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Aspartic Protease Inhibitors: Effective Drugs against the Human Fungal Pathogen *Candida albicans*

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Abstract: *Candida albicans* can invade humans and may lead to mucosal and skin infections or to deep-seated mycoses of almost all inner organs, especially in immunocompromised patients. In this context, both the host immune status and the ability of *C. albicans* to modulate the expression of its virulence factors are relevant aspects that drive the candidal susceptibility or resistance; in this last case, culminating in the establishment of successful infection known as candidiasis. *C. albicans* possesses a potent armamentarium consisting of several virulence molecules that help the fungal cells to escape from the host immune responses. There is no doubt that the secretion of aspartic proteases, designated as Saps, is one of the major virulence attributes produced by *C. albicans* cells, since these hydrolytic enzymes participate in a wide range of fungal physiological processes as well as in different facets of the fungal-host interactions. For these reasons, Saps clearly hold promise as new potential drug targets. Corroborating this hypothesis, the introduction of anti-human immunodeficiency virus drugs of the aspartic protease inhibitor-type (HIV PIs) have emerged as new agents for the inhibition of Saps. The introduction of HIV PIs has revolutionized the treatment of HIV disease, reducing the opportunistic infections, especially candidiasis. The attenuation of candidal infections in HIV-infected individuals might not solely has not only resulted from improved immunological status, but also as a result of direct inhibition of *C. albicans* Saps as well as the blockage of several biological processes controlled by these proteolytic enzymes. The present article will discuss the updates on the functional implications of HIV PIs on the development of candidiasis.

Keywords: *Candida albicans*, secreted aspartic proteases, aspartic protease inhibitors, alternative chemotherapy, candidiasis, virulence.

1. *CANDIDA ALBICANS*: AN OPORTUNISTIC FUNGAL PATHOGEN

Candida albicans is a polymorphic fungus (Fig. **1**), which belongs to the normal microbial flora of human beings, colonizing the skin and the gastrointestinal and urogenital tracts. Under normal circumstances, the yeast lives as a harmless commensal on mucosal surfaces in healthy individuals, existing in equilibrium with the microbial flora, the host's epithelial tissues, the host's immune defenses and other local environmental conditions. However, disturbance of this balance can result in uncontrolled growth of *C. albicans* lead to infections of either the skin or mucosal surfaces and more aggressively the invasion of deeper mucosal tissue, culminating in the dissemination to other organs. Hence, *C. albicans* causes several types of infections in predisposed patients (Table **1**), ranging from superficial to life threatening diseases [1-4]. Impressive numbers were published regarding *Candida* infection: (i) in North American hospitals, *Candida* spp. cause around 10% of intensive care unit-acquired bloodstream infections and represent the third or fourth commonest bloodstream pathogen with a mortality rate of 30-50% [5-9]; (ii) approximately 75% of all women experience vulvovaginal candidiasis during their lifetime and a significant proportion (5%) of all adult women suffer from recurrent infections [10, 11]; and (iii) in HIV-positive individuals, oral and esophageal candidiasis is considered as an acquired immunodeficiency syndrome (AIDS)-defining illness with 80-90% of these individuals, without intervention with highly active antiretroviral therapy (HAART), suffering recurrent episodes during the course of their illness [12, 13]. How the transition from a harmless commensal to an aggressive pathogen is triggered is not fully understood. However, *C. albicans* possesses a potent arsenal of molecules (virulence attributes) that help it to adapt, to interact with host structures and to circumvent the host immune responses. Furthermore, this fungus has an impressive ability to quickly alter its gene expression in response to environmental stimuli, such as changes in nutrient availability, pH, osmolarity, temperature or attack by cells of the immune system [14-16]. As a consequence, *C. albicans* presents an extraordinary ability to successfully infect virtually every anatomical site of the human body, being one of the most relevant and the most frequently isolated fungal pathogen in hospitals, which is directly

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associated with a prolonged hospital stay, especially in the intensive care unit, and a resulting rise in care costs [17-21].

