroporation in yeast, with the potential to minimize off-target effects. Therefore, this technique can also be effectively applied to the functional genomics of *C. neoformans*. With such molecular tools, it is possible to specifically disrupt candidate drug resistance genes and determine their importance in the pathobiology of *Cryptococcus*. These strategies will potentially facilitate the development of drugs targeting these gene products or their receptors.

Conclusions and Prospects

Drug-resistant *Cryptococcus*, with an escalating threat to global *Cryptococcus* control, mandates the development and application of new tools. WGS helps enormously delineate the cryptococcal genes related to drug resistance. However, complementary evaluation of these putative genes is often required to verify their role in drug resistance. Therefore, CRISPR-Cas9 technology can be applied to construct gene mutants. The discovery of new targets should be applied to the study of new anti-cryptococcal drugs to solve the problem of drug resistance endangering current cryptococcal treatment.

Funding

This work was supported by the grants from the National Key Research and Development Program (No. 2021YFC2302100), the National Key Research and Development Program (No. 2020YFC2005404), the Beijing Key Clinical Specialty Project (No. 2020ZDZK1), the National Capacity Building Project for Multidisciplinary Diagnosis and Treatment of Major Diseases (No. 2019YLFW), the Capital Health Development Scientific Research Unit Matching Fund (No. 2020-2Z-2086), and the "Sailing" Plan for Clinical Technology Innovation Project in Beijing (No. XMLX202115).

Conflict of Interest

The authors declare that they have no conflict of interest.

References

1. Francisco EC, de Jong AW, Hagen F. Cryptococcosis and *Cryptococcus*. *Mycopathologia* 2021, 186: 729–731
2. Rajasingham R, Smith RM, Park BJ, Jarvis JN, Govender NP, Chiller TM, Denning DW, *et al.* Global burden of disease of HIV-associated cryptococcal meningitis: an updated analysis. *Lancet Infect Dis* 2017, 17: 873–881
3. Brown AJP, Cowen LE, di Pietro A, Quinn J. Stress adaptation. *Microbiol Spectr* 2017, 5: 10.1128/microbiolspec.FUNK-0048-2016
4. Robbins N, Caplan T, Cowen LE. Molecular evolution of antifungal drug resistance. *Annu Rev Microbiol* 2017, 71: 753–775
5. Sun S, Coelho MA, David-Palma M, Priest SJ, Heitman J. The evolution of sexual reproduction and the mating-type locus: links to pathogenesis of *cryptococcus* human pathogenic fungi. *Annu Rev Genet* 2019, 53: 417–444
6. Ni M, Feretzaki M, Li W, Floyd-Averette A, Mieczkowski P, Dietrich FS, Heitman J. Unisexual and heterosexual meiotic reproduction generate aneuploidy and phenotypic diversity de novo in the yeast *Cryptococcus neoformans*. *PLoS Biol* 2013, 11: e1001653
7. Lee Y, Puumala E, Robbins N, Cowen LE. Antifungal drug resistance: molecular mechanisms in *Candida albicans* and beyond. *Chem Rev* 2021, 121: 3390–3411
8. Paugam A, Dupouy-Camet J, Blanche P, Gangneux JP, Tourte-Schaefer C, Sicard D. Increased fluconazole resistance of *Cryptococcus neoformans* isolated from a patient with AIDS and recurrent meningitis. *Clin Infect Dis* 1994, 19: 975–976
9. Mondon P, Petter R, Amalfitano G, Luzzati R, Concia E, Polacheck I, Kwon-Chung KJ. Heteroresistance to fluconazole and voriconazole in *Cryptococcus neoformans*. *Antimicrob Agents Chemother* 1999, 43: 1856–1861

