ANTIFUNGAL DRUG DISCOVERY: NEW THEORIES AND NEW THERAPIES

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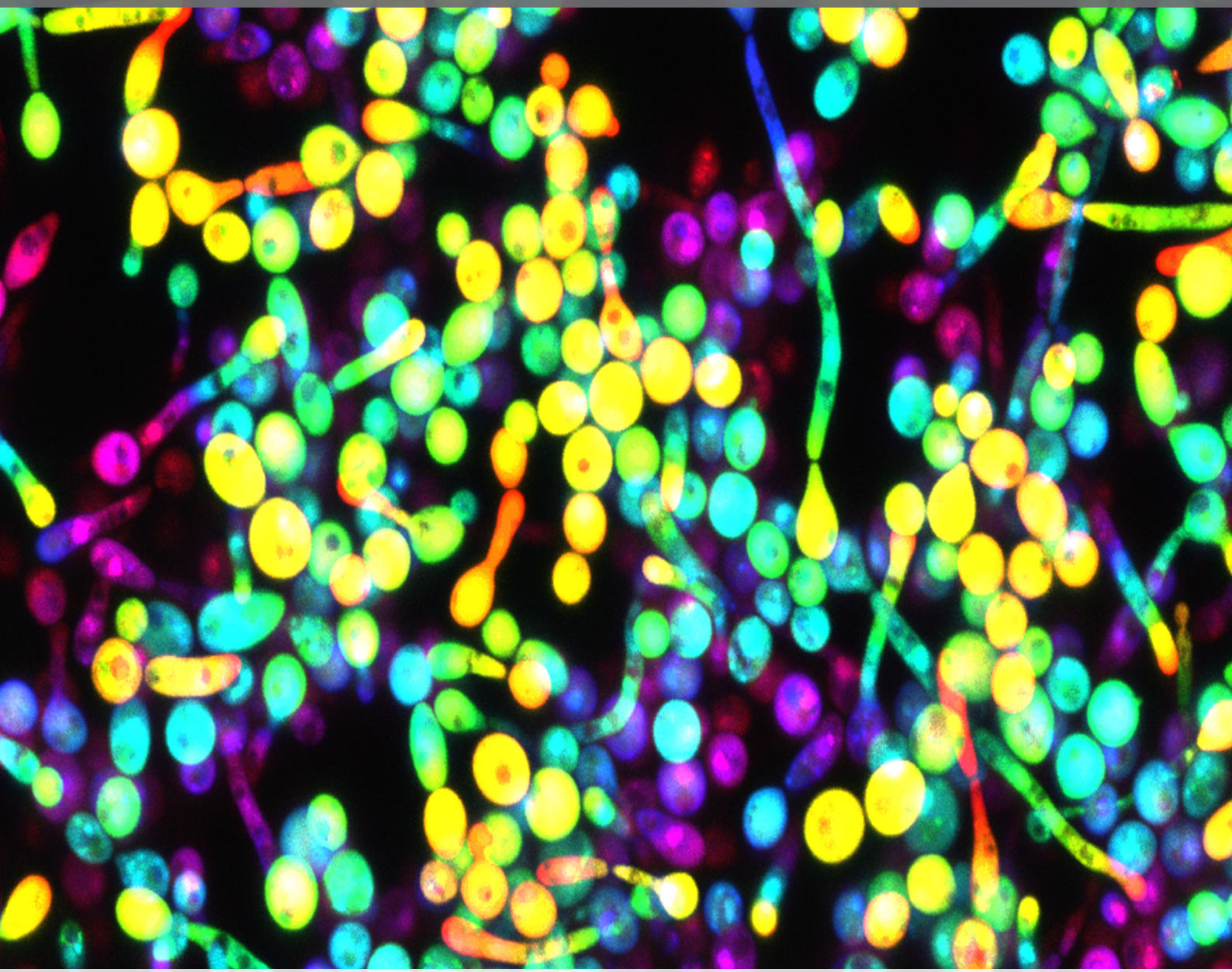

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ANTIFUNGAL DRUG DISCOVERY: NEW THEORIES AND NEW THERAPIES

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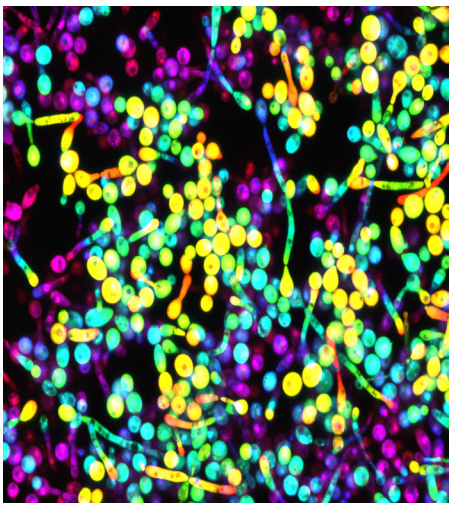

Confocal laser scanning microscopic image of Candida albicans biofilm, showing yeast and hyphal elements. C. albicans biofilms are highly resistant to antifungal agents. Image by C. J. Seneviratne

Fungal infections such as candidoses can range from superficial mucous membrane infection to life-threatening systemic mycoses. Candida infections are a significant clinical problem globally due to rapid rise in compromised host populations including HIV/AIDS, organ transplant recipients and patients on chemotherapy. In addition, sharp increase in aging populations which are susceptible to fungal infections is expected in next few decades. Antifungal drugs are relatively difficult to develop compared to the antibacterial drugs owing to the eukaryotic nature of the cells. Therefore, only a handful of antifungal agents are currently available to treat the myriad of fungal infections. Moreover, rising antifungal resistance and host-related adverse reactions have limited the antifungal arsenal against fungal pathogens. In this research topic, we tried to update the theoretical aspects pertaining to the antifungal drug discovery i.e. proposed novel mechanisms, new drug targets and pathways. In addition, invited authors explored the new antifungal drugs derived from natural and synthetic sources which are currently under development. Contributors were encouraged to bring new insight into the antifungal drug discovery. We hope the reader may arrive at a general consensus on the possible strategies to combat ever increasing ubiquitous fungal infection in this new century.