Table 1. Predisposing Factors for Developing Candidiasis

Populations/Conditions
Severely impaired immunity (e.g., neutropenia)
Cancer patients receiving radiotherapy or chemotherapy (e.g., leukemia)
Disruption of natural barriers (e.g., trauma, burn injury or abdominal surgery)
Indwelling catheters
Diabetes
Acute renal failure (e.g., dialysis requirement)
Recipients of bone marrow or solid organ transplants
Antimicrobial therapy
Steroid use
Malnutrition
Total parenteral nutrition
HIV infection

2. VIRULENCE FACTORS

Polymorphism of *C. albicans* (Fig. **1**), enabling morphological plasticity in response to environmental changes, is known to contribute to its virulence process. Yeast-to-hyphae differentiation (dimorphism) of *C. albicans* is associated with destruction and invasion of host structures (extracellular matrices, cells and tissues) [22]. Interestingly, the transcriptional programs associated with dimorphism genes also influence and are linked to the expression of other virulence factors [14]. In this sense, a number of fungal attributes, such as the expression of adhesion factors (e.g., surface mannoproteins), directed growth/thigmotropism, stress adaptation, metabolic flexibility, ability of phenotypic switching, capability to form biofilm in numerous substrates, and the secretion of hydrolytic enzymes such as lipases, phospholipases and proteases are directly implicated in the infectious process [16, 23-28].

2.1. Aspartic Proteases

The secretion of aspartic proteases has long been recognized as a virulence associated trait of this fungal pathogen [29]. The secreted aspartic proteases (Saps) of *C. albicans*, which are encoded by a family of 10 homologous genes (*SAPs1-10*), are known to contribute to the fungal pathogenicity due to the participation in several facets of the infective process, including (i) degradation of tissue barriers (e.g., extracellular matrix/basal membrane and surface membrane proteins) during invasion; (ii) destruction of host defense molecules by cleavage of different classes of immunoglobulins, complement proteins, proteinaceous protease inhibitors, cytokines and antimicrobial peptides; (iii) nutrition and (iv) adherence to both abiotic substrates (e.g., medical devices like catheters, prosthetic valves, artificial dentures and others) and biotic surfaces (e.g., cells and tissues) [26, 30-32] (Fig. **2**). Due to their wide substrate specificity and broad pH range (2.0-7.0), it is accepted that Saps contribute to the development of active *C. albicans* infections, making them interesting targets for new antifungal drugs [33-36]. This is supported by the fact that some of the HIV aspartic protease inhibitors (PIs) in clinical use have inhibitory effects against *C. albicans* both *in vitro* and *in vivo* [36-38]. In addition, the increasing emergence of strains of *C. albicans* resistant to the commonly used antifungal agents has made clinical management of candidiasis increasingly difficult and emphasizing the need for improved drug therapies [39-41].

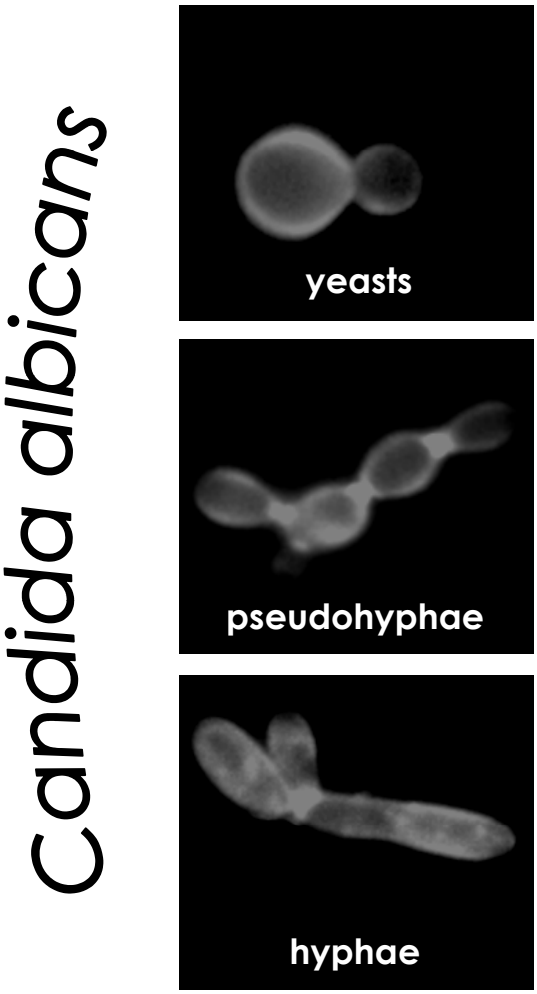

Fig. (1). Epifluorescence photocomposition of the different morphological growth forms of *Candida albicans* stained with calcofluor white.

