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REVIEW ARTICLE

Peptidases: promising antifungal targets of the human fungal pathogen, *Cryptococcus neoformans*

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Abstract

Cryptococcus neoformans is a globally important fungal pathogen, primarily inflicting disease on immunocompromised individuals. The widespread use of antifungal agents in medicine and agriculture supports the development of antifungal resistance through evolution, and the emergence of new strains with intrinsic resistance drives the need for new therapeutics. For *C. neoformans*, the production of virulence factors, including extracellular peptidases (e.g., CnMpr-1 and May1) with mechanistic roles in tissue invasion and fungal survival, constitute approximately 2% of the fungal proteome and cover five classes of enzymes. Given their role in fungal virulence, peptidases represent promising targets for anti-virulence discovery in the development of new approaches against *C. neoformans*. Additionally, intracellular peptidases, which are involved in resistance mechanisms against current treatment options (e.g., azole drugs), as well as capsule biosynthesis and elaboration of virulence factors, present additional opportunities to combat the pathogen. In this review, we highlight key cryptococcal peptidases with defined or predicted roles in fungal virulence and assess sequence alignments against their human homologs. With this information, we define the feasibility of the select peptidases as "druggable" targets for inhibition, representing prospective therapeutic options against the deadly fungus.

Key words: proteases, antifungals, anti-virulence, *Cryptococcus neoformans*, protease inhibitors, peptidases, virulence factors

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1. Introduction

Cryptococcus neoformans is an encapsulated human fungal pathogen responsible for 15% of HIV/AIDS-related deaths worldwide (Sloan and Parris 2014; Rajasingham et al. 2017). The fungus is found ubiquitously within the environment, generally associated with pigeon droppings and various tree species (e.g., eucalyptus), infecting hosts when spores or desiccated fungal cells are inhaled (May et al. 2016). Once inside the lungs, alveolar macrophages phagocytose fungal cells; however, within immunocompromised individuals, fungal cells can escape from the protective barrier of the innate immune system and disseminate throughout the body (Vu et al. 2019). During dissemination, cryptococcal cells can invade the central nervous system following crossing of the blood–brain barrier (BBB) using three proposed mechanisms: (*i*) transcytosis (i.e., yeast cells transit directly through endothelial cells), (*ii*) transcellular traversal (i.e., penetration of pathogens between brain

microvascular endothelial cells), and (*iii*) Trojan Horse-like mechanism (i.e., carriage inside an infected macrophage) (Sorrell et al. 2016; Santiago-Tirado et al. 2017; Casadevall et al. 2018). If left untreated or in the presence of antifungal resistant strains, cryptococcosis leads to meningoencephalitis or inflammation of the meninges, the major cause of mortality and morbidity in immunocompromised individuals. Notably, within immunocompetent individuals, the fungus may be cleared upon inhalation; cause bloodstream, pulmonary, or cutaneous infections; or lay dormant and become activated if the immune status of the host changes (Min et al. 2020; Qu et al. 2020).

Treating cryptococcosis varies depending on infection severity and host immune status, with reliance on three distinct classes of antifungals and their mechanisms of action: (*i*) polyenes (pore formation and leakage of cellular components), (*ii*) flucytosine (inhibition of DNA and RNA synthesis), and (*iii*) azoles (ergosterol synthesis inhibition and cell wall integrity impairment) (Perfect 2017; Geddes-McAlister and Shapiro 2019; Bermas and Geddes-McAlister 2020). The widespread use of antifungal agents in medicine and agriculture promotes the development of antifungal resistance through the evolution of resistant strains (Geddes-McAlister and Shapiro 2019). Moreover, the emergence of new fungal strains and species with intrinsic resistance towards common antifungals presents a growing threat. In general, the degree of resistance developed by fungal pathogens varies depending on the class of antifungals administered, and a primary challenge for the development of new antifungals is target similarity between eukaryotes (i.e., fungus and human).

To overcome these challenges, novel therapeutic strategies are needed. One strategy involves the targeting of *C. neoformans* virulence factors (e.g., growth temperature, polysaccharide capsule, melanin and extracellular enzymes) as potential anti-virulence targets (Siscar-Lewin et al. 2019; Vu et al. 2019; Aaron et al. 2020). This approach has several advantages, including that virulence factors are well-characterized and disarming these factors, instead of killing the pathogen itself, can reduce rates of antifungal resistance by imposing a weaker selective pressure. Various studies have assessed the possibility of targeting extracellular enzymes to reduce or inhibit virulence of *Cryptococcus* spp. (Olszewski et al. 2004; Shi et al. 2010; Vu et al. 2014). Likewise, *C. neoformans* produces an array of extracellular and intracellular peptidases associated with virulence and drug resistance, and exploration of the relevance behind targeting such proteins using inhibitors, conveys promising results (Muller and Sethi 1972; Brueske 1986; Bien et al. 2009; Kryštůfek et al. 2021). However, validation of peptidases as therapeutic targets is difficult because of a limited understanding of precise mechanisms of action, kinetic characteristics, biological roles, and homology with human enzymes. In this review, we present a distribution of peptidases within *C. neoformans* and describe representative enzymes with relevance in virulence, host invasion, and drug resistance. Moreover, through protein alignment strategies, we identify human homologs of the fungal peptidases and highlight commonalities and differences between the sequences, including conservation of active sites. With this information, we propose promising new protease inhibitor targets that may be leveraged as alternative therapeutic options in the future.

2. Peptidases: roles in fungal virulence and antifungal resistance

Peptidases account for almost 6% of total proteins in the human genome and constitute 1–5% of the genomes for bacteria, viruses, and fungi (Barrett 2004). These enzymes are used as virulence factors by many pathogenic microorganisms, including *C. neoformans*, and they constitute promising targets to combat antifungal resistance, as they impose a weaker selective pressure, restricting the evolution of resistance. For *C. neoformans,* the proteome consists of 7,429 proteins (Uniprot database 31 August 2021; uniprot.org) with 158 peptidases reported, representing approximately 2% of the total proteome (UP000010091) (**Fig. 1**).

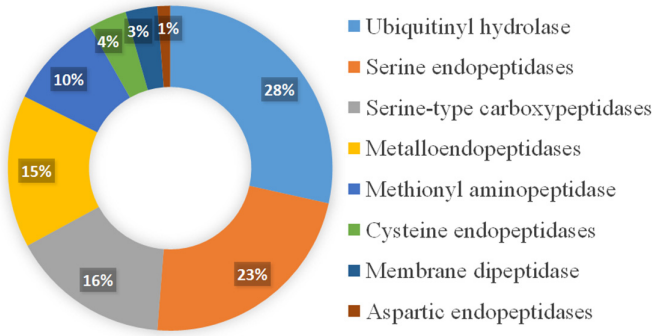

Fig. 1. Distribution of characterized peptidases from *Cryptococcus neoformans*. Figure was prepared using data from proteome UP000010091 and Uniprot statistics (uniprot.org).