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EDITORIAL
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Editorial: Antifungal Drug Discovery: New Theories and New Therapies

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The Editorial on the Research Topic

Antifungal Drug Discovery: New Theories and New Therapies

Medically important fungal infections can be broadly classified into superficial surface infections and invasive mycoses (Samaranayake and MacFarlane, 1990; Roemer and Krysan, 2014). Superficial surface infections include mucosal candidiasis, dermatophyte infections whereas invasive mycoses affect sterile body sites such as bloodstream, central nervous system, kidney, lungs, and liver. Rise of fungal infections has caused a substantial morbidity and mortality globally (Vallabhaneni et al., 2016). It is reported that mortality among patients with invasive candidiasis is as high as 40%, even when patients receive antifungal therapy (Kullberg and Arendrup, 2015).

Antifungal drugs are relatively difficult to develop compared to antibacterial drugs owing to the eukaryotic nature of the cells. Only a few classes of antifungal drugs, such as polyenes, azoles, echinocandins, allylamines, and flucytosine, are available to treat the myriad of fungal infections (Sanglard et al., 2009). Of the current antifungal agents, none have all the characteristics of an ideal agent (Wong et al., 2014). Antifungal resistance and host-related adverse reactions further limit the existing antifungal arsenal against fungal pathogens (Chandrasekar, 2011). Rising drug resistance is an inevitable problem, particularly for fluconazole, a drug of choice for candidiasis in AIDS patients (Siikala et al., 2010; Rautemaa and Ramage, 2011). Drug resistance has also been reported for recently introduced echinocandin antifungal agents (Seneviratne et al., 2008a; Ben-Ami et al., 2011; Clancy and Nguyen, 2011). Moreover, some fungal species are inherently resistance to existing antifungals (Sanglard; Kołaczkowska and Kołaczkowski, 2016). In addition, biofilm mode of fungal growth is known to be highly resistant to antifungal agents (Chandra et al., 2005; Seneviratne et al., 2008b). Hence, the development of more effective and safe antifungal agents is a top priority in the health care field. Therefore, this special research topic aimed to address the new theories and therapies pertaining to antifungal drug discovery, covering aspects of clinical relevance and novel antifungal strategies.

Majority of the articles published under this research topic belongs to the *Candida* species, which is a group of major fungal pathogens in humans. *Candida* species are commensal fungi that inhabit various niches of the human body, including the oral cavity, gastrointestinal tract, vagina, and skin (Samaranayake and MacFarlane, 1990; Mayer et al., 2013). However, under certain circumstances, *Candida* can cause infections, or candidiasis, ranging from superficial mucous membrane infections to life-threatening systemic diseases. *Candida albicans* is the most prevalent fungal pathogen in lethal blood stream infections of humans (Seneviratne et al., 2011). *C. albicans* infections are a significant clinical problem especially in compromised host populations undergoing HIV/AIDS treatment, chemotherapy or organ transplantation. Moreover, sharp increase in aging populations which are susceptible to fungal infections is expected in the next few decades. The currently available antifungal agents are not always effective against *C. albicans,* which remains a ubiquitous pathogen in nosocomial diseases, causing severe mucosal infections such as oral

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candidiasis, onycomycoses, vulvovaginal candidiasis, and systemic mycoses with high mortality rates (Kojic and Darouiche, 2004; Zaoutis et al., 2005; Concia et al., 2009).

At the start of the research topic, clinical relevance of oral candidiasis has been discussed in order to provide a glimpse of human fungal infections (Patil et al.). Biofilm formation of the fungal pathogen is a significant problem in medical-device associated infections and directly related to therapeutic failure (Williams and Ramage, 2015). As conventional antifungal agents are ineffective against fungal biofilms, alternative strategies are needed. Novel antifungal compounds that target fungal biofilm formation and the host inflammatory response such as myriocin, fulvic acid, and acetylcholine have been discussed in the research topic as candidate dual action therapeutics to treat opportunistic fungal infections (Borghi et al.). Microbial biotransformation has emerged as an important tool for obtaining novel substances which possess antifungal activity. Implication of endophytic fungi as cell factories for producing new antifungal molecules and *in silico* approach using databases of 3D molecular structures are also discussed (Bianchini et al.). Oshima and colleagues introduce an interesting concept of biogenics for oral candidiasis. Biogenics advocates the use of beneficial bioactive substances produced by probiotic bacteria, whose activities are independent of the viability of probiotic bacteria in human bodies. Ravikumar and colleagues examine various immune-enhancing strategies for the invasive fungal diseases caused by *Candida* and *Aspergillus* species. These novel approaches include cytokine therapy, granulocyte transfusion, antibody-based therapy, natural killer cell treatment and adoptive T cell transfer. Molecules such as phenolic compounds, derived from natural sources and exhibiting considerable antifungal properties are a source for the development of novel anti-candidal therapy (Teodoro et al.). Therefore, potential use, proposed mechanisms of action and limitations of phenolic acids have been discussed.

Candida bloodstream isolates derived from Hong Kong have shown to possess virulence attributes such as biofilm formation, hemolysin production, proteinase activity as well as perturbations in their antifungal sensitivity in the presence of serum, which may contribute to treatment complication in candidemia (Seneviratne et al.). One of the major mechanisms contributing to multi-drug resistance in *C. albicans* is the plasma membrane drug-efflux system. Therefore, application of inhibitors of drug-efflux pumps has been suggested as a strategy to increase the susceptibility of *C. albicans* to antifungals. Szczepaniak et al. developed a new fluorescence method that allows *in vivo* activity evaluation of compounds inhibiting *C. albicans* transporters. They demonstrated that fluorescence labeling with diS-C3(3) potentiometric dye enables a real-time observation of the activity of *C. albicans* Cdr1 and Cdr2 transporters. The new method was able to demonstrate the different specificities of enniatin A and beauvericin toward drug-efflux pumps. In another study investigators have developed three structurally related chemo-sensititzers i.e., oxathiolone fused chalcone derivatives to successfully restore the sensitivity of fluconazole resistant *C. albicans* strains. The mechanism of action is a possible non-competitive inhibition of drug-efflux pumps Mdr1, Cdr1, and Cdr2. However, more research is warranted in this area to fully establish the role of chemo-sensitizers in clinical use.

Antimicrobial peptide isolates from various sources are also a promising source to develop novel antimycotic agents. A study under this research topic has shown anti-*Candida* activity of antimicrobial peptide produced by *Enterococcus faecium* (Roy et al.). It appears to target chitin in the cell wall of *Candida* species. Host derived molecules like histatin 5 protects human oral mucosa against the transformation of commensal *C. albicans* into a pathogenic invader. A work by Moffa and colleagues demonstrated that coating with histatin 5 reduces *C. albicans* colonization of epithelial cell surfaces and also protects the basal cell layers from undergoing apoptosis. Hence, there is a possibility of using host derived antifungal molecules to prevent *Candida* infections, which may be a useful strategy in compromised host populations.