10. Sionov E, Lee H, Chang YC, Kwon-Chung KJ. *Cryptococcus neoformans* overcomes stress of azole drugs by formation of disomy in specific multiple chromosomes. *PLoS Pathog* 2010, 6: e1000848
11. Ngamskulrungroj P, Chang Y, Hansen B, Bugge C, Fischer E, Kwon-Chung KJ. Characterization of the chromosome 4 genes that affect fluconazole-induced disomy formation in *Cryptococcus neoformans*. *PLoS ONE* 2012, 7: e33022
12. Rodero L, Mellado E, Rodriguez AC, Salve A, Guelfand L, Cahn P, Cuenca-Estrella M, *et al.* G484S amino acid substitution in lanosterol 14-α demethylase (*ERG11*) is related to fluconazole resistance in a recurrent *Cryptococcus neoformans* clinical isolate. *Antimicrob Agents Chemother* 2003, 47: 3653–3656
13. Sionov E, Chang YC, Garraffo HM, Dolan MA, Ghannoum MA, Kwon-Chung KJ. Identification of a cryptococcus neoformans cytochrome P450 lanosterol 14α-demethylase (Erg11) residue critical for differential susceptibility between fluconazole/voriconazole and itraconazole/posaconazole. *Antimicrob Agents Chemother* 2012, 56: 1162–1169
14. Paul S, Doering TL, Moye-Rowley WS. *Cryptococcus neoformans* Yap1 is required for normal fluconazole and oxidative stress resistance. *Fungal Genet Biol* 2015, 74: 1–9
15. Albehaijani SHI, Macreadie I, Morrissey CO, Boyce KJ. Molecular mechanisms underlying the emergence of polygenetic antifungal drug resistance in *msh2* mismatch repair mutants of *Cryptococcus*. *JAC Antimicrob Resist* 2022, 4: dlac033
16. Boyce KJ, Wang Y, Verma S, Shakya VPS, Xue C, Idnurm A. Mismatch repair of DNA replication errors contributes to microevolution in the pathogenic fungus *Cryptococcus neoformans*. *mBio* 2017, 8: e00595-17
17. Basso Jr LR, Gast CE, Bruzual I, Wong B. Identification and properties of plasma membrane azole efflux pumps from the pathogenic fungi *Cryptococcus gattii* and *Cryptococcus neoformans*. *J Antimicrob Chemother* 2015, 70: 1396–1407
18. Orsi CF, Colombari B, Ardizzoni A, Peppoloni S, Neglia R, Posteraro B, Morace G, *et al.* The ABC transporter-encoding gene *AFR1* affects the resistance of *Cryptococcus neoformans* to microglia-mediated antifungal activity by delaying phagosomal maturation. *FEMS Yeast Res* 2009, 9: 301–310
19. Posteraro B, Sanguinetti M, Sanglard D, La Sorda M, Boccia S, Romano L, Morace G, *et al.* Identification and characterization of a *Cryptococcus neoformans* ATP binding cassette (ABC) transporter-encoding gene, CnAFR1, involved in the resistance to fluconazole. *Mol Microbiol* 2003, 47: 357–371
20. Chang M, Sionov E, Khanal Lamichhane A, Kwon-Chung KJ, Chang YC. Roles of three *Cryptococcus neoformans* and *Cryptococcus gattii* efflux pump-coding genes in response to drug treatment. *Antimicrob Agents Chemother* 2018, 62: e01751-17
21. Krysan DJ. Toward improved anti-cryptococcal drugs: novel molecules and repurposed drugs. *Fungal Genet Biol* 2015, 78: 93–98
22. Vincent BM, Lancaster AK, Scherz-Shouval R, Whitesell L, Lindquist S. Fitness trade-offs restrict the evolution of resistance to amphotericin B. *PLoS Biol* 2013, 11: e1001692
23. Carolus H, Pierson S, Lagrou K, Van Dijck P. Amphotericin b and other polyenes-discovery, clinical use, mode of action and drug resistance. *J Fungi (Basel)* 2020, 6: 321
24. Mesa-Arango AC, Scorzoni L, Zaragoza O. It only takes one to do many jobs: amphotericin B as antifungal and immunomodulatory drug. *Front Microbio* 2012, 3: 286
25. Gray KC, Palacios DS, Dailey I, Endo MM, Uno BE, Wilcock BC, Burke MD. Amphotericin primarily kills yeast by simply binding ergosterol. *Proc Natl Acad Sci USA* 2012, 109: 2234–2239