Table 1. Representative cryptococcal peptidases related to virulence and antifungal resistance.

Peptidase	MW (kDa)	Family	Cellular localization	Pathogenic role	Reference
Carboxypeptidase D (CNAG_00919)	62	Serine peptidase (EC: 3.4.16.–)	Extracellular	Host protein degradation, melanin synthesis[a]	Steen et al. (2002); Clarke et al. (2016)
Cerevisin (CNAG_04625)	53.6	S8B subtilisin-like serine peptidase (EC: 3.4.21.–)	Extracellular	Melanin synthesis	Eigenheer et al. (2007); Clarke et al. (2016)
May1 (CNAG_05872)	51.3	A1 Pepsin-like aspartyl peptidase (EC:3.4.23.–)	Extracellular	Immune system evasion, brain invasion, biofilm formation[a]	Clarke et al. (2016)
Pqp1 (CNAG_00150)	100.9	S8 subtilisin-like (EC: 3.4.21.–)	Membrane-associated	Quorum sensing	Homer et al. (2016)
Rim13 (CNAG_05601)	89.6	C2A cysteine peptidase (EC: 3.4.22.–)	Intracellular	Capsule synthesis	Caza and Kronstad (2019)
CnMpr-1 (CNAG_04735)	70	M36 metallo-peptidase (EC: 3.4.24.–)	Extracellular	Brain invasion	Vu et al. (2014)
Stp1 (CNAG_05742)	65.1	Metallo-peptidase (EC: 3.4.24.85)	Intracellular	Antifungal resistance	Bien et al. (2009)

[a]Putative.

Cryptococcal peptidases compromise several classes, including: (*i*) endopeptidase, which cleaves peptide bonds within a polypeptide or protein (43%); (*ii*) ubiquitynil hydrolase, members of the ubiquitin-proteasome pathway (28%); (*iii*) carboxypeptidase, which cleaves a peptide bond at the carboxy-(C)-terminal of a protein or peptide (16%); (*iv*) aminopeptidase, which cleaves amino acids from the amino-(N)-terminus of proteins or peptides (10%); and (*v*) dipeptidase, which cleaves bound pairs of amino acids of proteins or peptides (3%). These classifications support the diverse roles and importance of peptidases within the human fungal pathogen. Specifically, for *C. neoformans*, extracellular peptidases from different classes assist with a variety of pathogenic roles, from host protein degradation to tissue invasion, and intracellular or membrane-associated peptidases are involved in antifungal resistance and quorum sensing to regulate virulence (**Table 1**).

2.1. Melanin production, thermotolerance, and oxidative damage protection

Melanin is a heterogeneous pigment formed by the oxidative polymerization of indolic or phenolic compounds, protecting cryptococcal cells against oxygen- and nitrogen-derived oxidants produced by host effector cells (Wang and Casadevall 1994). Beyond absorption of free radical fluxes, melanin also contributes to acquired resistance against antifungals, such as Amphotericin B and Caspofungin (Ikeda et al. 2003; van Duin et al. 2020). Furthermore, cryptococcal cells are mesophilic (i.e., grow well at moderate temperatures) and, thus, not well adapted to survive inside the mammalian host where temperature is high (e.g., 37 °C in humans) (Maliehe et al. 2020). Melanin protects fungal cells from elevated temperatures and permits harvesting of host thermal energy to fulfil fungal energetic needs (Dadachova et al. 2007). In *C. neoformans*, melanin is produced by oxidation of L-3,4-dihyroxyphenylalanine (L-DOPA) via laccase, initiating a series of spontaneous reactions that polymerize the pigment (Eisenman and Casadevall 2012).

2.1.1. Serine-type carboxypeptidase D

During melanin formation, an elevation of enzymes (e.g., hydroxylases or laccases) is observed, as well as an increase in peptidase activity, like carboxypeptidase D (CNAG_00919) and subtilisin-like serine peptidases (Clarke et al. 2016). Although, precise mechanisms of peptidase involvement in melanin production are unclear, subtilisin-like serine peptidases may impact the maturation of enzymes related with phenolic metabolism. For instance, given the role of subtilisin members from higher eukaryotes (e.g., mammals or yeasts) as proprotein convertases (e.g., kexin family or S8B subfamily), a family of proteins that activate other proteins, this may support a connection to melanin production (Gongora et al. 2021). Conversely, carboxypeptidase D, which possesses broad specificity and is responsible for exopeptidase activity outside the cell, may provide free amino acids, such as tyrosine (precursor of L-DOPA), from extracellular protein sources and contribute to melanin synthesis (Clarke et al. 2016).

Carboxypeptidase D (EC: 3.4.16.–) is a serine peptidase belonging to the S10 family (Rawlings et al. 2018). Although a homology search indicated that human carboxypeptidase is closely related to the fungal enzyme, the human carboxypeptidase shares 99.5% sequence identity (including the signal peptide) with the heavy polypeptide chain of human lysosomal protective protein (EC:3.4.16.5). This information indicates that carboxypeptidase is an isoform of the polypeptide chain (**Fig. 2**). Considering these results, we selected human lysosomal protective protein (a heterodimer lysosomal carboxypeptidase related to the regulation of stability and activity of ß-galactosidase and neuraminidase) as the closest human protein to fungal carboxypeptidase D for further analysis (Galjart et al. 1991). These enzymes share high sequence identity (41.7%); however, overlapping regions contain only one amino acid from the active center of the human peptidase (S178), suggesting that these enzymes belong to the same family but are not homologs (**Fig. 2**). In fact, human lysosomal protective protein is also known as carboxypeptidase C, carboxypeptidase L, and cathepsin A (CTSA). These results indicate that the fungal and host proteins share higher sequence similarity on exosites than at the active sites, possessing different catalytic features and specificities, supporting the design of competitive inhibitors. Additionally, both enzymes are situated in different cellular locations (e.g., endoplasmic reticulum, lumen, or membrane for the human enzyme vs. extracellular space for the fungal enzyme), which highlights carboxypeptidase D as a feasible target against cryptococcosis.

