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Diagnostics and drugs against the pathogenic fungus Cryptococcus neoformans

Diagnostika a léčiva proti patogenní houbě Cryptococcus neoformans

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V Praze, dne 12.12.2024 Vojtěch Valerián

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Declaration

I declare that I have prepared the thesis independently and that I have listed all the information sources and literature used. Neither this thesis nor any substantial part of it has been submitted for another or the same academic degree. I have used artificial intelligence, or tools supported by it, to write the thesis as follows: to gain feedback on the form of the text, and to improve the formulation of ideas and their clarity. To gain inspiration and insight into related topics and literature, I used the Perplexity tool to search for relevant information and sources. This information was then critically evaluated and adapted to suit the needs of the thesis. All data obtained was processed and incorporated into the thesis to meet the requirements of academic honesty and originality.

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Abstract

Fungal pathogens pose a complex issue in the department of infectious diseases. Yet their importance is often underestimated. Among the most dangerous are *Cryptococcus neoformans, Candida albicans,* and *Aspergillus fumigatus.* This thesis focuses on the opportunistic pathogen *C. neoformans* responsible for severe infection known as cryptococcal meningitis. This disease has a high mortality rate among fungal pathogens and primarily affects individuals with suppressed immune systems, such as those with AIDS, transplant recipients, or patients undergoing immunosuppressive therapy. This thesis aims to introduce *C. neofomans* as a pathogen, present current options in the diagnosis and treatment of cryptococcosis, and look into possible future options to upgrade the current state of cryptococcal treatment.

Keywords*:* *C. neoformans*, cryptococcosis, diagnostics, treatment

Abstrakt

Houbové patogeny představují složitou problematiku v oblasti infekčních onemocnění. Jejich význam je však často podceňován. Mezi nejnebezpečnější patří *Cryptococcus neoformans, Candida albicans* a *Aspergillus fumigatus*. Tato práce se zaměřuje na oportunní patogen *C. neoformans*, který je zodpovědný za závažnou infekci známou jako kryptokoková meningitida. Toto onemocnění má mezi houbovými patogeny vysokou úmrtnost. Především postihuje osoby s potlačeným imunitním systémem, jako jsou osoby s AIDS, příjemci transplantovaných orgánů nebo pacienti podstupující imunosupresivní léčbu. Cílem této práce je představit *C. neofomans* jako patogen, prezentovat současné možnosti diagnostiky a léčby kryptokokózy a podívat se na možné budoucí možnosti modernizace současného stavu léčby kryptokoků.

Klíčová slova*: C. neoformans*, kryptokokóza, diagnostika, léčba

List of used abbreviations

5-FC	Flucytosine	
5-FU	Fluorouracil	
AMB	Amphotericin B	
BBB	Blood brain barrier	hematoencefalická bariéra
CD14	Cluster of differentiation 14	
CNS	Central nervous system	centrální nervová soustava
CrAg	Cryptococcal antigen	kryptokokální antigen
CSF	Cerebrospinal fluid	mozkomíšní mok
CT	Computed tomography scan	výpočetní tomografie
DAMB	Amphotericin B deoxycholate	
ECM	Extracellular matrix	extracelulární matrix
ELISA	Enzyme-Linked Immunosorbent Assays	
FLC	Fluconazole	
GMX	Glucuronoxylomannan	glucoronoxylomannan
GMXGal	Glucoronoxylomanoglatan	glucoronoxylomanoglatan
GPI	Glycosylphosphatidylinositol	
HIV	Human immunodeficiency virus	virus lidské imunitní nedostatečnosti
HSP90	Heat-shock protein 90	
IFD	Invasive fungal disease	Invazivní houbové onemocnění
IFN-gamma	Interferon Gamma	
LAMB	Liposomal AMB	
PAFE	Post-antifungal effect	
PCR	polymerase chain reaction	polymerázová řetězová reakce
ROS	Reactive oxygen species	kyslíkové radikály
TLR2	Toll-like receptor 2	
WHO	World health organization	světová zdravotnická organizace
Covid -19	Coronavirus disease 2019	
AIDS	Acquired Immune Deficiency Syndrome	

Contents

1 Introduction

Diseases caused by fungal pathogens spread worldwide, causing approximately 1,5-2 million deaths annually, with many more patients left put in critical condition. Public health authorities often underestimate or pay less attention to them than they should. Most deaths from fungal diseases are avoidable if the proper treatment is provided (Bongomin et al., 2017). The challenging aspect of fungal infections is that they are often a cofactor or consequence of other illnesses such as Human Immunodeficiency Virus (HIV), cancer, or organ transplant, which makes their management even more complex. A well-known example of an illness that is followed by fungal disease is Coronavirus disease 2019 (Covid-19) (Passarelli et al., 2020).

The World Health Organization (WHO) has developed a fungal priority pathogens list to guide research, development, and public health initiatives. This list categorizes pathogens into three priority groups based on mortality and other criteria. The Critical Group contains: *Cryptococcus neoformans*, *Candida auris. Candida albicans*, *Aspergillus fumigatus* (Parums, 2022).

Awareness must be raised about the impact of rising global temperatures, as climate change is likely to increase pathogen spread from tropical and subtropical areas into the mid-latitude regions. This shift poses challenges to public health. Dispersion of the pathogens is expected, therefore action should be taken beforehand. While viral and bacterial diseases receive the most attention as the potential cause of plagues and pandemics, fungi inarguably pose equal or even greater threats. Currently, there are no vaccines available yet for fungal pathogens. The arsenal of antifungal agents is minimal, and fungi possess unique survival strategies. They can live saprotrophically, which enables producing large quantities of infectious spores, and do not require host-to-host contact to establish infection (Garcia-Solache & Casadevall, 2010; Nnadi & Carter, 2021).

The arsenal of antifungal drugs is limited, and pathogenetic fungi exhibit significant drug resistance, making this issue even more critical in years to come. Antifungal resistance can be either acquired when exposed to the effect of a specific drug. or intrinsic resistance is independent of contact with the drug. Specific genera and species can reveal obtained resistances such as fluconazole resistance in *Candida krusei*, amphotericin B resistance in *Aspergillus terreus*, or echinocandin resistance in *Cryptococcus* species(Denning, 2022). It appears that the success rate of fluconazole treatment is decreasing (Naicker et al., 2020).

There are a few questions that should be focused on shortly. What new drugs will be available? Can existing drugs be repurposed for antifungal use?

The thesis is organized into three main parts. The first part covers the life cycle and infection cycle, morphology, and its importance as part of the virulence factors of *Cryptococcus neoformans*. The second part focuses on current diagnostic approaches, such as culture-based, serological, and molecular-based methods. The third part highlights the advantages and disadvantages of commonly used antifungal drugs such as fluconazole, amphotericin B, and flucytosine. Furthermore, the possibilities for future treatment regarding new candidates for cryptococcal treatment are discussed.