26. Kelly SL, Lamb DC, Taylor M, Corran AJ, Baldwin BC, Powderly WG. Resistance to amphotericin B associated with defective sterol delta 8→7 isomerase in a *Cryptococcus neoformans* strain from an AIDS patient. *FEMS Microbiol Lett* 1994, 122: 39–42
27. Chang YC, Bien CM, Lee H, Espenshade PJ, Kwon-Chung KJ. Sre1p, a regulator of oxygen sensing and sterol homeostasis, is required for virulence in *Cryptococcus neoformans*. *Mol Microbiol* 2007, 64: 614–629
28. Chun CD, Liu OW, Madhani HD. A link between virulence and homeostatic responses to hypoxia during infection by the human fungal pathogen *Cryptococcus neoformans*. *PLoS Pathog* 2007, 3: e22
29. Song MH, Lee JW, Kim MS, Yoon JK, White TC, Floyd A, Heitman J, *et al.* A flucytosine-responsive Mbp1/Swi4-like protein, Mbs1, plays pleiotropic roles in antifungal drug resistance, stress response, and virulence of *Cryptococcus neoformans*. *Eukaryot Cell* 2012, 11: 53–67
30. Jung KW, Yang DH, Maeng S, Lee KT, So YS, Hong J, Choi J, *et al.* Systematic functional profiling of transcription factor networks in *Cryptococcus neoformans*. *Nat Commun* 2015, 6: 6757
31. Vermes A. Flucytosine: a review of its pharmacology, clinical indications, pharmacokinetics, toxicity and drug interactions. *J AntiMicrob ChemoTher* 2000, 46: 171–179
32. Loyse A, Dromer F, Day J, Lortholary O, Harrison TS. Flucytosine and cryptococcosis: time to urgently address the worldwide accessibility of a 50-year-old antifungal. *J AntiMicrob ChemoTher* 2013, 68: 2435–2444
33. Hope WW, Tabernero L, Denning DW, Anderson MJ. Molecular mechanisms of primary resistance to flucytosine in *Candida albicans*. *Antimicrob Agents Chemother* 2004, 48: 4377–4386
34. Perfect JR, Dismukes WE, Dromer F, Goldman DL, Graybill JR, Hamill RJ, Harrison TS, *et al.* Clinical practice guidelines for the management of cryptococcal disease: 2010 update by the infectious diseases society of america. *Clin Infect Dis* 2010, 50: 291–322
35. Whelan WL. The genetic basis of resistance to 5-fluorocytosine in *Candida* species and *Cryptococcus neoformans*. *CRC Crit Rev Microbiol* 1987, 15: 45–56
36. Billmyre RB, Applen Clancey S, Li LX, Doering TL, Heitman J. 5-luorocytosine resistance is associated with hypermutation and alterations in capsule biosynthesis in *Cryptococcus*. *Nat Commun* 2020, 11: 127
37. Liljelund P, Lacroute F. Genetic characterization and isolation of the *Saccharomyces cerevisiae* gene coding for uridine monophosphokinase. *Molec Gen Genet* 1986, 205: 74–81
38. Chang YC, Lamichhane AK, Cai H, Walter PJ, Bennett JE, Kwon-Chung KJ. Moderate levels of 5-fluorocytosine cause the emergence of high frequency resistance in cryptococci. *Nat Commun* 2021, 12: 3418
39. Aguilar-Zapata D, Petraitiene R, Petraitis V. Echinocandins: the expanding antifungal armamentarium. *Clin Infect Dis* 2015, 61: S604–S611
40. Thompson JR, Douglas CM, Li W, Jue CK, Pramanik B, Yuan X, Rude TH, *et al.* A glucan synthase *FKS1* homolog in *Cryptococcus neoformans* is single copy and encodes an essential function. *J Bacteriol* 1999, 181: 444–453
41. Huang W, Liao G, Baker GM, Wang Y, Lau R, Paderu P, Perlin DS, *et al.* Lipid flippase subunit Cdc50 mediates drug resistance and virulence in *Cryptococcus neoformans*. *mBio* 2016, 7: e00478-16
42. Toh EA, Ohkusu M, Shimizu K, Yamaguchi M, Ishiwada N, Watanabe A, Kamei K. Creation, characterization and utilization of *Cryptococcus neoformans* mutants sensitive to micafungin. *Curr Genet* 2017, 63: 1093–1104
43. Fu C, Donadio N, Cardenas ME, Heitman J. Dissecting the roles of the calcineurin pathway in unisexual reproduction, stress responses, and virulence in *Cryptococcus deneoformans*. *Genetics* 2018, 208: 639–653
44. Kraus PR, Fox DS, Cox GM, Heitman J. The *Cryptococcus neoformans* MAP