Candida glabrata is an emerging human fungal pathogen. A study examined the role of glucose sensing mechanism in *C. glabrata* using SNF3 (Sucrose Non Fermenting 3) knockout strains. Mutation results in higher susceptibility to amphotericin B in low glucose environment (0.1%), but showed no effect on biofilm formation capability. Going beyond *Candida* species, a study of dermatophyte fungus *Trichophyton rubrum* investigated the role of Hsp90 in its pathogenicity and drug susceptibility. Chemical inhibition of Hsp90 resulted in increased susceptibility of the fungus to itraconazole and micafungin. The synergism observed between the inhibition of Hsp90 and the effect of itraconazole or micafungin in reducing the fungal growth is of great interest as a novel and potential strategy to treat dermatophytoses.

This specific research topic on antifungal drug discovery provides a detailed overview of potential novel antifungal strategies, promising new discoveries and their clinical implications, particularly that of *Candida* species.

AUTHOR CONTRIBUTIONS

CS and ER contributed to the Editorial.

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Antifungal Susceptibility in Serum and Virulence Determinants of *Candida* Bloodstream Isolates from Hong Kong

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Candida bloodstream infections (CBI) are one of the most common nosocomial infections globally, and they account for a high mortality rate. The increasing global prevalence of drug-resistant *Candida* strains has also been posing a challenge to clinicians. In this study, we comprehensively evaluated the biofilm formation and production of hemolysin and proteinase of 63 CBI isolates derived from a hospital setting in Hong Kong as well as their antifungal susceptibility both in the presence and in the absence of human serum, using standard methodology. *Candida albicans* was the predominant species among the 63 CBI isolates collected, and non-*albicans Candida* species accounted for approximately one third of the isolates (36.5%). Of them, *Candida tropicalis* was the most common non-*albicans Candida* species. A high proportion (31.7%) of the CBI isolates (40% of *C. albicans* isolates, 10% of *C. tropicalis* isolates, 11% of *C. parapsilosis* isolates, and 100% of *C. glabrata* isolates) were found to be resistant to fluconazole. One of the isolates (*C. tropicalis*) was resistant to amphotericin B. A rising prevalence of drug-resistance CBI isolates in Hong Kong was observed with reference to a previous study. Notably, all non-*albicans Candida* species, showed increased hemolytic activity relative to *C. albicans,* whilst *C. albicans*, *C. tropicalis,* and *C. parapsilosis* exhibited proteinase activities. Majority of the isolates were capable of forming mature biofilms. Interestingly, the presence of serum distorted the yeast sensitivity to fluconazole, but not amphotericin B. Taken together, our findings demonstrate that CBI isolates of *Candida* have the potential to express to varying extent their virulence attributes (e.g., biofilm formation, hemolysin production, and proteinase activity) and these, together with perturbations in their antifungal sensitivity in the presence of serum, may contribute to treatment complication in candidemia. The effect of serum on antifungal activity warrants further investigations, as it has direct clinical relevance to the treatment outcome in subjects with candidemia.

Keywords: *Candida*, antifungal susceptibility, virulence factors, clinical isolates, plasma protein binding

INTRODUCTION

Candida is an opportunistic pathogen that can cause life-threatening systemic and bloodstream infections in humans (Calderone and Clancy, 2002). It is the fourth leading cause of bloodstream infection in the United States, accounting for approximately 9% of the total bloodstream infections, following coagulase-negative *Staphylococci*, *Staphylococcus aureus,* and *Enterococcus* species (Wisplinghoff et al., 2004). In recent reports, *Candida* spp. remains the leading fungal cause of central line-associated bloodstream infections (Hidron et al., 2008; Sievert et al., 2013). Despite the advent of many new antifungal agents, the incidence of *Candida* bloodstream infection (CBI) has been steady over the past decades (Pfaller and Diekema, 2007). In addition to its high incidence, the attributable mortality rate and the associated cost burden are substantial (Wilson et al., 2002; Warnock, 2007). In Hong Kong, an epidemiological study (Yap et al., 2009) revealed a high prevalence, associated mortality, and morbidity of CBI.

Of the *Candida* species, *Candida albicans* is by far the predominant species of CBI (Pfaller et al., 2001, 2011; Labbé et al., 2009). However, recently, the incidence of CBI caused by non-*albicans* species (NAC) has increased and some of the common species isolated are *Candida tropicalis, C. parapsilosis, C. glabrata*, *C. guilliermondii*, *C. dubliniensis,* and *C. krusei* (Falagas et al., 2010). The key virulence factors of *Candida* that are associated with bloodstream infections include hemolysin production, proteinases production and biofilm formation (Calderone and Fonzi, 2001; Lim et al., 2012). Hydrolytic enzymes, such as proteinases, of *Candida* species sequester nitrogen from proteins of the host and facilitates tissue invasion (Staib, 1966; Schaller et al., 2005), whereas, hemolysin is needed to acquire iron from the hosts (Nayak et al., 2013). However, it should be noted that the relevance of secreted aspartyl proteinases to the fungal virulence is questionable as shown in data from animal studies (Correia et al., 2010).

Biofilm formation is another feature that contributes to *Candida* pathogenicity in catheter-related bloodstream infection (Shin et al., 2002). *Candida* biofilm is known to be highly resistant to antifungal agents, and it is thus a key attribute to the mortality in bloodstream infections (Seneviratne et al., 2008a). In addition, rising drug resistance among *Candida* species has posed a great challenge to clinicians, especially when treating bloodstream infections (Pfaller et al., 2011). Furthermore, there are only a few studies in the literature that examine the antifungal susceptibility and virulence attributes of CBI such as biofilm formation in Asian populations (Shin et al., 2002; Seneviratne et al., 2011; Tay et al., 2011; Kaur et al., 2014; Tellapragada et al., 2014).

In general, the pharmacologic effect of protein-bound drugs is lower than their unbound counterparts. The protein binding of a drug influences the amount of free unbound drug at the site of infection, as well as its pharmacokinetics and pharmacodynamics (Ashley et al., 2006). This is particularly important for drugs targeting bloodstream infections where the drug is intrinsically exposed to the serum proteins. However, studies on *Candida* bloodstream isolates rarely attempted to capture the latter, real-life scenario by evaluating the *in vitro* minimum inhibitory concentration (MIC) of antifungals against these isolates in the presence of serum.