This thesis aims to summarize diagnostic and treatment options for cryptococcosis and present future opportunities for further research.

2 Cryptococcus neoformans

The first detailed description of cryptococcosis was made by Otto Busse in 1894. However, the name of the agent behind the disease is responsible F. Sanfelice, whose results were published later (Knoke' & Schwesinger, 1994).

C. neoformans survive in the environment, both within the soil and in trees. Soil is usually contaminated with bird guano, particularly from pigeons, which plays a significant role in the global dispersion of this fungal pathogen (Figure 1). However, birds are not the only participants in global spread. Possible hosts and reservoirs range from insects to amoebas, cats, goats, and koalas. Interactions with other microorganisms and fungi are also possible. In humans, *C. neoformans* is responsible for pulmonary infection by inhaling spores through the pulmonary system. Once in the lung, the cryptococcal cells can evade the host's immune defenses, especially in individuals with compromised immunity. The pathogen then enters the blood and then the central nervous system (CNS), which causes the most severe cases of cryptococcosis, called cryptococcal meningitis (Lin & Heitman, 2006).

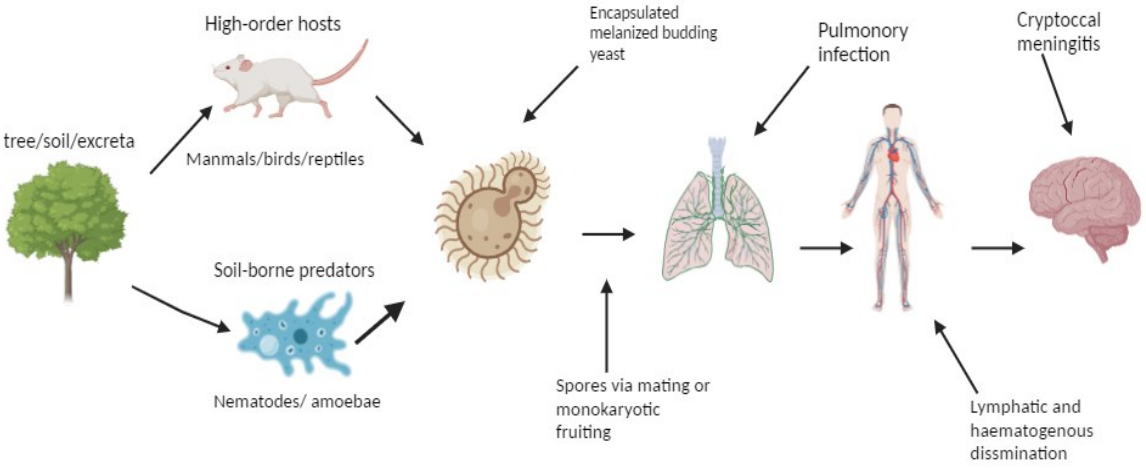

Figure 1: Transmission pathway of the fungi *Cryptococcus neoformans*. *C. neoformans* can survive in diverse environments including the soil, trees, and bird guano. These habitats facilitate interaction with animals or microbial predators. This contributes to survival and dissemination. Through inhalation of yeast cells or spores humans are invaded with the pathogen and a pulmonary form of the disease is formed. If the host is immunocompromised, either initially or due to subsequent conditions, the fungus can spread beyond the lungs, entering the bloodstream and crossing the blood-brain barrier (BBB) via microcapillaries to reach the central nervous system (CNS). CNS infection represents the most severe and life threatening form of the disease (Idnurm et al., 2005). Created with BioRender.com.

2.1 The life cycle

C. neoformans exists mainly in the form of budding yeast. It is also capable of a dimorphic transition to filamentous growth by two distinct pathways: mating and monokaryotic fruiting. Those pathways are shown in Figure 2.

The mating pathway is initiated under nutrient-limiting conditions. Two opposite haploid mating-type cells fuse to form dikaryotic filaments. Basidium is formed from the filaments followed by the process of nuclear fusion. Then mitosis and meiosis occur resulting in the production of basidiospores by budding. The second morphogenetic pathway, known as monokaryotic fruiting begins when haploid spores produce filaments and basidiospores in response to conditions such as nitrogen starvation, and water deprivation. In this pathway cells of one mating type form diploid monokaryotic hyphae, which generate a basidium, which triggers meiosis cell division. Formulation of blastospores and chlamydospores can also occur. Monokaryotic fruiting concludes with sporulation (Idnurm et al., 2005).

C. neoformans can produce large polyploid cells known as titan cells. Studies indicate that titan cells exhibit resistance to oxidative and nitrosative stresses and phagocytosis by host macrophages. The amount of titan cells correlates with the cryptococcal burden in the host's lungs. Titan cell formation was stimulated by coinfection with strains of opposite mating type. The creation of a titan cell is influenced by multiple factors such as host, nutrients, temperature, and pheromone (Okagaki et al., 2010a)**.** Titan cells represent an important virulence factor as they provide pathogens within the host until the immune system is compromised (Crabtree et al., 2012).