2.1.2. Kexin-like cerevisin

Another candidate peptidase important in melanin production includes cerevisin (CNAG_04625), a secreted serine peptidase identified in the cryptococcal secretome but at baseline levels, suggesting strict substrate specificity and regulation (Clarke et al. 2016). Although a connection with melanin

A

ENTRY	PROTEIN NAMES	IDENTITY
A0A7I2V4Q9	**Carboxypeptidase**	39.1%
P10619	**Lysosomal protective protein**	41.7%
A0A7I2V3M2	**Carboxypeptidase**	41.7%
U3KQU6	**Carboxypeptidase**	41.7%

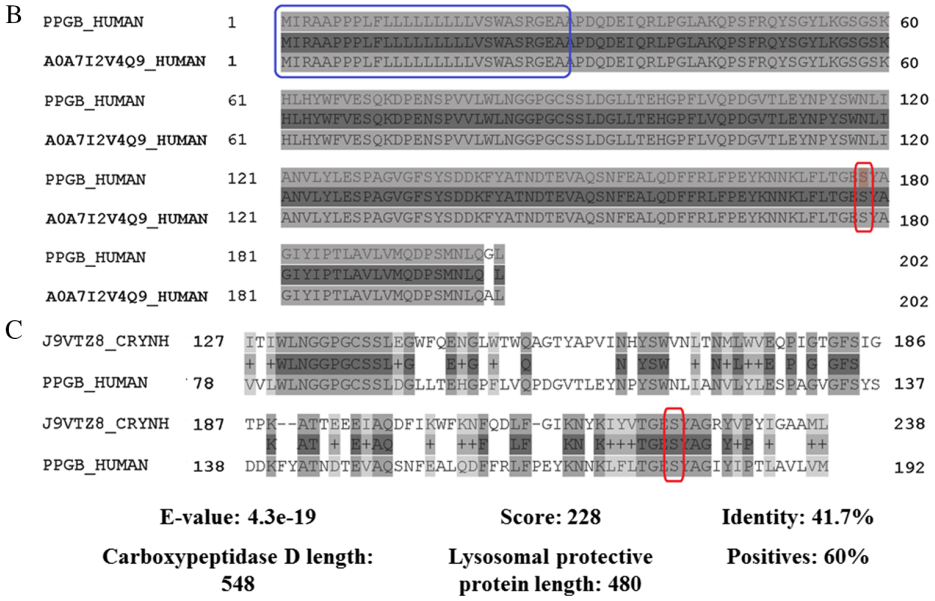

Fig. 2. Homology search results between carboxypeptidase D and human proteins. (A) Similar human proteins designated by sequence identity sorted by alignment score against carboxypeptidase D. (B) Alignment results between carboxypeptidase from *Homo sapiens* and heavy polypeptide chain of lysosomal protective protein from *H. sapiens*. (C) Alignment results between carboxypeptidase D from *Cryptococcus neoformans* and lysosomal protective protein from *H. sapiens*. Dark boxes indicate conserved amino acids, light boxes indicate amino acids from the same group, blue squares contain signal peptide, and red squares contain amino acids from lysosomal protective protein active center. Alignment was performed using BLAST (basic local alignment search tool).

production exists, exact mechanisms of peptidase function and contributions to pigment formation are unknown. Cerevisin (EC: 3.4.21.) is a subtilisin-like serine peptidase belonging to the kexin family (S8B subfamily). Sequence identity with a human homolog, proprotein convertase subtilisin kexin type-9 (PCSK-9) (EC: 3.4.21.61), is 31.7%. PCSK-9 is involved in plasma cholesterol homeostasis regulation by proteolytic degradation of intracellular acidic compartments of lipoprotein and apolipoprotein receptors (e.g., LDLR, LRP1/APOER or LRP8/APOER2) (**Fig. 3**) (Poirier et al. 2008). The alignment parameters between cerevisin and PCSK-9 are high (e.g., E-value and score) and all amino acids from the active center are conserved, indicating homology between the proteins (**Fig. 3**) (Pearson 2013). Although this suggests that more research is needed to identify opportunities for leverage of cerevisin as a novel target for inhibition to treat cryptococcal infections, compounds interacting on exosites (e.g., noncompetitive inhibitors) could be also explored.

2.2. Biofilm formation

Biofilm structures offer many microorganisms, including *C. neoformans*, resistance to the host immune response, environmental stresses, and antimicrobial therapy, as well as serving a role in

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ENTRY	PROTEIN NAMES	IDENTITY
Q8NBP7	**Proprotein convertase subtilisin/kexin type 9**	31.0%
A0A669KBG0	**Proprotein convertase 9**	31.0%
B4DMI2	**cDNA FLJ57533. highly similar to Proprotein convertase subtilisin/kexin type 9**	35.7%
Q16549	**Proprotein convertase subtilisin/kexin type 7**	26.0%

B

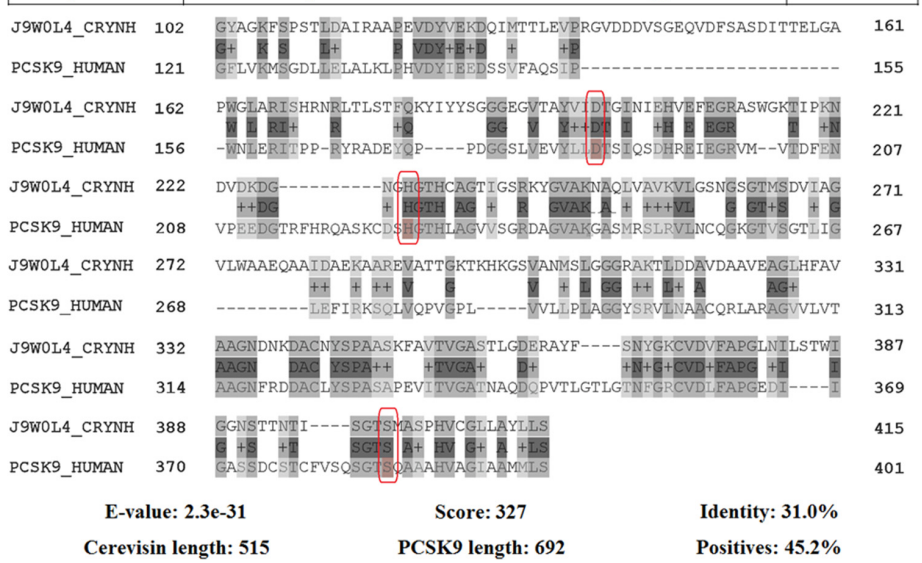


Fig. 3. Homology search results between cerevisin and human proteins. (A) Similar human proteins designated by sequence identity sorted by alignment score to cerevisin. (B) Alignment results between cerevisin from *Cryptococcus. neoformans* and proprotein convertase subtilisin/kexin-type 9 from *Homo sapiens*. Dark boxes indicate conserved amino acids, light boxes indicate amino acids from the same group, and red squares indicate amino acids from active center of PCSK9. Alignment was performed using BLAST (basic local alignment search tool).

chronic infections (Martinez and Casadevall 2006, 2007). Additionally, cryptococcal cells form biofilms on medical devices, including ventricle–arterial shunt catheters, peritoneal dialysis fistula, cardiac valves, and prosthetic joints, within a clinical setting, posing a substantial threat to patient health (Braun et al. 1994; Banerjee et al. 1997; Johannsson and Callaghan 2009). Notably, after biofilm formation, *C. neoformans* secretes peptidases (e.g., methionine aminopeptidase and elastinolytic metallopeptidase) involved in fungal invasion, degrading host protein components of the basement membrane (e.g., fibronectin) and extracellular matrix (Santi et al. 2014).