In the present study, we comprehensively evaluated 63 isolates from candidemic patients for their pathogenic attributes such as hemolysin and proteinase production, and biofilm formation as well as the susceptibility to the two most commonly used antifungals, amphotericin B (a fungicidal agent) and fluconazole (a fungistatic agent). Moreover, taking the foregoing research gap into consideration, we also evaluated the MIC of these antifungal agents in a serum-laced environment. Our study demonstrated that CBI isolates are able to express pathogenic attributes to varying extent; furthermore, the susceptibility of these isolates against fluconazole is influenced in the presence of serum.

MATERIALS AND METHODS

Species Identification of *Candida* Bloodstream Infection Isolates

Anonymous archival collection of *Candida* isolates was used in the study with the approval of exemption from the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (HKU/HA HKW IRB). It has been accepted by the funding authority, the Research Office of the Food and Health Bureau, the Government of the Hong Kong Special Administrative Region (Health & Medical Research Fund, Project no.: 12111512). This study included 63 CBI isolates derived from two hospitals i.e., Queen Mary Hospital (23 isolates) and Queen Elizabeth Hospital (40 isolates) in Hong Kong. The *Candida* strains were isolated from patients before any antifungal medication was administered. Species identification of *Candida* isolates was performed by two standard culture-dependent methods, namely CHROMagar (CHROMagar™ *Candida*) and commercially available identification kit API 32C AUX method (bioMérieux SA, France; Odds and Bernaerts, 1994). In brief, CHROMagar differentiates various species of *Candida* by formation of specific colored colonies when incubated at 37°C for 48–72 h. API 32C AUX assay is a carbohydrate assimilation test which identifies the species based on their sugar metabolism.

Antifungal Susceptibility Testing

Antifungal susceptibility testing of the CBI isolates in planktonic mode was performed using Clinical Laboratory Standards Institute method (CLSI) protocol M27-A3 (broth microdilution assay; Seneviratne et al., 2008b; Fothergill, 2012). Two-fold dilution series of amphotericin B and fluconazole was prepared in RPMI 1640 medium. For the serum induction experiment, RPMI 1640 supplemented with 50% (v/v) human serum (Sigma) was used (Wiederhold et al., 2007). Inocula from 24 h *Candida* cultures were harvested and suspended in RPMI with turbidity equivalent to McFarland standard 0.5 (1×10^6 cells/ml) and then diluted to approximately 0.5×10^3–2.5×10^3 cells/ml. The test was performed in pre-sterilized, flat-bottom 96-well polystrene plates (Iwaki, Japan). *C. albicans* ATCC 90028 was used as quality control strain. Plates were incubated at 37°C for 48 h. MIC was defined as the lowest concentration of the drug that completely inhibits the growth according to the CLSI criteria.

Hemolysin Assay

Hemolysin assay for *Candida* strains was performed according to a previously validated protocol by our group (Luo et al., 2001). In brief, Sabouraud dextrose agar supplemented with 7% sheep blood and 3% glucose was used to determine the hemolysin production by the CBI isolates. Suspension of yeast (1×10^8 cells/ml) was prepared in phosphate buffered saline (PBS; pH 7.2, 0.1 M) and 10 µl was spot-inoculated on sheep blood agar plates, incubated at 37°C in 5% CO_2 for 48 h. The diameters of the colony and the transparent halo were measured by computerized image analyzer (Qwin, Leica, UK). The hemolysin index (Hi) was calculated by dividing the diameters of the colony and the transparent halo. The assay was performed on two separate occasions as quadruplicates for all isolates.

Proteinase Assay

The activity of secreted aspartyl proteinases was determined by the bovine serum albumin (BSA) plate assay with some modifications to the previous methods (Staib, 1966; Wu et al., 1996). Suspensions equivalent to 0.5 McFarland standard (1×10^6 cells/ml) were prepared from 18-h yeast cultured in Sabouraud dextrose agar (SDA) and 10 µl was spotted on 1% BSA plates. The plates were incubated at 37°C for 120 h. *C. albicans* ATCC 90028 and *C. parapsilosis* ATCC 22019 were used as positive and negative controls. The plates were stained with staining solution containing 1.25% of naphthalene black in 90% methanol/water (v/v) for 5 min and decolorized in 90% methanol/water (v/v) for 48 h. The diameters of the colony and the transparent halo were measured using the computerized image analyzer (Qwin, Leica, UK). Proteinase production index (Ppr) was calculated by dividing the diameters of the transparent halo and the colony by the diameter of the colony. The assay was performed on two separate occasions as quadruplicates for all isolates.

Biofilm Formation and XTT Reduction Assay

Biofilm formation of CBI isolates was analyzed by previously validated method by our group (Seneviratne et al., 2008b). In brief, a loopful of 18 h culture grown at 37°C in SDA was harvested and suspended overnight in yeast nitrogen base medium (YNB) supplemented with 50 mM glucose in a rotary shaker at 80 rpm overnight at 37°C. Yeast cells in the late exponential phase of growth were extracted and washed twice with PBS. Then, the cells were re-suspended in YNB supplemented with 100 mM glucose with turbidity equivalent to 4 McFarland standard. *C. albicans* ATCC 90028 was used as a control for comparison. Hundred microliters of the yeast suspension was transferred to the 96-well polystrene plate and incubated at 37°C for 90 min (adhesion phase) in an orbital shaker rotating at 80 rpm. Then, the medium was aspirated and the biofilms were washed twice with 100 µl of PBS to remove unattached cells. After washing, 200 µl of YNB medium with 100 mM glucose was added to each well. The plates were incubated at 37°C in a rotary shaker at 80 rpm for 48 h, with a change of the growth medium at 24 h. After the 48 h incubation period, the growth medium was pipetted out and the biofilms were washed twice with 200 µl of PBS before quantifying with XTT reduction assay (Ramage et al., 2001). In brief, 200 µl of the XTT solution was added to the wells and the plate was incubated in the dark at 37°C for 3 h. The XTT solution consisted of 40 µl of XTT stock solution (1 mg/ml in PBS) and 2 µl of menadione (0.4 mM in acetone) topped up to 200 µl in PBS. After incubation, 100 µl of the colored solution was aspirated from all the wells, transferred to Eppendorf tubes and centrifuged at 8000 rpm for 10 min. The centrifuged solution was transferred to a different microtitre plate and the optical density (OD) of the change in color was measured using a plate reader (SpectraMAX 340 Tunable Microplate Reader; Molecular Devices Ltd., Sunnyvale, CA) at 490 nm. This test was performed in duplicates.