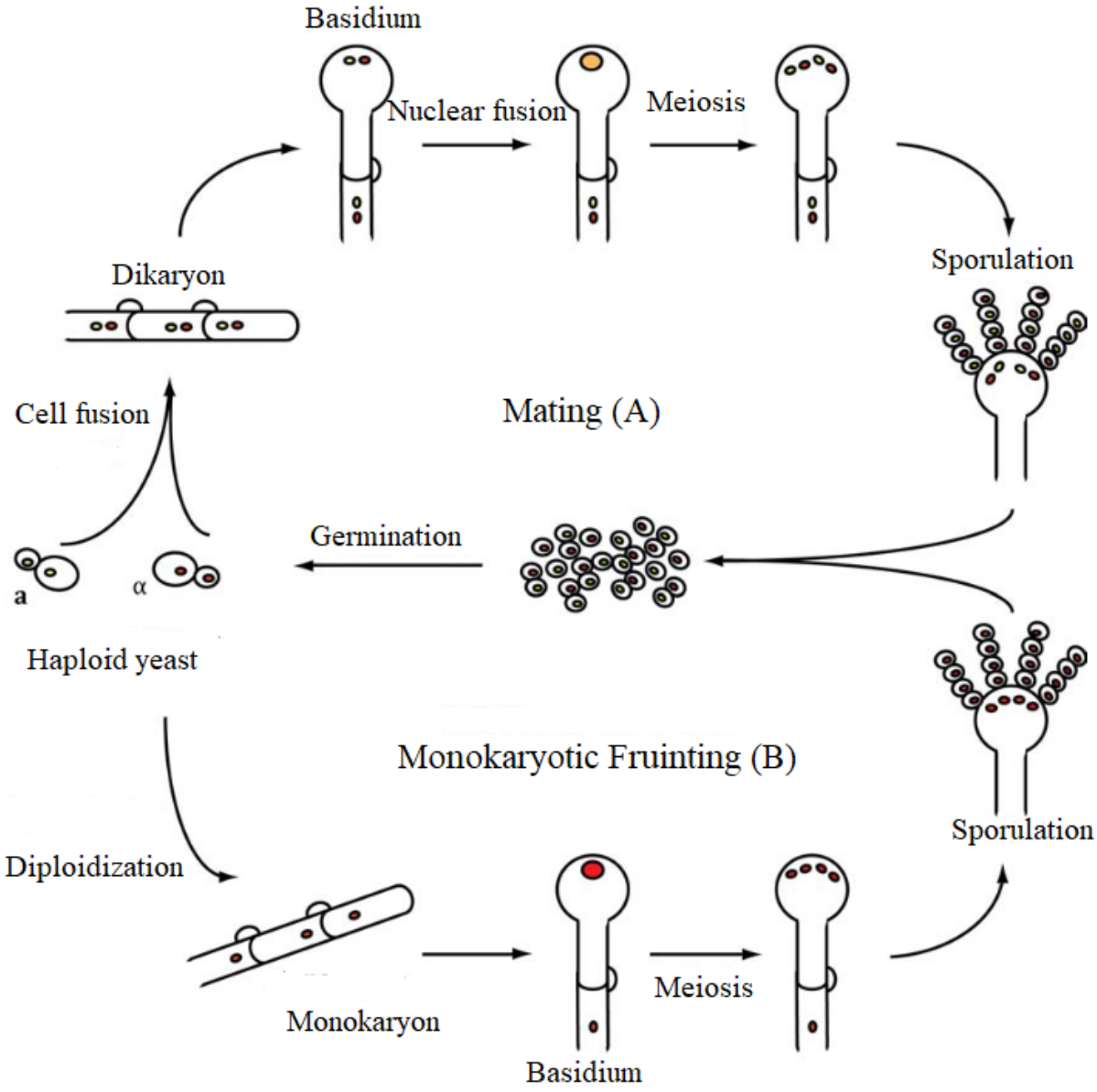

Figure 2: Reproductive processes of *Cryptococcus neoformans*. This figure illustrates the two main reproductive processes of *Cryptococcus neoformans*: A) sexual reproduction (mating) and (B) asexual reproduction (monokaryotic fruiting) (Voelz, 1988).

2.2 Infection in human host

Infection with *C. neoformans* is presumed to follow the inhalation of yeast cells into the lung from environmental sources that were mentioned earlier leading to cryptococcal pneumonia. In immunocompetent hosts, the initial infection is usually contained in a lung granuloma, triggering an antibody-mediated immune response. If the host was immunocompromised by other illnesses such as those with HIV, COVID-19, or undergoing organ transplant the infection could disseminate and cause significant problems for the host's body. Dissemination sites include CNS, where the disease manifests itself as cryptococcal meningitis, in blood as cryptococcemia, skin, and occasionally other organs (Zhou et al., 2024),

Despite the difficulties posed by host immunity, *C. neoformans* has evolved multiple mechanisms to overcome two critical barriers: evading the immune system and crossing the blood-brain barrier (BBB). The pathogen's entry into the brain involves specific mechanisms, followed by various virulence factors to ensure its survival and persistence.

2.2.1 Blood brain barrier

C. neoformans can cross the BBB with 3 distinct and different mechanisms: Trojan horse, transcytosis, and paracellular crossing. The Trojan horse mechanism involves phagocytes especially macrophages, which act as carriers for cryptococcal cells to be able to cross BBB using cellular migration (Alanio et al., 2015; Charlier et al., 2009; Vu et al., 2013). Transcytosis is a form of cellular transport in which exocellular cargo is endocytosed, transported through the cell cytoplasm in vesicles, and secreted at opposite membrane surface (Chang et al., 2011; Charlier et al., 2009).The paracellular crossing mechanism binds the host´s plasminogen to *C. neoformans* and is converted to the serine protease plasmin (Kim, 2008). Plasmin degrades the extracellular matrix (ECM), facilitating fungal crossing of the BBB. Cryptococcal urease, through the production of ammonia, and other secreted cryptococcal proteases can also damage the BBB by disrupting tight junctions. This damage further aids *C. neoformans* in breaching the barrier and invading the brain (Stie et al., 2009; Stie & Fox, 2012).

2.2.2 Cloak of Cryptococcus Neoformans

A closer look at the structure of the cell can reveal the arsenal of virulence factors for the fungi. The cloak of *C. neoformans* consists of three layers: capsule, cell wall, and plasmatic membrane. These three layers add complexity to the virulence of the cell and are also responsible for the difficulties in the drug design.

2.2.2.1 The capsule

The outermost structure of the *C. neoformans* cell is called the capsule. It is a hallmark feature and critical virulence factor. Studies show that it also helps to evade the host immune system. The two most prevalent polysaccharides that are in the capsule are glucoronoxylomannan (GMX) and glucoronoxylomanoglatan (GMXGal) with traces of mannoproteins (Cherniak et al., 1988; Vartivarian et al., 1989). These structural components contribute to its protective functions and enhance the fungus's survival in the host.

2.2.2.2 The cell wall

This cell structure is essential for the survivability of the cryptococcal cell. It protects the inner structures of the cell from various types of stresses such as osmotic pressure or some outer environmental dangers. One of the significant challenges the fungus faces is the host's immune system. The barrier that is provided by the cell wall is more than useful. The wall itself provides a structural scaffold for the capsule. The fungal cell wall consists of alpha and beta-linked glucans, chitin, chitosan, glycoproteins, and the pigment melanin (Wang et al., 1995; Reese et al., 2007). Electron microscopy reveals distinct layers within the wall: an inner and outer layer. The inner layer consists primarily of beta-glucans and chitin, with mannoproteins and melanin present, though they are found throughout the entire cell wall. The outer layer mainly contains alpha- and beta-glucans. (Vartivarian et al., 1989; McFadden et al., 2007).

The pathogenetic importance of the cell wall is mainly attributed to melanin and chitosan. Melanin is a negatively charged polymeric and hydrophobic pigment made from phenolic or indolic precursors (Nosanchuk and Casadevall 2006). Melanization serves as a tool to enhance the cryptococcal survival against predators such as amoebas and nematodes. Melanization also increases resistance to antifungal compound caspofungin (Martinez & Casadevall, 2006).Chitosan, which is produced by chitin synthase and chitin deacetylases, plays a key role in immune evasion by the host (Baker et al., 2007, 2011; Hembach et al., 2020)

2.2.2.3 The plasmatic membrane

Fungal membranes consist of molecules such as sterols, glycerophospholipids, and sphingolipids. Fungal membranes differentiate from those of mammals in containing ergosterol in place of cholesterol. Two major classes of antifungal drugs have exploited this feature: Amphotericin B, which binds ergosterol, and azoles, which inhibit its synthesis. *C. neoformans* also produces glycosylated ergosterols, termed sterolglycosides (Santiago-Tirado & Doering, 2016; Singh et al., 2017).