3. HIV PIs: ANTI-*C. ALBICANS* PROPERTIES

Inhibitors of HIV-encoded aspartic protease (Table **2**), combined with nucleoside analogs with antiretroviral activity, caused profound and sustained suppression of viral replication, increased CD4+ T lymphocyte cell counts, stimulated the survival and activation of neutrophils, monocytes and endothelial cells, reduced morbidity and mortality, promoted an improvement in the quality and prolongation of life, arresting the progression of the HIV/AIDS

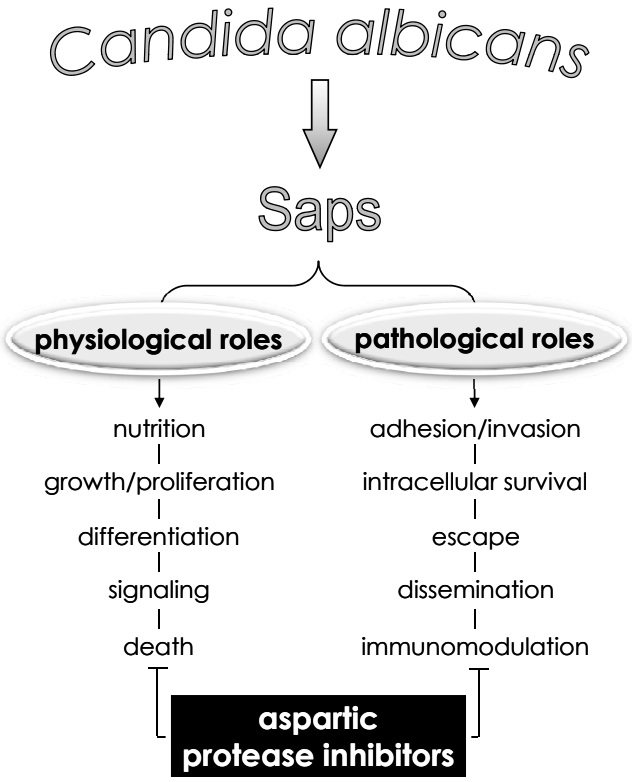

Fig. (2). Possible roles played by Saps produced by *Candida albicans*. Saps are able to cleave different host components such as serum proteins, antimicrobial peptides, surface molecules and structural proteinaceous compounds. The degradation of host proteins can help the microorganisms in several steps of their life cycle and pathogenesis including dissemination, adhesion, escape, nutrition and immunomodulation of the host immune responses. These proteases can also contribute to maintaining basic metabolic processes in *C. albicans* cells, which govern crucial events like proliferation, differentiation as well as signaling and death pathways. Aspartic protease inhibitors are able to block one or several of these fundamental events, reducing the ability of this fungus in causing infections.

condition, with a dramatic fall in HIV-related opportunistic infections [42-45]. In this sense, clinical evidences showed that the inclusion of aspartic PI in the HAART, the therapeutic scheme used to treat HIV-infected individuals, severely diminished the frequency of oropharyngeal candidiasis, being considered a clinical marker for disease progression [46-49]. For instance, in a multinational cohort study involving 6,941 HIV-positive individuals from Australia and ten European countries, when comparing the periods 1997-2001 and 1994-1996, there was a significant HIV PI-induced decrease in the progression of candidiasis from 17.0% to 5.7% [50]. The positive impacts of HAART on quality of life, mental health, labor productivity and economic well-being for people living with HIV/AIDS have also been documented [51-54]. Moreover, several works have demonstrated the direct antifungal actions of the HIV PIs against *C. albicans* [reviewed in 33 and 35]. The attenuation of candidal infections in HIV-infected individuals in the HAART era might not solely have resulted from improved immunological status, but also as a result of direct inhibition of *Candida* Saps by the HIV PIs contained in HAART [33], since the production of proteases by *C. albicans* and HIV-1 belongs to the same catalytic type (Table **3**).

Published works demonstrated that indinavir, saquinavir, ritonavir, nelfinavir and amprenavir (Fig. **3**) were able to restrain the proteolytic activity of purified Sap1, Sap2 and Sap3 in a concentration-dependent manner [55-61]. The binding of the HIV PIs to the active sites of Saps produced by *C. albicans* blocks the binding of substrate to the enzyme that results in an incapability of the microorganism to obtain peptides and amino acids to its nutrition, leading to a reduction or a complete interruption in the proliferation rate. Corroborating this finding, HIV PIs were capable in arresting the proliferation and development of *C. albicans* yeasts when cultured in defined medium supplemented with large protein (e.g., albumin or hemoglobin) as a unique source of nitrogenous compound [35, 57, 58, 61]. In addition, the yeast-to-hyphae differentiation process, which is a hallmark event during the pathogenesis of *C. albicans* [62], was delayed by the treatment with indinavir [57] and inhibited by amprenavir in a dose-dependent way [35, 61].