Genotyping of the *Candida* Isolates by Random Amplification of Polymorphic DNA (RAPD)

The genetic similarities of the *C. albicans* and *C. tropicalis* isolates were examined by DNA fingerprinting through RAPD analysis. Genomic DNA of the isolates was extracted using the QIAamp DNA Mini Kit (Qiagen, Germany) according to the instructions of the manufacturer. The PCR master mix was prepared with 2 µL (100 ng/µL) of genomic DNA, 5 µL 10X PCR buffer (200 mM Tris/HCl, pH 8.4, 500mM KCl), 200 µM dNTPs, 25 mM MgCl2, 1 µM primer (T3B, 5′-AGG TCG CGG GTT CGA ATC C-3′; Thanos et al., 1996) and 1.5U Taq Polymerase (Invitrogen). PCR was performed by a thermal cycler (GeneAmp PCR System 9700, Applied Biosystems), with the first five cycles at 94°C for 5 min, followed by 35 cycles of denaturation (94°C, 30 s), annealing (52°C, 2 min) and elongation (72°C, 2 min), and lastly, final elongation at 72°C for 10 min. Positive control (genomic DNA of *C. albicans* SC5314) and negative control (water) were added in each PCR run. Gel electrophoresis of the PCR products was performed in 1% agarose gel at constant voltage of 150V for approximately 1 h. The bands were visualized by UV light (ChemiDoc Imaging System, Bio-Rad, USA) after staining with ethidium bromide. The bands of the isolates were analyzed and dendrogram was constructed by the unweighted pair group method in the program GelJ (Heras et al., 2015).

Statistical Analysis

One-way ANOVA with Bonferroni's corrections were used for multiple comparisons of hemolysin index, proteinase production index and optical densities of the XTT reduction assay in Prism 6 (GraphPad Software, La Jolla, CA). A *p*-value of 0.05 or lower was considered to be significant.

RESULTS

Species Distribution of the Isolates

Of the 63 *Candida* bloodstream isolates included in the study, *C. albicans* was the most commonly detected species ($n = 40$), followed by *C. tropicalis* ($n = 10$), *C. parapsilosis* ($n = 9$), *C. glabrata* ($n = 2$), *C. guilliermondii* ($n = 1$) and *C. dubliniensis* ($n = 1$; **Table 1**).

Antifungal Susceptibility Testing

The planktonic cells of all the isolates were susceptible to amphotericin B, except for a single isolate of *C. tropicalis* exhibiting marginal resistance with 2 μg/ml as MIC (**Table 2**). A total of 31.7% of the CBI isolates was resistant to fluconazole (MIC > 32 μg/ml). Of the *C. albicans* isolates, 16 (40%) were resistant to fluconazole. For the NAC, all the *C. glabrata* isolates were resistant to fluconazole, whilst all the *C. guilliermondii* and *C. dubliniensis* were susceptible. However, it has to be aware that the low number of isolates of these three species may not be representative.

Interestingly, serum-laced AST media did not alter the activity of amphotericin B. On the other hand, 9 out of 63 isolates showed an increase in MIC of fluconazole in serum-laced media (**Table 3**). Seven isolates exhibited four-fold raise in MIC (S18, S25, M4, M5, M6, M8, and M16) and two isolates exhibited three-fold increase (S29 and S14). On the contrary, a few isolates (S15, S17, S36, and S11) showed three-fold reduction in the MIC in the serum-laced medium (**Table 4**).

Hemolysin Activity

The mean hemolysin index of the *C. albicans* isolates was the lowest among all the species tested (1.592 ± 0.129). It was significantly lower than the mean hemolysin index of *C. tropicalis* and *C. glabrata*. Only two out of nine *C. parapsilosis* isolates produced hemolysin on the blood agar, while all isolates of other species exhibited hemolytic activity (**Table 5**).

Proteinase Activity

No proteinase activity was observed in the *C. glabrata*, *C. guilliermondii,* and *C. dubliniensis* isolates (**Table 5**). Proteinase activity was observed among the remaining species (*C. albicans*, *C. tropicalis,* and *C. parapsilosis*) and no statistical significant difference was observed between the mean proteinase indices of these three species.

Biofilm Formation and XTT Assay

C. albicans formed significantly more robust biofilms when compared to NAC (**Figure 1**). Of the two *C. glabrata* isolates, one produced very minimal biofilm, which gave the optical density (OD) of 0.138 as examined by XTT reduction assay and was 10 times less than the average optical density of the *C. albicans* biofilm (OD = 1.087). *C. guilliermondii* and *C. dubliniensis* produced moderate biofilms (OD = 0.9).

TABLE 1 | Species distribution of the *Candida* bloodstream infection isolates.

Species	No. of isolates (%)
C. albicans	40 (63.5)
C. tropicalis	10 (15.9)
C. parapsilosis	9 (14.3)
C. glabrata	2 (3.2)
C. guilliermondii	1 (1.6)
C. dubliniensis	1 (1.6)
Total	63 (100)

TABLE 3 | Fluconazole susceptibility of *Candida* bloodstream isolates that showed increase in MIC under 50% serum induction.

Isolates	MIC (μg/ml)	
	RPMI	RPMI + 50% serum
***C. albicans*[a]**		
S18	8	128
S25	8	128
S29	16	128
M4	4	64
M5	2	32
M6	2	32
M8	8	128
***C. tropicalis*[b]**		
S14	8	64
M16	8	128

MIC, minimum inhibitory concentration.
[a] *17.5% (7 out of 40) of C. albicans isolates.*
[b] *20% (2 out of 10) of C. tropicalis isolates.*

TABLE 2 | Antifungal susceptibility of *Candida* bloodstream isolates.

Species	No. of isolates	Amphotericin B		Fluconazole	
		Susceptible MIC < 2 μg/ml	Resistant MIC ≥ 2 μg/ml	Susceptible MIC ≤ 32 μg/ml	Resistant MIC > 32 μg/ml
C. albicans	40	40 (100%)	0 (0%)	24 (60%)	16 (40%)
C. tropicalis	10	9 (90%)	1 (10%)	9 (90%)	1 (10%)
C. parapsilosis	9	9 (100%)	0 (0%)	8 (88.9%)	1 (11.1%)
C. glabrata	2	2 (100%)	0 (0%)	0 (0%)	2 (100%)
C. guilliermondii	1	1 (100%)	0 (0%)	1 (100%)	0 (0%)
C. dubliniensis	1	1 (100%)	0 (0%)	1 (100%)	0 (0%)
Total	63	62 (98.4%)	1 (1.6%)	43 (68.3%)	20 (31.7%)

MIC, minimum inhibitory concentration.