3 Diagnostic methods for cryptococcosis and cryptococcal meningitis

3.1 Methods

This chapter should give a summary of diagnostic options that can be used to obtain fast and precise diagnoses for patients infected with cryptococcal cells. Each method comes with its advantages and disadvantages and possible future direction in the diagnostic department of research. At the end of the chapter, there is a figure and table that summarize this chapter.

3.2 Cultivation methods

Fungal culture remains the golden standard in diagnostics of fungal diseases. When it comes to cryptococcal infection the best samples are taken from cerebrospinal fluid (CSF), blood, and urine. The sample is then put on a plate with specialized media that promotes rapid fungal growth. The advantages of this include its high specificity and sensitivity of the test. This helps to perfectly isolate the organism from the sample which helps with identification in patients. The disadvantage of culture-based methods is their time-consuming nature. Additionally, not all samples taken from the patient can have viable organisms. Despite the limitations, cultivation methods are the backbone of current fungal diagnostic methods (Kwizera et al., 2024).

3.3 Microscopic examination

India ink preparation involves mixing India staining with CSF to stain the sample. This staining technique allows the visualization of the characteristic capsule of *C. neoformans* under a microscope. The advantage related to this examination comes in its time effectiveness. This examination is done in minutes. It is a very simple and affordable technique. The downside of the research is that this method cannot differentiate dead and live cells. However, a major limitation is that it cannot distinguish between live and dead cells. Additionally, due to its relatively low sensitivity, India ink preparation cannot be used alone for accurate diagnosis. It should be combined with other diagnostic tests to ensure a reliable diagnosis (Abassi et al., 2015).

3.4 Antigen detection

Cryptoccocal antigen (CrAg) is used in antigen diagnostic methods. Latex agglutination assays can detect capsular polysaccharide antigens. The antigen detections is possible in serum or CSF samples. This method combines advantages from the previously mentioned tests such as high sensitivity and also quick results. The disadvantage is in false positive results that can occur and also in needing well-equipped laboratories that may not be in affected regions (Heelan et al., 1991).

Enzyme Immuno eassay is another antigen technique for instance Enzyme-Linked Immunosorbent Assays (ELISA) that detects specific antigens in serum or CSF. The assay involves coating a microplate with antibodies specific to the cryptococcal antigen. The benefit of this method is its high sensitivity and specificity. Other important advantages are quantitative results and the versatility of this method. Disadvantages come with the cost of the method. With antigen tests, there is always an issue with false positives or false negatives results (Panackal et al., 2014).

Lateral Flow Assays are rapid tests that utilize capillary action to move a liquid sample along a test strip containing specific antibodies for *C. neoformans* antigens. When the sample is applied, if the target antigen is present, it binds to the antibodies on the strip and produces a visible line or color change at a designated test line. The advantages of the method are rapid results and availability in low-resource settings. Disadvantages are lower sensitivity and also it has qualitative results only (Kamble et al., 2021a, 2021b).

3.5 Molecular techniques

The essential technique in the molecular examination is the polymerase chain reaction (PCR) method targeting the DNA sequence of *C. neoformans*. This provides fast results directly from clinical samples of patients. The benefit of PCR lies in its sensitivity that is close to 100% and as mentioned before in swiftly obtained results. The drawbacks of PCR are the same as in antigen testing. The main drawback that should be addressed is the affordability and accessibility of molecular tests, especially PCR, in developing countries, which are affected by fungi pathogens the most (Bialek et al., 2002; Christo et al., 2016).

3.6 Imaging techniques

While imaging techniques such as computed tomography scan (CT) scans are not part of diagnostics they add an extra layer to clinical studies and help understand better the status of affected patients when the proper treatment is prescribed (Dantas et al., 2023).

3.7 Overview of diagnostic methods

In summary, the diagnosis of *C. neoformans* infections involves a combination of traditional and molecular methods, each with its advantages and disadvantages. The choice of diagnostic method depends on the availability of resources, the need for rapid diagnosis, and the clinical presentation of the patient. The following table (Table 1) and diagram (Figure 3) provide a summary of the diagnostic chapter.

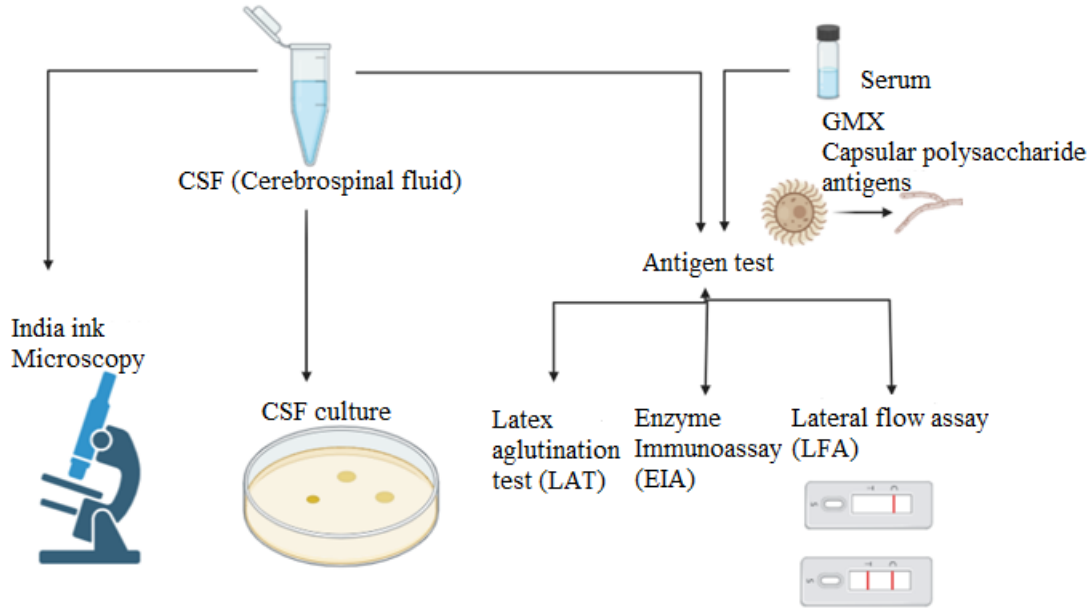

Figure 3: Overview of diagnostic methods used for cryptococcal diagnosis This figure illustrates the variety of diagnostic choices used for the diagnosis of cryptococcosis and cryptococcal meningitis. On the top, there are the two main samples that are used as diagnostic materials and then which method uses which material (Zhao et al., 2023). Created by Biorender.com