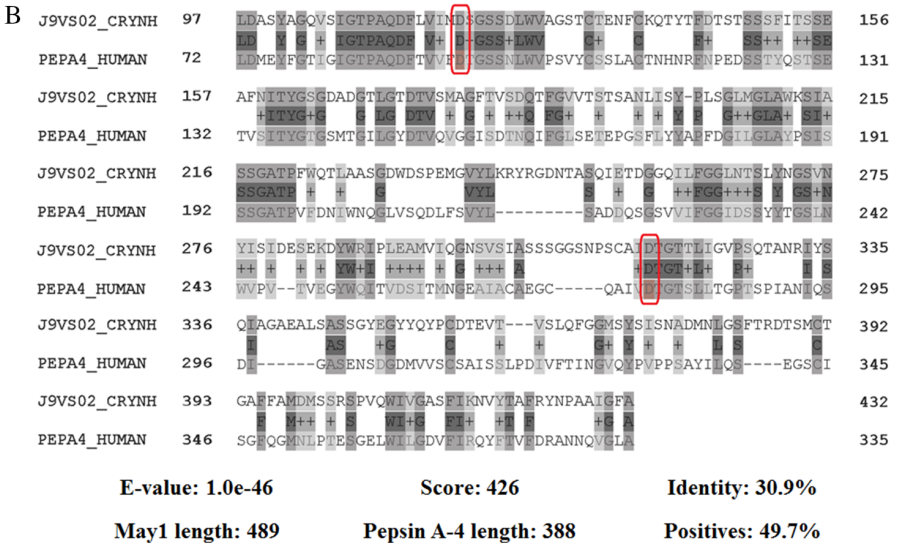
2.2.1. Pepsin-like May1

During biofilm formation, *C. neoformans* alters protein production to modulate metabolic activity, dormancy, and stress response (Santi et al. 2014). Although peptidases are present at high levels in a biofilm state, a direct relationship between these enzymes and biofilm production has not been well established (Santi et al. 2014). However, previous reports demonstrate that other fungal pathogens (e.g., *Candida albicans*) secrete aspartic peptidases (e.g., Sap5 and Sap6) at specific stages of biofilm formation to mediate adhesion of fungal cells to surfaces and one another (Kumar et al. 2015; Winter et al. 2016). Aspartic peptidases can also contribute to the breakdown of processing of molecules (e.g., adhesion molecules) important for biofilm formation, such as described in the bacterial pathogen *Staphylococcus* spp. (Koziel and Potempa 2013; Paharik et al. 2017; Martínez-García et al. 2018). Furthermore, some HIV aspartic peptidase inhibitors constitute effective drugs at preventing

A

ENTRY	PROTEIN NAMES	IDENTITY
B7ZW10	**Pepsin A**	30.9%
P0DJD7	**Pepsin A-4**	30.9%
P0DJD8	**Pepsin A-3**	30.9%
A0A1S5UZ02	**Pepsin A**	30.9%

B



E-value: 1.0e-46 **Score: 426** **Identity: 30.9%**

May1 length: 489 **Pepsin A-4 length: 388** **Positives: 49.7%**

Fig. 4. Homology search results between May1 and human proteins. (A) Similar human proteins designated by sequence identity sorted by alignment score for May1. (B) Alignment results between May1 from *Cryptococcus neoformans* and Pepsin A-4 from *Homo sapiens*. Dark boxes indicate conserved amino acids, light boxes indicate amino acids from the same group, and red squares indicate amino acids from active center of Pepsin A-4. Alignment was performed using BLAST (basic local alignment search tool).

biofilm formation and (or) at disrupting established biofilms in *C. albicans* when combined with major antifungals used to treat infections in the clinic fungi (Lohse et al. 2020). Such observations highlight the importance of serine aspartic peptidases during biofilm formation in fungi.

C. neoformans secretes several peptidases, including major aspartyl peptidase 1 (May1), involved in virulence, and possibly degradation of host proteins (Clarke et al. 2016). May1 (CNAG_05872) is an extracellular aspartyl peptidase belonging to the A1 family (Rawlings et al. 2018). Protein sequence alignment of May1 identifies the human protein, Pepsin A-4, sharing sequence identity at 30.9% (Fig. 4). Although these peptidases share the same catalytic amino acids, they share less than 80% of their sequence length, indicating no homology between the proteins (Pearson 2013). These results suggest that May1 could be an ideal antifungal target given the lack of a human homolog; however, both enzymes share active center loop and catalytic amino acids, suggesting similar catalytic properties. Taken together, this information supports the use of noncompetitive inhibitors as potential antifungal compounds, to avoid undesired off-target effects.

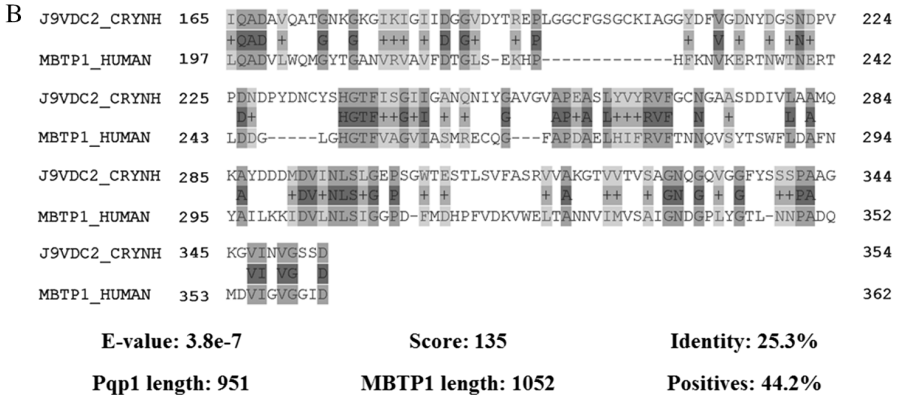
2.3. Quorum sensing

Quorum sensing is traditionally defined as a bacterial communication mechanism in which secreted signaling molecules impact population function and gene expression (Bassler and Miller 2013). This phenomenon is associated with virulence in some fungi, and in *C. neoformans* a quorum sensing-like peptide 1 (Qsp1) regulates virulence and cell wall function at high cell densities (Homer et al. 2016).

A

ENTRY	PROTEIN NAMES	IDENTITY
Q14703	**Membrane-bound transcription factor site-1 protease**	25.3%
B4DR21	**cDNA FLJ58156. highly similar to Membrane-bound transcription factor site-1 protease**	25.3%
Q8NBP7	**Proprotein convertase subtilisin/kexin type 9**	27.0%

B



E-value: 3.8e-7 **Score: 135** **Identity: 25.3%**

Pqp1 length: 951 **MBTP1 length: 1052** **Positives: 44.2%**

Fig. 5. Homology search results between Pqp1 and human proteins. (A) Similar human proteins designated by sequence identity sorted by alignment score towards Pqp1. (B) Alignment results between Pqp1 from *Cryptococcus neoformans* and MBTP1 from *Homo sapiens*. Dark boxes indicate conserved amino acids and light boxes indicate amino acids from the same group. Alignment was performed using BLAST (basic local alignment search tool).