kinase Mpk1 regulates cell integrity in response to antifungal drugs and loss of calcineurin function. *Mol Microbiol* 2003, 48: 1377–1387
45. Saito H, Posas F. Response to hyperosmotic stress. *Genetics* 2012, 192: 289–318
46. Singh SD, Robbins N, Zaas AK, Schell WA, Perfect JR, Cowen LE. Hsp90 governs echinocandin resistance in the pathogenic yeast *Candida albicans* via calcineurin. *PLoS Pathog* 2009, 5: e1000532
47. Cao C, Wang Y, Husain S, Soteropoulos P, Xue C. A mechanosensitive channel governs lipid flippase-mediated echinocandin resistance in *Cryptococcus neoformans*. *mBio* 2019, 10: e01952-19
48. Glazier VE, Kaur JN, Brown NT, Rivera AA, Panepinto JC. Puf4 regulates both splicing and decay of *HXL1* mRNA encoding the unfolded protein response transcription factor in *Cryptococcus neoformans*. *Eukaryot Cell* 2015, 14: 385–395
49. Kalem MC, Subbiah H, Leipheimer J, Glazier VE, Panepinto JC. Puf4 mediates post-transcriptional regulation of cell wall biosynthesis and caspofungin resistance in *Cryptococcus neoformans*. *mBio* 2021, 12: e03225-20
50. Koselny K, Green J, Favazzo L, Glazier VE, DiDone L, Ransford S, Krysan DJ. Antitumor/antifungal celecoxib derivative AR-12 is a non-nucleoside inhibitor of the ANL-family adenylating enzyme acetyl CoA synthetase. *ACS Infect Dis* 2016, 2: 268–280
51. Koselny K, Green J, DiDone L, Halterman JP, Fothergill AW, Wiederhold NP, Patterson TF, ***et al.*** The celecoxib derivative AR-12 has broad-spectrum antifungal activity *in vitro* and improves the activity of fluconazole in a murine model of cryptococcosis. *Antimicrob Agents Chemother* 2016, 60: 7115–7127
52. Martinez LR CA. Biofilm formation by *Cryptococcus neoformans*. *Microbiol Spectr* 2015, 3: 135-47
53. Ramage G, Rajendran R, Sherry L, Williams C. Fungal biofilm resistance. *Int J Microbiol* 2012, 2012: 1–14
54. Aguiar Cordeiro R, Mourão CI, Rocha MF, Farias Marques FJ, Teixeira CE, Oliveira Miranda DF, Neto LV, ***et al.*** Antifolates inhibit *Cryptococcus* biofilms and enhance susceptibility of planktonic cells to amphotericin B. *Eur J Clin Microbiol Infect Dis* 2013, 32: 557–564
55. Kim J, Cho YJ, Do E, Choi J, Hu G, Cadieux B, Chun J, ***et al.*** A defect in iron uptake enhances the susceptibility of *Cryptococcus neoformans* to azole antifungal drugs. *Fungal Genet Biol* 2012, 49: 955–966
56. Kim SY, Ko YJ, Jung KW, Strain A, Nielsen K, Bahn YS. Hrk1 plays both Hog1-dependent and -independent roles in controlling stress response and antifungal drug resistance in *Cryptococcus neoformans*. *PLoS ONE* 2011, 6: e18769
57. Beom JY, Jung JA, Lee KT, Hwangbo A, Song MC, Lee Y, Lee SJ, ***et al.*** Biosynthesis of nonimmunosuppressive FK506 analogues with antifungal activity. *J Nat Prod* 2019, 82: 2078–2086
58. Lin CJ, Chang YL, Yang YL, Chen YL. Natural alkaloid tryptanthrin exhibits novel anticryptococcal activity. *Med Mycol* 2021, 59: 545–556
59. Ko HT, Hsu LH, Yang SY, Chen YL. Repurposing the thrombopoietin receptor agonist eltrombopag as an anticryptococcal agent. *Med Mycol* 2020, 58: 493–504
60. Huang DS, LeBlanc EV, Shekhar-Guturja T, Robbins N, Krysan DJ, Pizarro J, Whitesell L, ***et al.*** Design and synthesis of fungal-selective resorcylate aminopyrazole hsp90 inhibitors. *J Med Chem* 2020, 63: 2139–2180
61. Cordeiro RA, Evangelista AJJ, Serpa R, Marques FJF, Melo CVS, Oliveira JS, Franco JS, ***et al.*** Inhibition of heat-shock protein 90 enhances the susceptibility to antifungals and reduces the virulence of *Cryptococcus neoformans/Cryptococcus gattii* species complex. *Microbiology* 2016, 162: 309–317