Diagnostic Method	Sensitivity	Specificity	Time to Result	Advantages	Disadvantages
Fungal Culture	High	High	Days	Gold standard; isolates organism	Time-consuming; requires viable organisms
India Ink Microscopy	Low (<90%)	Moderate	Minutes	Quick; inexpensive	Low sensitivity; cannot differentiate cells
Latex Agglutination Test	High (>90%)	Moderate	Hours	Quick results; good sensitivity	False positives possible
PCR	Very High (~100%)	Very High (100%)	Hours	Fast; high accuracy	Higher costs; requires technical expertise
Imaging	Not applicable	Not applicable	Variable	Aids in clinical assessment	Not definitive for diagnosis
Enzyme Immunoassays	High	High	Hours	Quantitative results; versatile	Costly; complex; risk of false results
Lateral Flow Assays	Moderate	Moderate	Minutes	Rapid; easy to use; cost-effective	Lower sensitivity; qualitative results only

Table 1: Summary of all methods and their advantages and disadvantages This table shows all the methods mentioned and all of the features important in *C. neoformans* diagnostics (Dantas et al., 2023).

4 Cryptococcus neoformans infection treatment

4.1 Antifungal treatment

When focused on the treatment of fungal diseases, multiple factors are considered. The key factor in drug design is that they should be harmless for the immunocompromised and as little toxic as possible. Importance is given to the fact that the drugs will also work properly for immunocompetent individuals. If the disease reaches the brain, we have to have good access to the brain through the BBB. The emerging fungal problems are connected to the resistance against various drugs so thus treatment has to constantly evolve and progress. The therapies that will be stated in the next part of this thesis are divided into current therapy and possible future therapies.

4.2 Treatment Overview

The therapy will likely be differentiated into 3 parts: induction, consolidation, and maintenance therapy. Induction and consolidation therapy is the initial therapy against all forms of cryptococcal infections in all disease progression. The drugs used in treatment are amphotericin B (AMB) and its most used form is liposomal amphotericin B (LAMB), flucytosine. The maintenance therapy is induced with fluconazole or voriconazole (Ngan et al., 2022).

C. neoformans infection is treated with prescription antifungal medication for at least 6 months, often longer. The type of treatment depends on the severity of the infection and the parts of the body affected. (Guo et al., 2016).

Severe lung infections or central nervous system infections require initial treatment with AMB in combination with flucytosine, followed by fluconazole. The type, dose, and duration of antifungal treatment may differ for certain groups of people, such as pregnant women, children, and those in resource-limited settings. Some patients may also need surgery to remove fungal growths.

5 Drugs used in treatment

5.1 Amphotericin B/ Liposomal Amphotericin B

Amphotericin B (AMB) is a polyene antifungal agent with a broad spectrum of activity against yeast and molds, as well as the parasite *Leishmania sp*. The liposomal formulation Amphotericin B (LAMB) targets ergosterol, a prevalent sterol in cell membranes in many fungi and protozoa. AMB binds to ergosterol in the fungal cell membrane, leading to the formation of pores, ion leakage, and ultimately fungal cell death. The precise mechanism by which AMB is transferred from the liposome through the fungal cell wall to the fungal membrane is not known (Gray et al., 2012)

The first known version of amphotericin was amphotericin B deoxycholate (DAMB). It was developed in the 1950s. This version was the only version that could treat fungal illness. For many decades DAMB was the only antifungal agent available for the treatment of invasive fungal diseases. However, the significant dose-limiting toxicity of DAMB is most notably nephrotoxicity and infusion-related reactions. Infusion-related toxicity is a recognized side effect of DAMB, causing acute fevers and chills, possibly due to a proinflammatory cytokine response mediated by toll-like receptor 2 (TLR2) and cluster of differentiation 14 (CD14). That provided an impulse to develop new less toxic formulations, LAMB is consistently the least nephrotoxic of all commercially available lipid formulations of AMB (Sau et al., 2003).

Liposomal amphotericin B is a unique lipid formulation of AMB that has been used for nearly 20 years to treat a broad range of fungal infections. The unilamellar lipid structure of LAMB has two major components. The first is hydrogenated soy phosphatidylcholine, which comprises the majority of the lipid bilayer. It has the advantage of a gel to liquid-crystal phase transition point of 37°C meaning it is not readily hydrolyzed at body temperature The second component, cholesterol, was added as it binds AMB and further facilitates the retention of AMB within the liposome bilayer (Stone et al., 2016).

Resistance to AMB is rare and often caused by a decrease in the amount of ergosterol in the plasma membrane or a change in the target sterol, which leads to a decrease in the binding of AMB. Some fungal cells have a mutation in the ergosterol biosynthesis pathway, producing ergosterol-like compounds instead of ergosterol, which have a lower binding affinity for AMB (Stone et al., 2016).