HIV PIs perturbed the ultrastructural architecture of *C. albicans* cells, inducing several and drastic alterations on the surface layer. For instance, the treatment with amprenavir [61] promoted the removal of the amorphous layer that covers the entire surface of *C. albicans*, turning the rough surface into a smooth one, as evidenced by scanning electron microscopy. Moreover, amprenavir-treated yeasts had invaginations and deformations in the cell shape [61]. These irreversible effects corroborate the anti-proliferative properties of the HIV PIs on *C. albicans* growth and development [35]. Surface-located molecules can act as adhesive structures [63]; consequently, their inhibition (synthesis and/or exposition) can diminish the ability of *C. albicans* cells to interact with host structures. In this line of thinking, HIV PIs reduced the expression/exposition of surface molecules, including Saps, mannose- and sialic acid-rich glycoconjugates and sterol [57, 61]. In addition, HIV PIs significantly diminished the secretion of hydrolytic enzymes to the extracellular environment, including Saps, phospholipase and esterase [35, 56, 61, 64]. Overall, the removal of these surface structures culminates in the inability of this fungus to adhere to abiotic (e.g., plastic and acrylic materials), which inhibits the biofilm formation [35, 61, 65], and biotic substrates (e.g., different epithelial cell lineages) [35, 55, 59, 66-68].

Two HIV PIs, indinavir and ritonavir, promoted a therapeutic effect in an experimental model of vaginal candidiasis, with an efficacy comparable to that of fluconazole, a recognized anti-*C. albicans* drug [56]. Also, the anti-Sap effect of HIV PI was associated with clinical resolution of oral candidiasis in HIV-positive patients [69]. In that elegant study, Cassone and co-workers [69] carried out a controlled, randomized, longitudinal study in which therapy-naive HIV-positive subjects receiving PI-HAART were matched with subjects under an non-nucleoside reverse transcriptase inhibitors (NNRTI)-HAART regimen, all

Table 2. Current Licensed HIV PIs

Generic Name	Trade Name	Abbreviation	Manufacturer	FDA Approval
Amprenavir	Agenerase	APV	GlaxoSmithKline	1999 (April)
Atazanavir	Reyataz	ATV	Bristol-Meyers Squibb Company	2003 (June)
Darunavir	Prezista	DRV	Tibotec Pharmaceuticals	2006 (June)
Fosamprenavir	Lexiva	F-APV	GlaxoSmithKline	2003 (October)
Indinavir	Crixivan	IDV	Merck	1996 (March)
Lopinavir	Kaletra	LPV	Abbott Laboratories	2000 (September)
Nelfinavir	Viracept	NFV	Pfizer	1997 (March)
Ritonavir	Norvir	RTV	Abbott Laboratories	1996 (March)
Saquinavir	Invirase[a] Fortovase[b]	INV	Roche	1995 (December)
Tipranavir	Aptivus	TPV	Boehringer Ingelheim Pharmaceuticals	2005 (June)

[a]hard and [b]soft gelatin capsules.

Table 3. MEROPS Identification for HIV-1 Protease and Saps from *Candida albicans*.

Organism	Protease Name	MEROPS Name	Catalytic Type	Clan	Family	Subfamily	Identifier
Human immunodeficiency virus 1	HIV-1 protease	HIV-1 retropepsin	Aspartic endoprotease	AA	A2	A	A02.001
Candida albicans	Secreted aspartic proteases (SAPs)	Candidapepsin SAP1	Aspartic endoprotease	AA	A1	A	A01.014
		Candidapepsin SAP2					A01.060
		Candidapepsin SAP3					A01.061
		Candidapepsin SAP4					A01.062
		Candidapepsin SAP5					A01.063
		Candidapepsin SAP6					A01.064
		Candidapepsin SAP7					A01.065
		Candidapepsin SAP8					A01.066
		Candidapepsin SAP9					A01.067
		Candidapepsin SAP10					A01.085