62. Nooney L, Matthews RC, Burnie JP. Evaluation of Mycograb®, amphotericin B, caspofungin, and fluconazole in combination against *Cryptococcus neoformans* by checkerboard and time-kill methodologies. *Diagnostic Microbiol Infect Dis* 2005, 51: 19–29
63. Alspaugh JA. Virulence mechanisms and *Cryptococcus neoformans* pathogenesis. *Fungal Genet Biol* 2015, 78: 55–58
64. Nakamura S, Ikeda-Dantsuji Y, Jin L, Higashi Y, Abe M, Inukai T, Nagi M, ***et al.*** Macrolides inhibit capsule formation of highly virulent *Cryptococcus gattii* and promote innate immune susceptibility. *Antimicrob Agents Chemother* 2019, 63: e02364-18
65. Antachopoulos C, Walsh TJ. Immunotherapy of *Cryptococcus* infections. *Clin Microbiol Infect* 2012, 18: 126–133
66. Specht CA, Lee CK, Huang H, Hester MM, Liu J, Luckie BA, Torres Santana MA, ***et al.*** Vaccination with recombinant *Cryptococcus* proteins in glucan particles protects mice against *Cryptococcosis* in a manner dependent upon mouse strain and *Cryptococcal* species. *mBio* 2017, 8: e01872-17
67. van Duin D, Casadevall A, Nosanchuk JD. Melanization of *Cryptococcus neoformans* and *Histoplasma capsulatum* reduces their susceptibilities to amphotericin B and caspofungin. *Antimicrob Agents Chemother* 2002, 46: 3394–3400
68. Wang Y, Casadevall A. Susceptibility of melanized and nonmelanized *Cryptococcus neoformans* to the melanin-binding compounds trifluoperazine and chloroquine. *Antimicrob Agents Chemother* 1996, 40: 541–545
69. Chayakulkeeree M, Sorrell TC, Siafakas AR, Wilson CF, Pantarat N, Gerik KJ, Boadle R, ***et al.*** Role and mechanism of phosphatidylinositol-specific phospholipase C in survival and virulence of *Cryptococcus neoformans*. *Mol Microbiol* 2008, 69: 809–826
70. Jong A, Wu CH, Chen HM, Luo F, Kwon-Chung KJ, Chang YC, LaMunyon CW, ***et al.*** Identification and characterization of *CPS1* as a hyaluronic acid synthase contributing to the pathogenesis of *Cryptococcus neoformans* infection. *Eukaryot Cell* 2007, 6: 1486–1496
71. Olszewski MA, Noverr MC, Chen GH, Toews GB, Cox GM, Perfect JR, Huffnagle GB. Urease expression by *Cryptococcus neoformans* promotes microvascular sequestration, thereby enhancing central nervous system invasion. *Am J Pathol* 2004, 164: 1761–1771
72. Cuomo CA, Rhodes J, Desjardins CA. Advances in *Cryptococcus* genomics: insights into the evolution of pathogenesis. *Mem Inst Oswaldo Cruz* 2018, 113: e170473
73. Rhodes J, Beale MA, Vanhove M, Jarvis JN, Kannambath S, Simpson JA, Ryan A, ***et al.*** A population genomics approach to assessing the genetic basis of within-host microevolution underlying recurrent cryptococcal meningitis infection. *G3 (Bethesda)* 2017, 7: 1165–1176
74. Wongsuk T, Homkaew A, Faksri K, Thongnak C. Multi-locus sequence typing and whole genome sequence analysis of *Cryptococcus neoformans* isolated from clinical specimens in Vajira Hospital, Bangkok, Thailand. *Mycopathologia* 2020, 185: 503–514
75. Davidson RC, Cruz MC, Sia RAL, Allen B, Alspaugh JA, Heitman J. Gene disruption by biolistic transformation in serotype D strains of *Cryptococcus neoformans*. *Fungal Genet Biol* 2000, 29: 38–48
76. Morio F, Lombardi L, Butler G. The CRISPR toolbox in medical mycology: state of the art and perspectives. *PLoS Pathog* 2020, 16: e1008201
77. Zhang P, Wang Y, Li C, Ma X, Ma L, Zhu X. Simplified all-in-one CRISPR-Cas9 construction for efficient genome editing in *Cryptococcus* species. *JoF* 2021, 7: 505
78. Wang P. Two distinct approaches for CRISPR-Cas9-mediated gene editing in *Cryptococcus neoformans* and related species. *mSphere* 2018, 3: e00208-18
79. Wang Y, Wei D, Zhu X, Pan J, Zhang P, Huo L, Zhu X. A 'suicide' CRISPR-Cas9 system to promote gene deletion and restoration by electroporation in *Cryptococcus neoformans*. *Sci Rep* 2016, 6: 31145