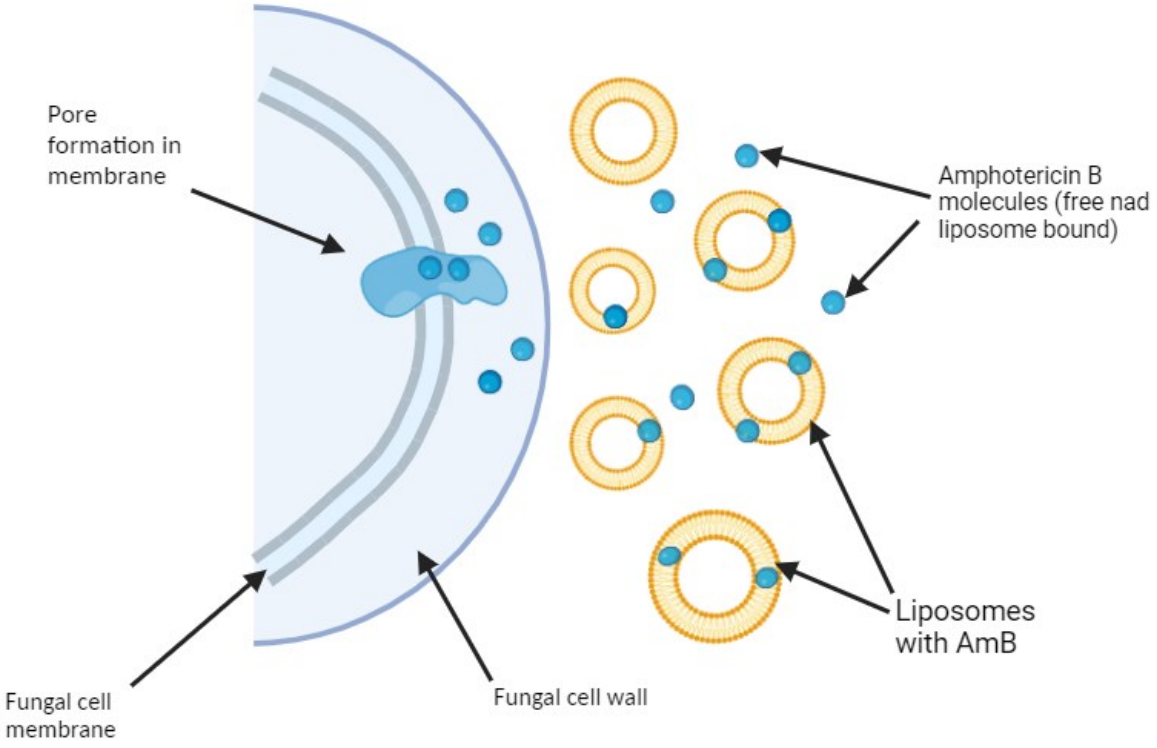

Figure 4: Illustration presenting liposomal Amphotericin B and it´s mechanism of action: Free molecules of AMB, which are protein-bound and liposome-associated circulate in the bloodstream. The liposomal vesicles with LAMB preferably attach to the fungal cell wall. Molecules of amphotericin are released and form pores in the cell membrane. The precise process of transferring molecules through the cell wall and membranes is unknown (Stone et al., 2016). Created with Biorender.com.

5.2 Flucytosine

The synthesis of fluorinated pyrimidines was first documented in 1957, with flucytosine emerging as a synthetic fluorinated analog of cytosine (Bender et al., 1957). The specific compound used for cryptococcal treatment is known as flucytosine (5-FC).

In *Cryptococcus* cells, 5-FC is imported through an energy-dependent process mediated by the cytosine permease enzyme. Once inside the fungal cell, 5-FC is rapidly deaminated by the cytosine deaminase enzyme, producing its active form, 5-fluorouracil (5-FU). The 5-FU is subsequently metabolized into 5-fluorouridine triphosphate, which incorporates into fungal RNA, replacing uridylic acid. This substitution disrupts RNA synthesis and inhibits protein production. Additionally, 5-FU is converted into 5-fluorodeoxyuridine monophosphate, which blocks thymidylate synthetase activity, thereby halting DNA synthesis (Sigera & Denning, 2023). These dual mechanisms account for the antifungal activity of 5-FC and they are shown in (Figure 5).

Unlike fungal and prokaryotic cells, human cells lack the cytosine deaminase enzyme needed to convert 5-FC into its active form. This selective activation makes 5-FC safe for human use at pharmacological doses. However, adverse effects may occur when plasma concentrations exceed 100 μg/ml or in cases of renal dysfunction. Toxicity can also arise from the conversion of 5-FC to 5-FU by the human gut microflora (O'Connor et al., 2013)

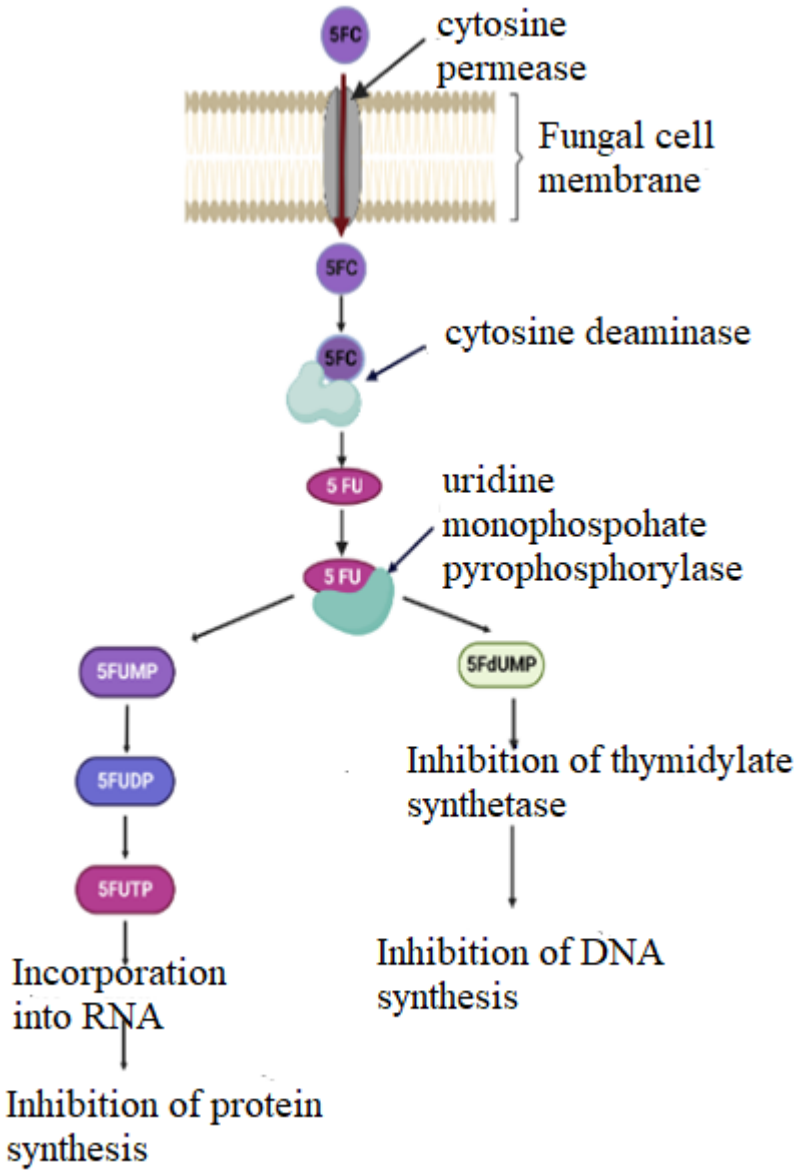

Figure 5: Schematic showing the action mechanism of 5-FC. The step by step graphic shows the process of flucytosine affecting cryptococcal cell from passing through the fungal cell membrane to inhibition of synthesis of either protein or DNA.
(Sigera & Denning, 2023).