patients being followed up for oral candidiasis incidence, oral *Candida* carriage, immunoreconstitution and Sap levels in the oral cavity. The results demonstrated that PI-HAART regimen was extremely beneficial against oral candidiasis, since of the 80% Sap-positive subjects at the beginning of the treatment more than half no longer presented Sap in their saliva after two weeks of treatment and almost all subjects converted to Sap negativity after 30 days of treatment. Contrarily, the Sap positive subjects receiving NNRTI-HAART maintained their positivity after 30 days and the majority had Sap in the saliva for the whole duration (180 days) of follow-up [69]. Interestingly, the combination of classical antifungal agents (e.g., fluconazole) and HIV PI (e.g., saquinavir) both at sub-inhibitory concentrations was effectively demonstrated against strains of *C. albicans* isolated from HIV-positive patients [70]. Collectively, these findings emphasize the idea that HIV PI treatment had advantageous clinical efficacy against candidiasis.

4. NEW APPROACHES FOR IMMINENT PROSPECTS

The inhibitory effects of HIV PIs both in *in vitro* and *in vivo* experimental models were observed at concentrations (micromolar range) higher than those needed for HIV-1 protease inhibition (nanomolar or subnanomolar). This probably reflects a much lower affinity of these drugs for Sap than that for HIV-1 protease [33, 71]. Another explanation is that, in contrast to the very small and structurally simplified HIV-1 protease (a homodimeric enzyme of the A2 protease family) (Table **3**), Saps are larger, more complex and monomeric enzymes with 2-fold internal