In addition, Qsp1 promotes melanin synthesis, macrophage phagolysosome accumulation, and modulates secreted peptidase activity (e.g., serine-, aspartyl-, and metallo-peptidase) (Homer et al. 2016). This role connects back to the example of May1, an aspartyl peptidase with defined roles in biofilm formation (Clarke et al. 2016), an important example of high cell density structure. Notably, Qsp1 also decreases serine and metallopeptidase activity levels, confirming roles beyond virulence and cell wall function, such as tissue invasion that will be further described below (Homer et al. 2016). Qsp1 is expressed as a pro-peptide, activated extracellularly by peptidase, Pqp1 (CNAG_00150), and internalized, where it exerts its functions as a target of several transcription factors implicated in virulence (Homer et al. 2016).

2.3.1. Subtilisin-like Pqp1

Pqp1 (EC: 3.4.21.–) is a cell-associated peptidase belonging to the subtilisin-like serine peptidase family (S8 family) (Rawlings et al. 2018). This peptidase possesses a low sequence identity with human proteins, with highest identity mapping to a membrane-bound transcription factor site-1 peptidase (MBTP1) (25.3%) (**Fig. 5**). MBTP1 is an intracellular furin-like peptidase (S8B subfamily) involved in cholesterol metabolism and located in *cis*- and *medial*-Golgi (Jaaks and Bernasconi 2017). Considering the relevance of Pqp1 in quorum sensing-like phenomenon to regulate virulence and extracellular peptidase activity (Homer et al. 2016), and its poor similarity with human proteins, Pqp1 arises as an enzyme of interest. Specifically, Pqp1 may serve not only as an anti-cryptococcal target but also to increase knowledge about the quorum sensing process in fungi.

2.4. Polysaccharide capsule synthesis

The polysaccharide capsule is an important virulence factor for *C. neoformans,* contributing to its immunomodulatory and antiphagocytic properties during infection (Bose et al. 2003). The structure is comprised of polysaccharides synthesized intracellularly, exported to the cell surface, and attached

to the cell wall (Yoneda and Doering 2006). In *C. neoformans*, the cyclic-AMP/protein kinase A (cAMP/PKA) signal transduction pathway is a key regulator of capsule elaboration (D'Souza et al. 2001; Kronstad et al. 2011; Caza and Kronstad 2019). Recently, we demonstrated a connection between *PKA* regulation and the ubiquitin-proteasome pathway (UPP), along with changes in abundance for ribosomal and translational proteins (Geddes et al. 2016). Interestingly, inhibition of the proteasome with Bortezomib affected fungal growth and virulence by impairing capsule formation (Geddes et al. 2016). These findings highlight the role of the proteolytic complex as a potential anti-cryptococcal
target and warrant further investigation.

2.4.1. Cysteine-like Rim13

Another important component involved in capsule production includes Rim101, a transcriptional regulator in *C. neoformans* involved in capsule synthesis, as well as sensing neutral and alkaline extracellular pH as part of the RIM pathway (O'Meara et al. 2010; Ost et al. 2015). This protein requires phosphorylation by *PKA* before its proteolytic activation by the Rim101 proteolysis complex, which includes Rim13 peptidase (CNAG_05601) (Caza and Kronstad 2019). Once Rim101 is cleaved, it translocates to the nucleus and regulates expression of genes encoding functions for cell wall integrity, metal homeostasis, and capsule attachment to the cell wall (Ost et al. 2015). Rim13 (EC: 3.4.22.–) is a cysteine peptidase belonging to the C2A subfamily, and although it possesses low sequence identity (25.7%) with human proteins, it has the same amino acid length and similar function of Calpain-7 (**Fig. 6**) (Rawlings et al. 2018). Calpain-7 is a calcium-regulated nonlysosomal thiol-protease involved in the regulation of epithelial cell migration (H. Liu et al. 2013). Importantly, the catalytic amino acids are slightly different, indicating that some enzymatic characteristics, such as binding affinities or specificity, differ between the fungal and human proteins. Such properties highlight an opportunity for selective competitive inhibitors as potential treatment options to avoid off-targets effects.

2.5. Central nervous system invasion

C. neoformans possesses specific mechanisms to allow evasion of the human immune system and, in consequence, persist in tissues and organs for prolonged periods in latency (Vu et al. 2019). Dissemination from the lungs to the brain requires migration of fungal cells and crossing of the BBB. This leads to disruption of brain homeostasis and can cause meningoencephalitis, the major pathogenic factor of this fungus (Zaragoza 2019). The mechanism of fungal cell migration, transcytosis, transits fungi directly through endothelial cells, binding to the luminal side of the BBB, facilitating endocytosis (Chen et al. 2003; Shi et al. 2012). This binding and invasion depends on host elements (e.g., CD44 and AnnexinA2), and cryptococcal factors, such as urease, phospholipase, secretion of hyaluronic acid, and peptidases (Santangelo et al. 2004; T.-B. Liu et al. 2013; Vu et al. 2013). For example, serine peptidases have been reported in BBB invasion, degrading human fibronectin, and promoting fungal invasion (Rodrigues et al. 2003; Xu et al. 2014). Moreover, connections between virulence factors, including biofilms and capsule synthesis, can be influenced by peptidases. This is observed for May1, which is active within the acidic environment of macrophages, aiding in fungal survival by degrading host proteins (Chen et al. 1996; Levitz et al. 1999; Clarke et al. 2016). These properties promote immune system evasion, and subsequent brain invasion, confirming the importance of this enzyme as a promising anti-cryptococcal target.

2.5.1. Metallopeptidase, CnMpr-1

CnMpr-1 (CNAG_04735) is a secreted metallopeptidase from *C. neoformans* required for specific association with BBB and posterior crossing (Vu et al. 2014; Pombejra et al. 2017). This enzyme alters the surface proteins of brain microvascular endothelial cells to promote internalization of fungal cells by inducing host cell surface ruffling (Vu et al. 2014; Pombejra et al. 2017). Additionally, CnMpr-1 engages

A

ENTRY	PROTEIN NAMES	IDENTITY
Q9Y6W3	Calpain-7	25.7%
Q7Z479	Calpain-7	25.7%
B2RAM2	cDNA. FLJ94999. highly similar to *Homo sapiens* calpain 7	25.5%