Genotyping of the *Candida* Isolates by (RAPD)

Genotyping was performed for C. albicans and C. tropicalis strains. It seemed that strains derived from Queen Mary Hospital (23 isolates) and Queen Elizabeth Hospital (40 isolates) are genetically quite similar (Supplementary Figures 1, 2). There was no clear genotype specially associated with a particular hospital. There was also no clear association between the genotype of the species with their phenotypic features of biofilm formation, hemolysin index and proteinase index.

DISCUSSION

Candidemia due to NAC has shown a steep rise in recent decades (Samonis et al., 2008; Rodríguez et al., 2010). In the present study, NAC accounted for a high proportion of all the CBI isolates collected (36.5%), of which *C. tropicalis* was the most common. These results reaffirm the findings of ours (Seneviratne et al., 2011) and a 9-year long study conducted by Yap et al. (2009), where NAC accounted for 46% of the 128 CBI isolates collected in Hong Kong, with *C. tropicalis* being the most common NAC. Similarly, *C. tropicalis* is also the most common NAC amongst the *Candida* bloodstream isolates collected in other regions of Asia (Chen et al., 1997, 2011; Jung et al., 2012; Chander et al., 2013; Kaur et al., 2014). These data are in contrast to those from Europe and the Northern and Latin America, where *C. glabrata* and *C. parapsilosis* were the most common NAC in bloodstream isolate (Pfaller et al., 2011). Clinicians should be mindful of the geographical variation in the prevalence of different NAC species, as they are often associated with higher mortality and resistance to antifungals (Pfaller et al., 2011; Silva et al., 2012).

The increased prevalence of fungal infections and the concomitant prescription of antifungals, have led to emergence of drug-resistant *Candida* strains in the communities worldwide (Arendrup et al., 2013). For instance, fluconazole-resistance is now widespread owing to increased use of antifungals (Anaissie

TABLE 4 | Fluconazole susceptibility of *Candida* blood isolates that showed decrease in MIC under 50% serum induction.

Isolates	MIC (μg/ml)	
	RPMI	RPMI + 50% serum
***C. albicans*[a]**		
S15	16	2
S17	32	4
***C. glabrata*[b]**		
S36	32	4
***C. tropicalis*[c]**		
S11	32	4

MIC, minimum inhibitory concentration.
[a]5% (2 out of 40) of C. albicans isolates.
[b]50% (1 out of 2) of C. glabrata isolates.
[c]10% (1 out of 10) of C. tropicalis isolates.

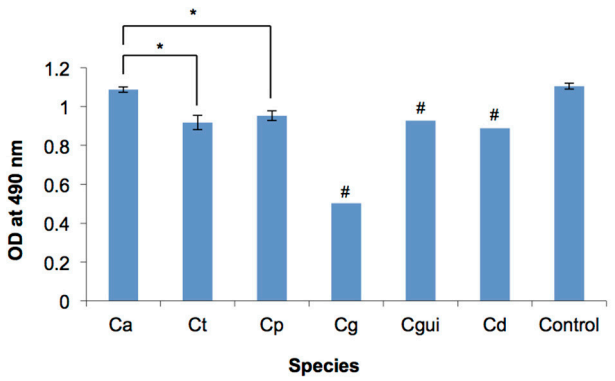

FIGURE 1 | Biofilm formation of the *Candida* bloodstream infection isolates measured by XTT reduction assay. OD, optical density; Ca, *C. albicans*; Ct, *C. tropicalis*; Cp, *C. parapsilosis*; Cg, *C. glabrata*; Cgui, *C. guillermondii*; Cd, *C. dubliniensis*; Control, *C. albicans* ATCC 90028; Error bars, standard deviation; # Standard deviations could not be determined due to the low number of isolates, *$p < 0.05$. The biofilm of each *Candida* bloodstream infection isolate was quantified by XTT reduction assay. The readings of isolates of each *Candida* species were averaged. Among all the *Candida* species tested, *C. albicans* biofilm was the most robust, whilst *C. glabrata* biofilm was the least robust. *C. albicans* biofilm was significantly more robust than those of *C. tropicalis* and *C. parapsilosis*. No significant difference was observed between the biofilm of *C. tropicalis* and *C. parapsilosis*. The optical density of each of the individual isolates is provided in Supplementary Table 1.

TABLE 5 | Hemolysin index and proteinase index of the *Candida* bloodstream infection isolates.

Species (n)	Hemolysin index mean ± SD	No. of hemolysin-positive isolate/total no. of isolates	Proteinase index mean ± SD	No. of proteinase-positive isolate/total no. of isolates
C. albicans (40)	1.592 ± 0.129	40/40	1.854 ± 0.262	31/40
C. tropicalis (10)	1.949 ± 0.206[a]	10/10	1.799 ± 0.130	8/10
C. parapsilosis (9)	1.778 ± 0.230	2/9	1.640 ± 0.101	6/9
C. glabrata (2)	2.058 ± 0.078[a]	2/2	1	0/1
C. guilliermondii (1)	1.727	1/1	1	0/1
C. dubliniensis (1)	2.074	1/1	1	0/1

n, number of isolates; SD, standard deviation; a, significant higher than C. albicans. The hemolysin index was calculated by dividing the diameter of the transparent hemolytic halo with that of the fungal colony. Similarly, the proteinase index was determined by dividing the diameter of the transparent proteolytic halo with that of the fungal colony. A value of one indicates the absence of enzyme activity, and is excluded in the calculation of the mean. The hemolysin and proteinase indices of each of the individual isolates are provided in Supplementary Table 1.

et al., 1996; Kanafani and Perfect, 2008). In a previous study we reported that all of the Hong Kong derived CBI isolates (including *C. tropicalis*) were susceptible to amphotericin B and fluconazole (Seneviratne et al., 2011). In contrast, in the present study, almost a third (31.7%) of the CBI isolates were resistant to fluconazole. Indeed, a single isolate of *C. tropicalis* showed marginal resistance (2 μg/ml) to amphotericin B (**Table 2**). Resistance to amphotericin B has been recorded rarely in the past, especially in *C. tropicalis* (Drutz and Lehrer, 1978). These data point toward a rather insidious emergence of drug-resistance in CBI in Hong Kong, and hence, the need for constant vigilance accompanied by clinical surveillance studies.