5.3 Fluconazole

Fifty-two different azoles are inhibited by the C-14 demethylation. This step is crucial for the synthesis of ergosterol, the essential sterol in the fungal membrane. Among those azoles is Fluconazole and voriconazole. Fluconazole is a fluorine-substituted, bis-triazole antifungal agent. Its mechanism of action, like that of other azoles, involves interruption of the conversion of lanosterol to ergosterol via binding to fungal cytochrome P-450 and subsequent disruption of fungal membranes. Fluconazole (FLC) was evaluated against Candida infections in leukemia, cancer and AIDS patients. One of the most exciting results with FLC has been its efficacy against cryptococcal meningitis in acquired immune deficiency Syndrome (AIDS) patients. FLC can be administered both orally and intravenously. FLC is also approved for initial and suppressive therapy of cryptococcal meningitis. It is hypothesized that the result and accumulation of 14-methylsterols and altered membrane fluidity caused increased activity of membrane-bound desaturase with a subsequent increase acids. This leads to disturbances in fungal membrane permeability. Other types of azoles are used against IDF for example Voriconazole. The side effects caused by the toxicity of fluconazole include nausea, vomiting, and abdominal distress, elevated liver function tests (Pasko et al., 1990; Richardson, 1990)

Voriconazole as mentioned earlier is a derivate of FLC. This is a broad-spectrum agent working as an inhibitor for cytochrome P450-dependent 14-lanosterol demethylation. This demethylation is crucial for ergosterol synthesis in cell wall. Limited information is available about the clinical usage of voriconazole against cryptococcosis. Effects that voriconazole has on *C. neoformans* such as viability, melanin production, polysaccharide release, and cell and capsule size are areas ripe for future studies in animal models of cryptococcosis. Furthermore, these results support continued clinical investigations of the use of voriconazole for cryptococcosis (Van Duin et al., 2004). Azoles as a group are certainly po int of interest for future antifungal research cause other forms of tetraazoles with broad spectrum of activity has been shown.

6 Emerging Treatments and Research

This chapter focuses on selecting potential additions to classic treatment and potentially enhancing the curability for patients. The drugs are variable from the repropose of others such as Sertraline or Miltefosine. At the end of the chapter, there is a picture (Figure 6) showing all the drugs mentioned in the chapters 5 and 6.

6.1 Interferon Gamma

Standard treatment with AMB and flucytosine can be enhanced with IFN shown promise with a significant increase of clearance of cryptococcal infection from the CSF of patients with HIV-associated cryptococcal meningitis. Two doses of IFN-gamma were as effective as a full 2-week course, and the addition of adjunctive IFN-gamma was not associated with any increase in drug-related adverse events. It is assumed that the mechanism of action comes from the activation of effector cells such as macrophage and microglia cells. This stimulation enhances intracellular killing and prefers Th2-type immune response protective rather than Th1. IFN-gamma produced either peripherally or in the CNS, has been shown to stimulate microglial cell activation and anticryptococcal activity. (Pappas et al., 2004).

Data from this demonstration study present idea that augmentation of host's immune system through incorporating of adjunctive immunotherapy can have positive effect on patients with invasive fungal infection (Jarvis et al., 2012).

6.2 Mycograb®

Mycograb® is genetically recombinant antibody. The target of this drug is heat shock protein 90 (HSP90) protein of *Candida albicans*. This sequence is conserved (KILKVIKK) with the corresponding protein in *C. neoformans*. In *C. neoformans*, hsp90 has not been extensively studied. Interactions with AMB suggest that Mycograb® is clinically significant and could potentially improve outcome of treatment in patients infected by *C. neoformans* because the levels of drugs required are therapeutically achievable. This combination might allow the dosage of one of the already used agents in treatment such as AMB or fluconazole therefore reducing the toxicity of the treatment. The combination of AMB and Mycograb® in patients with infection due to *C. neoformans* will be assessed soon in clinical trials (Nooney et al., 2005).

6.3 APX001 (Fosmanogepix)/APX001A (Manogepix)

APX001, the prodrug of APX001A, is a first-in-class broad-spectrum antifungal agent in clinical development for the treatment of life-threatening invasive fungal infections. The antifungal mechanism comes with APX001A ability to inhibit the fungal enzyme Gwt1 of the glycosylphosphatidylinositol (GPI) biosynthesis pathway by preventing inositol acylation during synthesis of GPI-anchored proteins. The disruption of the GPI biosynthesis creates pleotropic effects such as cell wall integrity compromission which leads to reduction in fungal virulence attributes. This ultimately leads to fungal growth defects. APX001A/APX001 has potent broad in vitro and in vivo activity against major fungal pathogens, including *Candida, Cryptococcus, Aspergillus, Scedosporium*, and *Fusarium* strains, regardless of their azole resistance and echinocandin resistance, which is consistent with the distinct mechanism of action Similarly, mouse pharmacokinetic studies of all three prodrugs suggest that exposures after oral dosing are equivalent to or better than those of APX001, raising the possibility that other Gwt1 inhibitors can also be developed for oral administration, enabling new and rapidly effective all-oral regimens for the treatment of cryptoccal meningitis either alone or in combination with FLC (Shaw et al., 2018; M. Zhao et al., 2019).

6.4 T-2307

T-2307 is a novel arylamidine and is now undergoing clinical trials. Previous reports show that T-2307 has broad-spectrum effect *in vitro* and *in vivo* against majority of fungal pathogens, including *Candida albicans, Aspergillus fumigatus*, and *Cryptococcus neoformans*. T-2307 can be promising new candidate for the treatment of cryptococcosis. T-2307 exhibits excellent antifungal activity against fluconazole-resistant *Candida spp*. It actively disrupts mitochondrial functions of yeast cells. Drugs with similar chemical structure such as pentamidine and furamidine are used against for example *Pneumocystis jirovecii* and antimony resistant leishmaniasis. Reports show that the pentamidine inhibits topoisomerases and disturbs the mitochondrial function(Nishikawa et al., 2017; Shibata et al., 2012).

6.5 Sertraline

Sertraline is another repurposed drug that might enhance treatment options for cryptococcosis especially the most dangerous form of cryptococcal meningitis. The benefit of sertraline is that it may avoid the creation of resistance in *C. neoformans*. Sertraline's addition in the treatment of cryptococcus provides the ability to accumulate in CNS to other antifungals given the fact that *C. neoformans* proliferates in the brain. The effect displays broad spectrum ability. Sertraline studies indicate influence on vesicle and transport disruption in fungi. Sertraline has shown antibacterial, antiparasitic, antiviral and antitumoral properties. Recent evidence indicates that the antifungal mechanism of sertraline is probably through perturbation of translation and inhibition of fungal protein synthesis. Further randomized clinical trials need to be performed to demonstrate the relevance of sertraline as an antifungal agent for meningitide cryptococcosis (Trevinõ-Rangel et al., 2016; Zhai et al., 2012)

6.6 Tamoxifen

Tamoxifen belongs to the category of selective oestrongen receptor modulator, which main usage comes in treating breast cancer. In repurpose trials, Tamoxifen has shown excellent bioavailability and is also concentrated within macrophages. It has been shown that tamoxifen appears to have a synergistic effect when combined with amphotericin and importantly enough there was no evidence of antagonism between tamoxifen and any of amphotericin, fluconazole, or flucytosine. When combined the three drugs synergistic interaction were preserved. In addition, combining the three drugs also seemed to deliver a synergistic interaction in one of the clinical strains where synergy had not been apparent for the dual drug combination (Hai et al., 2019).