B

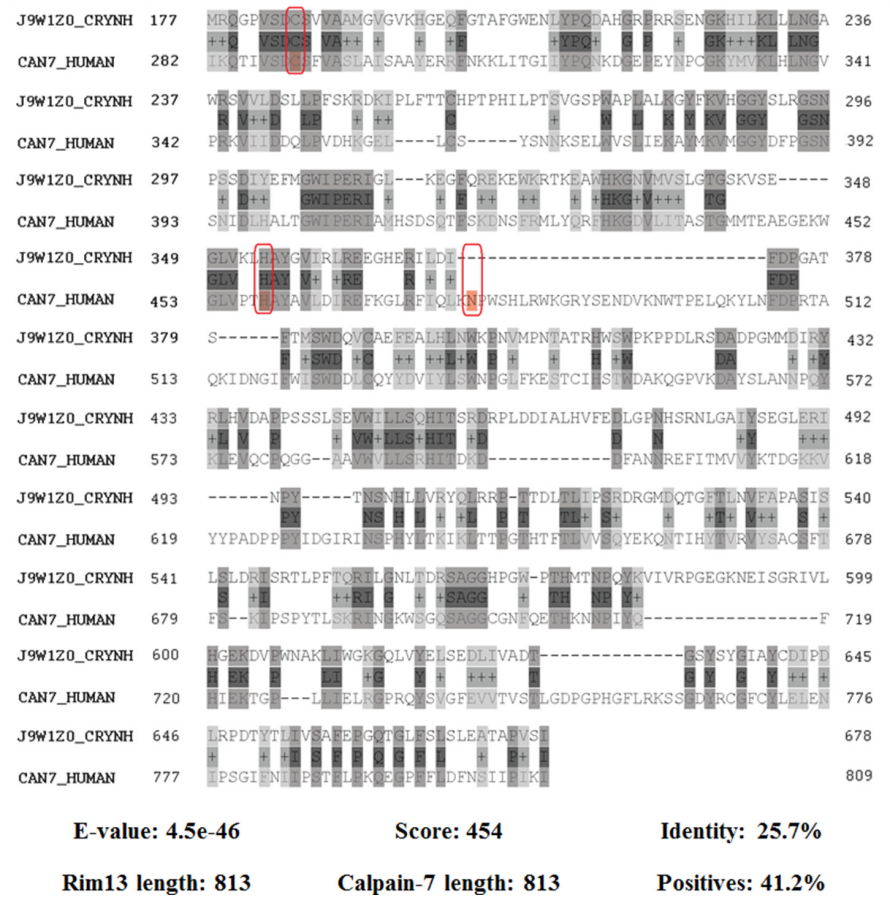


Fig. 6. Homology search results between Rim13 and human proteins. (A) Similar human proteins designated by sequence identity sorted by alignment score with Rim13. (B) Alignment results between Rim13 from *Cryptococcus. neoformans* and calpain-7 from *Homo sapiens*. Dark boxes indicate conserved amino acids, light boxes indicate amino acids from the same group, and red squares indicate amino acids from active center of calpain-7. Alignment was performed using BLAST (basic local alignment search tool).

with cytoskeleton-related proteins, such as AnnexinA2 (AnxA2), inducing transcytosis of cryptococcal cells across endothelial cells cytoplasm, and towards final exocytosis (Pombejra et al. 2017).

CnMpr-1 belongs to the M36 fungalysins family, which are found only in select fungal species, such as *Aspergillus fumigatus* and *C. neoformans* (Eigenheer et al. 2007; Fernández et al. 2013). Although several members of the fungalysins family have been discovered, until present, natural substrates or targets have yet to be identified. In humans, nonpeptidase Formin-1 (involved in microtubule binding) shows sequence identity of 34.5% towards CnMpr-1 with low alignment parameters and

A

ENTRY	PROTEIN NAMES	IDENTITY
Q68DA7	**Formin-1**	34.5%
A0A5F9ZHS8	**Formin-1**	34.5%
H0YM30	**Formin-1**	34.5%
Q9BZ29	**Dedicator of cytokinesis protein 9**	30.4%

B

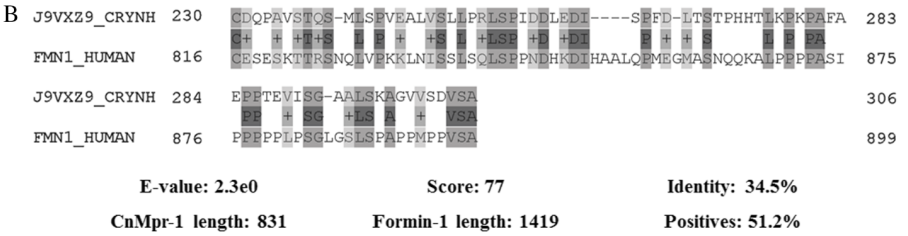


Fig. 7. Homology search results between CnMpr-1 and human proteins. (A) Similar human proteins designated by sequence identity sorted by alignment score for CnMpr1. (B) Alignment results between CnMpr-1 from *Cryptococcus. neoformans* and formin-1 from *Homo sapiens*. Dark boxes indicate conserved amino acids and light boxes indicate amino acids from the same group. Alignment was performed using BLAST (basic local alignment search tool).

diverging sequence length (Boekhorst and Snel 2007; Pearson 2013) (**Fig. 7**). Considering relevant roles of CnMpr-1 in brain invasion and low sequence identity with a human protein, CnMpr-1 highlights as a promising anti-cryptococcal druggable target.

2.6. Antifungal resistance

Ergosterol is an important component in the plasma membrane structure of fungal cells and, consequently, several antifungal therapies such as the azole class of antifungals target the ergosterol synthesis pathway (Gauwerky et al. 2009). Cryptococcal cells lacking ergosterol have a weaker plasma membrane structure and increased cellular permeability, leading to leakage of cellular contents and finally, disruption of fungal growth (Ghannoum and Rice 1999). Although azoles effectively treat cryptococcosis, in recent years, several *C. neoformans* isolates have developed resistance to these antifungals because of long-term treatment strategies and the fungistatic nature of azoles (i.e., inhibits fungal growth but does not kill the cells) (Smith et al. 2015; Hope et al. 2019; Bermas and Geddes-McAlister 2020).

2.6.1. Metalloprotease, Stp1

The sterol regulatory element-binding protein (SREBP) pathway is required in *C. neoformans* for host adaptation and virulence (Chang et al. 2007). Additionally, this factor stimulates ergosterol production in response to sterol depletion, for example when oxygen-dependent ergosterol synthesis is limited by hypoxia (Chun et al. 2007). Furthermore, SREBP factor (Sre1 in *C. neoformans*) is required for survival in the presence of ergosterol synthesis inhibitors, such as azole class drugs (Chang et al. 2007). Stp1 (CNAG_05742) is a site-2 peptidase relevant in ergosterol synthesis, as is required for Sre1 proteolytic activation, and therefore, antifungal resistance (Bien et al. 2009). Stp1 (EC: 3.4.24.85) is an intracellular metallopeptidase that coordinates the catalytic zinc ion and water molecule in its active site (Feng et al. 2007). Upon protein sequence alignment, the human membrane-bound transcription factor site-2 protease (MBTP2) is identified, sharing a sequence identity of 24.7% with Stp1, suggesting a lack of human homologs (**Fig. 8**) (Pearson 2013). Given the threat of antifungal resistance in treating infections, highlighting the potential to develop disruptive molecules for Stp1, specific to *C. neoformans,* suggests a new approach for combatting resistance.