Protein binding plays an important role in determining the pharmacodynamics of a drug. Various studies have shown that serum alters the MIC of antifungal drugs (Zhanel et al., 2001; Bekersky et al., 2002). Higher dose is required for highly protein-bound drugs to exhibit the same microbial killing efficiency when compared to low protein-bound drugs ("free drug hypothesis"; Drusano, 2004). Amphotericin B is a highly protein-bound drug (>95%) and it is anticipated that there would be an increase in MICs for *Candida in vitro* in the presence of serum proteins, while the MICs of fluconazole, which is a weakly-bound drug (11%), may remain unchanged (Humphrey et al., 1985; Bekersky et al., 2002; Ashley et al., 2006).

Other studies have shown that half maximal effective concentration (EC50) of amphotericin B significantly increased for *C. albicans* ATCC 90028 and *C. lusitaniae* in RPMI supplemented with 4 and 8% human serum albumin (Lewis et al., 2006). In contrast, some studies exhibited results contradictory to the free drug hypothesis (Zhanel et al., 2001; Zeitlinger et al., 2011; Elefanti et al., 2013). In the study by Zhanel et al. (2001), the MICs of amphotericin B of all the 10 isolates examined were not altered in RPMI with 80% fresh human serum; whereas, 64% of the isolates tested displayed increase in MIC of fluconazole in RPMI with 80% human serum, and the remaining isolates showed no change in MIC. In our study, all the isolated examined displayed no change in the MICs to amphotericin B in the presence of serum proteins. As for fluconazole, the MICs of the majority of the isolates remained unchanged, but 14.3 and 6.3% of the 63 isolates exhibited an increased and decreased MICs, respectively in the presence of serum proteins (**Tables 3, 4**). Our data, therefore, confirm the notion that the *in vitro* efficacy of an antifungal drug does not necessarily depend upon its protein binding capacity as suggested by others (Zhanel et al., 2001; Elefanti et al., 2013).

Hemolysin is produced by some species of *Candida* which destroy the circulating erythrocytes to acquire elemental iron from hemoglobin (Schaible and Kaufmann, 2004). In the present cohort, all the CBI isolates, except *C. parapsilosis*, exhibited hemolytic activity (**Table 5**). Interestingly, the hemolytic-positive isolates of NAC species exhibited higher hemolytic activities than *C. albicans.* This is in contrast to the studies of Luo et al. (2001) who reported that *C. albicans* as the most potent hemolytic species.

Secreted aspartyl proteinases of *Candida* are thought to degrade human proteins and provide nitrogen for the fungal growth (Naglik et al., 2003). Only *C. albicans*, *C. tropicalis,* and *C. parapsilosis*) in the present cohort demonstrated proteolytic activities, whilst *C. glabrata*, *C. guilliermondii,* and *C. dubliniensis* were devoid of such activity (**Table 5**).

Candida spp. are known to form highly organized biofilms, especially on indwelling catheters and other prosthetic devices (Seneviratne et al., 2008a). Different *Candida* species are also known to have both inter- and intra-species variations in biofilm development (Seneviratne et al., 2008a; Silva et al., 2010). In the present study, all the CBI isolates, except *C. glabrata*, were good biofilm formers, with *C. albicans* being superior to other species, followed by *C. tropicalis* and *C. parapsilosis* (**Figure 1**). It has been found that the mortality of CBI caused by biofilm-forming *Candida* spp. are higher than those caused by non-biofilm-forming counterparts (Tumbarello et al., 2012). Moreover, non-*albicans Candida* species isolated from bloodstream were found to be higher biofilm formers than those isolated from other sites (Shin et al., 2002). Patients treated with anti-biofilm antifungal agent (caspofungin), which demonstrates anti-biofilm efficacy *in vitro*, were more commonly associated with shorter post-CBI hospitalization than those treated with non-anti-biofilm antifungal agent (fluconazole; Tumbarello et al., 2012). Furthermore, the lower antifungal susceptibility associated with *Candida* biofilm is often implicated in treatment complication (Douglas, 2003; Seneviratne et al., 2008a). Our current finding adds to the evidence that biofilm formation is a major virulence factor that may lead to treatment complication of CBI. Genotyping of the *C. albicans* and *C. tropicalis* strains using RAPD showed that the strains derived from Queen Mary and Queen Elizabeth hospitals in Hong Kong are quite similar. There was no clear pattern of genotypic and phenotypic features of the *Candida* strains. This is possibly due to genetic and environmental relatedness of the strains in a single country. Other studies have shown geographical location is a major factor associated with genetic relatedness (Dassanayake et al., 2006). Therefore, future studies should aim to compare genotype of the *Candida* isolates with other regional countries.

In conclusion, the present study demonstrates that CBI isolates are to varying extents capable of expressing virulence attributes such as biofilm formation, hemolysin production and proteinase activity. *C. albicans* is the predominant pathogenic species in Hong Kong patients, while the proportion of NAC species remains high. Our current findings further demonstrate that *C. tropicalis* is the most common NAC isolated from CBI in Asia. Almost all the isolates we have evaluated are able to form mature biofilms. Antifungal resistance among CBI isolates, particularly for fluconazole is variably demonstrated amongst the isolates, a critical factor that should be borne in mind when managing candidaemic patients for effective care. Finally, this study indicates that the presence of serum may perturb the activity of some antifungal agents, a factor that needs to be considered when prescribing antifungals in candidemias.

AUTHOR CONTRIBUTIONS

CS, DT, and CL conceived and designed the study. DT and CL collected the isolates. CS, SR, and SW performed all the experiments, analyzed the data and wrote the manuscript. LS and LJ provided general guidance and revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00216

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Therapeutic Application of Synbiotics, a Fusion of Probiotics and Prebiotics, and Biogenics as a New Concept for Oral *Candida* Infections: A Mini Review

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Candida is a major human fungal pathogen causing infectious conditions predominantly in the elderly and immunocompromised hosts. Although *Candida* resides as a member of the oral indigenous microbiota in symbiosis, some circumstances may cause microbial imbalance leading to dysbiosis and resultant oral candidiasis. Therefore, oral microbial symbiosis that suppresses the overgrowth of *Candida* is important for a healthy oral ecosystem. In this regard, probiotics, prebiotics, and synbiotics can be considered a potential therapeutic and preventive strategy against oral candidiasis. Prebiotics have a direct effect on microbial growth as they stimulate the growth of beneficial bacteria and suppress the growth of pathogens. Probiotics render a local protective effect against pathogens and a systemic indirect effect on immunological amelioration. Synbiotics are fusion products of prebiotics and probiotics. This mini review discusses the potential use and associated limitations of probiotics, prebiotics, and synbiotics for the prevention and treatment of oral candidiasis. We will also introduce biogenics, a recent concept derived from the work on probiotics. Biogenics advocates the use of beneficial bioactive substances produced by probiotic bacteria, whose activities are independent from the viability of probiotic bacteria in human bodies.