6.6 AR-12

AR-12 has progressed as a targeted anticancer therapy. The structure of AR-12 on chemical level is an optimized scaffold for antifungal treatment as part of repurposing. The effect was seen against pathogenic yeast, molds, and dimorphic fungi. One of the most promising features of AR-12 is that in vitro it showed promising treatments against not only *C. neoformans* but also against extremely difficult molds. For example, treatment focuses on *Fusarium*. The spectrum of activity suggests that the scaffold should be optimized further. The existing resistance in *Candida albicans* for echinocandins and azole could also be solved with AR-12. The mechanism of action is that inhibits pump function or AR-12 decreases carbon flux through the ergosterol biosynthesis pathway, reducing the amount of FLU needed to achieve an antifungal effect. Further study of efflux pumps needs to be focused to understand the role of AR-12 (Koselny et al., 2016).

6.7 Miltefosine

Miltefosine (MFS) belongs to the alkylphosphocholine class of molecules, which are used in the treatment of cutaneous metastases of breast cancer and leishmaniasis. In repurposing studies it had shown effects with antifungal significance against Cryptococcus spp. Additionally, MFS also exhibits a dose-dependent prolonged post-antifungal effect (PAFE), further supporting its therapeutic potential. The spectrum of activity is large, which indicates a good candidate for future treatment (De Castro Spadari et al., 2018).

MFS mechanism of action against Cryptococcus involves interactions with ergosterol similar to AMB. It does not only effect cell wall structural components but other organelles of fungal cell s too. It mitochondria swelling and impairing of function can be seen. Damage is reflected in capsule synthesis, which is dependent on proper function of the mitochondria (De Castro Spadari et al., 2018).

In addition to structural damage of mitochondria. Production of ROS is initiated which induces oxidative stress. The accumulation of ROS leads to widespread damage to critical fungal components, including the plasma membrane, proteins, DNA, and mitochondria. This oxidative stress, coupled with mitochondrial dysfunction, triggers DNA fragmentation, chromatin condensation, and fungal cell apoptosis (De Castro Spadari et al., 2018).

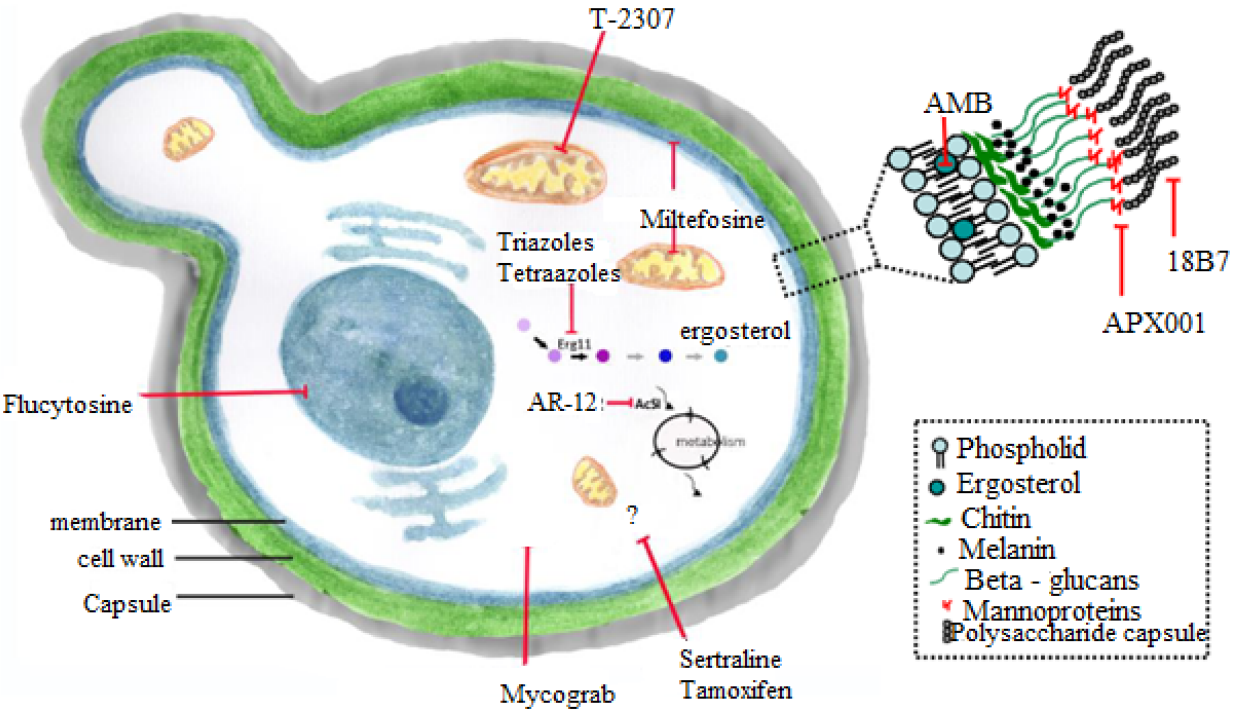

Figure 6: Cell with description and legend: This picture illustrates the structural components of a fungal cell with emphasis on its membrane, cell wall, and capsule, highlighting key antifungal drugs and their targets**.** (Spadari et al., 2020).

7 Conclusion

This thesis aimed to provide an overview of the current state of diagnostic and treatment options for *C. neoformans* and cryptococcal infections while exploring the possible opportunities for further research. *C. neoformans* pose thread as one of the most dangerous pathogens. This thread is even greater in regions with higher rates of immune suppressing diseases such as AIDS or in resource-limited developing countries. Addressing this fungal pathogen emergency requires more financial support as the crisis progresses.

The current state of diagnostics and diagnostical methods includes a wide variety of molecular, cultivation, and serological techniques with diverse levels of sensitivity and specificity. However, accessibility creates barriers with some costly options in the diagnostic department in developing countries. Research should focus on developing affordable, rapid, and reliable diagnostic options in resource-limited settings.

Similarly to diagnostics treatment options are heavily limited by the number of drugs on the market. These treatment options such as AMB, 5-FU, and FLC despite their efficacy come with limitations such as toxicity and cost. The emergence of resistance is also troubling. Future efforts should prioritize the development of less toxic antifungal agents, repurposing existing drugs, and exploring new therapeutic strategies, including adjunct therapies and immune-based approaches.

Rapid and accurate diagnosis, coupled with effective and accessible treatment, is crucial for improving clinical outcomes in cryptococcosis. Continued research and innovation are imperative to address the unmet needs in fungal diagnostics and therapeutics. By prioritizing affordability, accessibility, and safety, the global medical community can make significant strides in combating *C. neoformans* and other fungal pathogens, ultimately saving lives and improving public health outcomes.

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