A

ENTRY	PROTEIN NAMES	IDENTITY
O43462	**Membrane-bound transcription factor site-2 protease**	24.7%
B9ZVQ3	**Endopeptidase S2P**	24.7%
A8KA68	**Endopeptidase S2P**	24.7%
O43567	**E3 ubiquitin-protein ligase RNF13**	34.5%

B

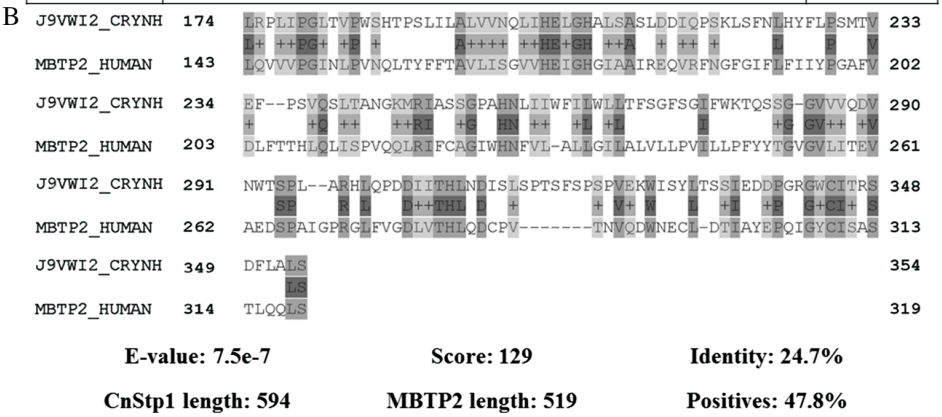


Fig. 8. Homology search results between CnStp1 and human proteins. (A) Similar human proteins designated by sequence identity sorted by alignment score for CnStp1. (B) Alignment results between CnStp1 from *Cryptococcus neoformans* and MBTP2 from *Homo sapiens*. Dark boxes indicate conserved amino acids and light boxes indicate amino acids from the same group. Alignment was performed using BLAST (basic local alignment search tool).

3. Cryptococcal peptidases as druggable targets

Peptidases constitute relevant enzymes in several virulent processes in *C. neoformans* and are also involved in drug resistance mechanisms (See **Table 1**). The roles and importance of peptidases for fungal pathogenesis have been demonstrated in vitro and using in vivo models of infection (Bien et al. 2009; Vu et al. 2014; Clarke et al. 2016). However, this approach is not applicable in clinical settings; therefore, the design, development, and application of peptidase inhibitors is a practical and feasible alternative. The role of peptidases as potential anti-cryptococcal targets while also highlighting their inhibitors as promising treatment options for further exploration, have been successfully demonstrated *in vitro* (Aaron et al. 2020; Lohse et al. 2020; Kryštůfek et al. 2021). Moreover, we recently explored the source of such peptidase inhibitors from the natural environment, including plants, invertebrate, and microbes, presenting the strong evolutionary advantage of natural versus synthetic compounds (Gutierrez-Gongora and Geddes-McAlister 2021). Further, we recently presented integration of cryptococcal research at the bench with in vivo models of infection to move applications to the clinic for diagnosis, treatment, and understanding of cryptococcosis (Muselius et al. 2021).

3.1. CnMpr-1 inhibitors prevent BBB crossing

CnMpr-1 (Section 2.5.1), an extracellular metallopeptidase used by cryptococcal cells for specific interactions with the BBB including crossing, invading brain tissue, and causing meningoencephalitis, lacks homology in humans, supporting it as a feasible anti-virulence target for developing therapeutics against cryptococcal meningitis (Vu et al. 2014; Pombejra et al. 2017). In this sense, abietic acid, diosgenin, and lupinine are three natural compounds obtained from plants with inhibitory activity against CnMpr1 (Aaron et al. 2020). In addition, these compounds block BBB crossing by *Cryptococcus* spp. without affecting endothelial membranes or mammalian cells.

Abietic acid is an abietane diterpenoid found primarily in pine resin, and although its biological activity has not been largely explored, some of its derivatives are known by their antimycotic and antiviral activities (Gonzalez et al. 2010). Other abietane diterpenoids, such as parvifloron D and indigoferabietone, have been described for their anti-cancer and anti-bacterial properties, respectively (Thangadurai et al. 2002; Burmistrova et al. 2015). Diosgenin is a plant steroidal sapogenin used in traditional medicine mainly because of its anti-inflammatory and anti-proliferative activities and anti-cancer properties (Jesus et al. 2016; Sethi et al. 2018). This compound also inhibits the activity of matrix metalloproteinase-2 (MMP-2) and MMP-9, which are involved in the proteolytic processing of matrix proteins (e.g., proteoglycans), adhesion molecules (e.g., integrins), and cell migration (e.g., syndecan) (Koshikawa et al. 2000; Ratnikov et al. 2002; Endo et al. 2003; Li et al. 2004; Chen et al. 2011). Although MMPs have been related with several diseases (e.g., cancer), minimal, if any, cytotoxic effects have been reported against mammalian cells (Bassi et al. 2005; Aaron et al. 2020). These results highlight the importance of further research, mainly focused on increasing specificity, to avoid unwanted off-target effects under normal conditions (e.g., no carcinogen cell presence). Additionally, lupinine, a quinolizidine alkaloid found primarily in the *Leguminosae* family, displays similar inhibitory properties as abietic acid and diosgenin against CnMpr-1 and shows no presentation of cytotoxic effects (Frick et al. 2017). Other compounds of quinolizidine alkaloid class have been reported as insulin secretion stimulants, which highlights their broad biomedical uses (López et al. 2004).

Importantly, these compounds (i.e., abietic acid, diosgenin, and lupinine) belong to different chemical classes but show similar enzymatic properties against CnMpr-1, suggesting different inhibitory mechanisms. Although it does not possess strong inhibitory activity against CnMpr-1 (half maximal inhibitory concentration (IC_{50}) values around 10 μM) compared with other clinically approved metallopeptidase inhibitors, CnMpr-1 does not currently have effective treatment options (Abbenante and Fairlie 2005). Therefore, these natural compounds constitute a new promising path to target cryptococcal peptidases for inhibition as an alternative strategy and suggest multiple opportunities to achieve the same goal.