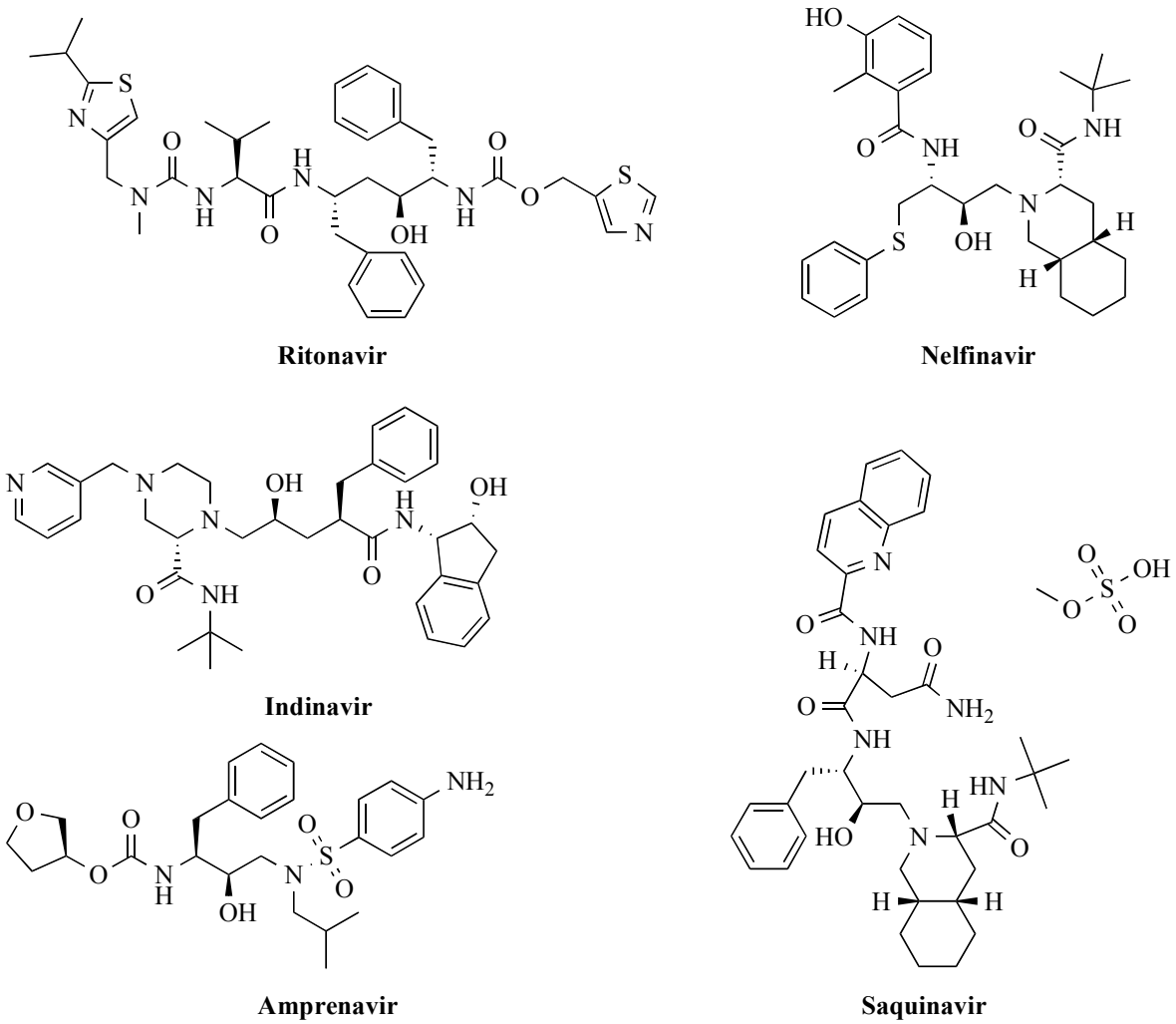

Fig. (3). Aspartic protease inhibitors approved for use in the anti-HIV chemotherapy and already tested on *Candida albicans*.

symmetry [72, 73]. They possess a relatively large active site which might be responsible for the broader substrate specificity and also their susceptibilities to distinct aspartic PIs [72]. Nonetheless, the above HIV PI concentrations may be achieved under current HAART regimens both in the blood [71], in human saliva (at least for indinavir) [74] and in lungs (at least for lopinavir) [75].

Two standpoints arose regarding the use of aspartic protease inhibitors against *C. albicans*: (i) the pivotal need for synthesis of new compounds, more effective and more specific to fit the active site of Saps, leading to a stronger inhibition of the proteolytic activity and (ii) the looking for novel target in the *Candida* cells to the binding of aspartic PIs. Regarding the last proposal, White and co-workers [76] identified an orthologous of the *Saccharomyces cerevisiae* Ddi1 protein as the only member of the aspartic protease family in *Leishmania* promastigote cells, being this protein as the major target to the binding of the HIV PIs. Ddi1 belongs to a family of proteins known as the ubiquitin receptors, which have in common the ability to bind ubiquitinated substrates and the proteasome [77]. The remarkable structural similarity between the central domain of Ddi1 and the retroviral proteases, in the global fold and in active-site detail, suggests that Ddi1 functions proteolytically during regulated protein turnover in the cell, being a key molecule in the maintenance of cellular homeostasis [77]. The Ddi1 protein of the yeast *S. cerevisiae* is involved in a wide range of functions, including protein targeting to the proteasome, control of cell cycle and suppression of protein secretion from the cell [78]. The search for Ddi1 homologous sequence in *C. albicans* can help in understanding the antifungal effect of HIV PIs.

5. CONCLUSIONS

Fungi are eukaryotic microorganisms that are closely related to humans at both cellular and biochemical levels, which make the treatment of mycosis difficult [79]. In addition, the emergence of drug resistance to antifungals and problems of toxicity and poor delivery of drugs at the target site in systemic infections generated the urgent necessity to find novel fungal targets as well as the discovery of more effective antifungal compounds [79]. In this scenario, Saps emerge as potential therapeutic target due to their participation in multiple relevant physiological and

pathological events performed by *C. albicans* (Fig. **2**). Inhibition of Sap produced by *C. albicans*, particularly Saps1-3, by the aspartic PIs used in the anti-HIV chemotherapy raised the curiosity of researchers around the world [80]. HIV PIs markedly improved the immunity of HIV-positive individuals and decreased significantly the infections caused by fungi, especially candidiasis. A huge number of studies revealed the capability of these bioactive pharmacological compounds to block crucial vital events of *C. albicans*, including nutrition, proliferation, growth, differentiation and interaction with host structures (e.g., cells and bioactive proteins), which culminated in the inability to sustain an infectious process (Fig. **2**). Looking to the future, the generation of more-specific Sap inhibitors with high selective toxicity against *C. albicans* would probably represent a therapeutic breakthrough in the fight against candidiasis and other diseases caused by aspartic protease-producing pathogens. Combination therapies that target not only essential genes but also important virulence factors that are essential for certain steps in infection (e.g., Saps) could be attractive in the treatment of *C. albicans* infections. Prospect research into the synergistic capabilities of inhibitors will help elucidate the most effective combination therapies to be used against candidiasis.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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ABBREVIATIONS

AIDS = Acquired immunodeficiency syndrome

HAART = Highly active antiretroviral therapy

HIV = Human immunodeficiency virus

NNRTI = Non-nucleoside reverse transcriptase inhibitors

PI = Protease inhibitor

Sap = Secreted aspartic protease

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