3.2. May1 inhibitors could prevent HIV coinfections

Cryptococcosis is the primary fungal-related cause of death among HIV/AIDS patients (Rajasingham et al. 2017). During the HIV replication cycle, proteins require proteolytic processing by HIV-1 peptidase, which belongs to the aspartic protease enzyme class (Dash and Rao 2001). Several anti-HIV treatment options rely on aspartic peptidase inhibitors (e.g., Indinavir, Raquinavir, or Ritonavir) to limit viral replication and reduce viral load and, notably, some also show anti-cryptococcal activity (Blasi et al. 2004; Sidrim et al. 2012). May1 is the major secreted aspartic peptidase of cryptococcal cells and involved in fungal virulence and immune system evasion (Clarke et al. 2016). Therefore, it seems possible that the anti-cryptococcal activity found in HIV-1 peptidase inhibitors may be associated with inhibition of this aspartic peptidase, a positive off-target effect.

Pepstatin A is a classic aspartic peptidase inhibitor and used as a treatment option against fungal-related diseases such as candidiasis and, more recently, as a model to create new derivatives, mainly by inhibition of secreted aspartic peptidases (Braga-Santos and Santos 2011). In this sense, Z-Pst–L-Glu–Hph–NH_2 is a pepstatin-derivative peptidase inhibitor that possesses very high inhibitory activity against May1 (K_i = 12 nM) and HIV-1 peptidase (K_i = 32 pM) using similar binding mechanisms (i.e., active site binding) (Kryštůfek et al. 2021). Notably, this compound inhibits both enzymes with similar potency compared with several clinically approved HIV-1 peptidase inhibitors (e.g., Ritonavir or Atazanavir) and presents very high specificity compared with human renin (K_i = 19 μM), its closest structural homologue (Abbenante and Fairlie 2005; Kryštůfek et al. 2021). Although pepstatin A affects *C. neoformans* and mammalian cell survival with similar potency,

suggesting off-target effects, this compound possesses lower cytotoxicity than some clinically applied peptidase inhibitors, including Darunavir, Amprenavir, and Indinavir (Callebaut et al. 2011). These properties support the clinical potential of pepstatin A and warrant further investigation. These results certainly draw attention to Z-Pst–L-Glu–Hph–NH_2 as a promising option to develop new treatments against both diseases and (or) to prevent co-infections.

4. Future Directions

In this review, we outlined the distribution of peptidases within the widespread, globally relevant human fungal pathogen, *C. neoformans*. We selected representative extracellular and intracellular enzymes with described roles in virulence (e.g., melanin and capsule production, biofilm formation, quorum sensing, and dissemination), as well as antifungal resistance. Using protein sequence alignment, we identified human homologs and demonstrated positioning of sequence identity, with an emphasis on catalytic regions, to define potential regions for competitive inhibition. This approach, which combines in vitro and in vivo data with in silico modeling, predictions, and assessment for the rational design of antimicrobial agents is at the forefront of researchers' minds. The goal is to discover and develop new strategies to overcome antimicrobial resistance and expand our current repertoire of antimicrobial agents (Durand-Reville et al. 2021). Importantly, this strategy brings together interdisciplinary researchers from fields such as microbiology, computer science, biochemistry, molecular biology, chemistry, immunology, and engineering to address the problem of limited therapeutics. However, a large portion of research efforts and funding is still dedicated towards bacterial infections, leaving fungal pathogens with many opportunities to circumvent our current arsenal of defense. Although the need for novel antifungal agents to combat fungal infections of humans, animals, and crops to ensure global health and food security is dire, much can be gleaned from advances made against bacterial pathogens, including the discovery and characterization of novel proteases and effective inhibitors (Fetzer et al. 2017). We also presented examples of peptidase inhibitors used in the clinic with predicted off-target effects against cryptococcal enzymes as well as alternative options with inhibitory activity and improved cytotoxicity effects compared with currently used clinical inhibitors. Together, these peptidase inhibitors may be optimized for fungal-specific activity or as a foundation for additional drug design.

Another critical component in our fight against fungal pathogens and subsequent infections is the acknowledgement that our identification and characterization of peptidases is far from complete. In *C. neoformans* alone, over 2,000 uncharacterized or hypothetical proteins remain (Uniprot, Fungidb: fungidb.org/fungidb/app), and the potential to discover many more enzymes involved in fungal virulence or antifungal resistance is high, which coincides with an opportunity to develop antifungal agents against these new targets. An important technology for the discovery of novel proteases is mass spectrometry-based proteomics, which detects and quantifies proteins produced under a variety of experimental conditions and has application within both the lab and the clinic (Aebersold and Mann 2016; Zhu et al. 2021). For many fungal pathogens, proteomics is a promising tool to further our understanding of pathogenesis, define signaling networks, explore interactions with the host, develop biomarkers for diagnostics, as well as assess treatment efficacy (Aebersold et al. 2016; Ball et al. 2019; Ball et al. 2020; Muselius et al. 2021; Retanal et al. 2021). Additionally, peptidases produced by the fungus can be measured within the intracellular (cellular proteome) and extracellular (secretome) environments for identification. This can be followed by molecular and biochemical experimentation for characterization, such as genetic manipulation to define a role for the corresponding gene, as well as pull-down or immunoprecipitation assays to identify direct and indirect interaction partners. Moreover, mass spectrometry-based proteomics can profile interactions between a protein and drug or inhibitor, assess binding capacities and detect off-target effects for an important and decisive role in drug discovery (Reinhard et al. 2015). Taken together, the potential for further

discovery of fungal peptidases relevant for virulence, as well as the opportunity to design and develop inhibitors for targeted and specific activity is promising.

5. Conclusion

To date, more than 7400 proteins have been reported in *C. neoformans* proteome where peptidases represent approximately 2% of defined proteins. However, given that approximately 31% of total cryptococcal proteins remain uncharacterized, further research is warranted, and more enzymes, specifically extracellular and intracellular peptidases have yet to be defined and characterized. Cryptococcal peptidases, mainly extracellular, are not only related with other virulence factors like thermotolerance, fungal growth, or melanin synthesis but are also involved in dissemination for brain invasion and immune system evasion. Although intracellular peptidases have been less studied, these enzymes constitute important elements involved in diverse biological processes, such as capsule formation and antifungal resistance. Moreover, some cryptococcal peptidases are implicated in quorum sensing, a phenomenon that is still unclear in fungi but highlights a remarkable process for regulating secreted peptidases and virulence. Overall, these studies show that proteolytic activity is not a random process in *C. neoformans* pathogenicity but is subject to tight control with many mechanisms remaining to be discovered. Furthermore, based on our analyses described herein, many of these enzymes show low sequence identity with human proteins, underscoring their role as prospective "druggable" anti-cryptococcal targets for further exploration.

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Author contributions

DG-G and JG-M conceptualized the topic. DG-G prepared the first manuscript draft. DG-G provided figures. DG-G and JG-M edited and prepared the final manuscript version. DG-G and JG-M have read and agreed to the published version of the manuscript.

Competing interests

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of this manuscript, or in the decision to publish the results.

Data availability statement

All relevant data are within the paper.

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