Keywords: probiotics, prebiotics, synbiotics, biogenics, oral candidiasis, lactobacilli

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INTRODUCTION

The indigenous microbiota on the surfaces of the skin and mucous membranes plays a role in preventing the invasion of foreign pathogenic microorganisms. The oral cavity possesses a diverse set of indigenous microbiota that perpetually interacts with the host mucosal surfaces. The oral microbiota predominantly comprises bacteria and a small proportion of fungi. *Candida* is the major fungus residing even in the healthy human oral cavity (Sardi et al., 2013). However, depending on circumstances, *Candida* can transform into a pathogen causing oral infections. Hence, when there is a collapse in the healthy microbial balance, i.e., dysbiosis, *Candida* can proliferate and cause a typical opportunistic infection. Oral candidiasis has been frequently observed in the elderly population due to problems associated with quality and the production of saliva, as well as decreased cell-mediated immunity (Scully et al., 1994). Systemic *Candida*

infections such as *Candida* pneumonia and candidemia due to intravascular indwelling catheters have also been observed in elderly populations (Eggimann et al., 2003). Recurrent oral candidiasis occurs frequently in HIV-positive and AIDS patients (Scully et al., 1994). The administration of antifungal drugs is generally the first-line therapy of candidiasis. However, the emergence of drug-resistant strains and frequent recurrence of the disease in affected individuals are increasing challenges in antifungal therapy (Pfaller and Diekema, 2007). This has prompted the need for an alternative therapeutic and prevention strategy. In this mini review, we will succinctly discuss the potential use of probiotics, prebiotics, and synbiotics as an alternative antifungal therapy. In addition, a new concept of biogenics will be introduced. Biogenics is a strategy to overcome the potential disadvantage of synbiotics, including difficulties in the colonization process of non-native probiotic bacteria. It also provides an additional advantage to produce functional foods with bioactive metabolites.

PROBIOTICS

The Definition and History

The term "probiotics," in contrast to antibiotics, was proposed by Lilly and Stillwell (1965), from the original ecological term, "probiosis" used by Kollath (1953), meaning a symbiotic relationship between organisms. Fuller (1989) defined a probiotic as "A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" (Fuller, 1989). Hence, at that time, probiotics were intended to be used only for the "intestinal microbiota." Subsequent studies revealed general health benefits of probiotics, such as an enhancement of the human immune system, preventive effects concerning urinary tract and respiratory tract infections and the allergic or atopic condition in infants (Gourbeyre et al., 2011). Hence, probiotics were redefined by Salminen et al. (1998) as "A viable microbial food supplement which beneficially influences the health of the host." According to the FAO/WHO, probiotics are defined as "live microorganisms when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2001).

Clinical Trials of Probiotics for Oral *Candida* Infections

There has been a gradual increase in the number of studies that focus on the application of probiotics on oral health (Haukioja, 2010). The majority of these studies have focused on two major dental diseases, dental caries and periodontitis (Krasse et al., 2005; Vivekananda et al., 2010; Cagetti et al., 2013). However, studies on the use of probiotics for oral candidiasis are sparse (**Table 1**). Ahola et al. (2002) and Hatakka et al. (2007) conducted double-blinded, randomized clinical trials using probiotic cheese on elderly populations with some oral health problems and carriers of oral *Candida* compared with a younger cohort (18–35 years of age). There was an observed trend that the probiotics could decrease the quantity of *Candida*. However, the effect was not significant (Ahola et al., 2002) or was small without an improvement in the mucosal symptom (Hatakka et al., 2007). On the other hand, studies conducted by Mendonça et al. (2012), Ishikawa et al. (2015), and Kraft-Bodi et al. (2015) reported a slight or moderate improvement of oral candidiasis when patients were treated with probiotics. Dos Santos et al. (2009) reported a drastic improvement of oral candidiasis upon probiotic treatment.

In Vivo Animal and *In Vitro* Studies of Probiotics for Oral *Candida* Infections

Several *in vivo* animal studies have been performed which have examined the effect of probiotics on oral *Candida* infections. However, the results remain controversial. Some reports suggested a local as well as systemic beneficial effect of probiotics on candidiasis (Wagner et al., 1997; Elahi et al., 2005; Matsubara et al., 2012), while others have not observed a positive effect (Zavisic et al., 2012). These diverse observations may result from differences in the administration technique employed. However, Kojima et al. (2015) demonstrated that the key factor for the effectiveness of probiotics may be the selection of an appropriate strain that works against *Candida.* A diverse set of *Lactobacilli* species has been used for the previous probiotic studies. The genome size of the *Lactobacillus* genus ranges from 1.23–4.91 Mb and the GC content spans 31.9–57.0% among different species (Caufield et al., 2015). In addition, the properties of strains within the same species of *Lactobacillus* have been shown to vary (Koll et al., 2008; Tiihonen et al., 2010). Some of these studies have selected probiotic (*Lactobacillus*) strains that are known to confer intestinal health benefits and presume a similar beneficial effect on oral infections or *Candida* infections. Therefore, it is important to demonstrate the *in vitro* activity of a probiotic strain against *Candida* and subsequently select an efficient strain for *in vivo* and clinical studies. Such studies are few and shown in **Table 1**.

Anti-*Candida* Products of Probiotics for Oral Candidiasis

Probiotic lactobacilli co-aggregate with *Candida* and produce antimicrobial substances that have a direct growth inhibitory effect on *Candida*. Some of these substances produced include organic acids (e.g., lactic acid and acetic acid), hydrogen peroxide (H_2O_2), bacteriocins, and uncharacterized low molecular weight substances with antifungal properties. Lactobacilli universally produce lactic acid that inhibits the metabolic activity of *Candida* sp. (Köhler et al., 2012), which has a weak antifungal activity (Zalán et al., 2010). It appears that lactobacilli do not produce effective concentrations of H_2O_2 against fungi (Shokryazdan et al., 2014), unlike other bacteria (Piard and Desmazeaud, 1991).

Lactic acid bacteria produce bacteriocins, proteinaceous antimicrobial substances with molecular weights of several thousand daltons or more. Bacteriocins can be divided into five classes according to their primary structure, molecular composition and properties (Chen and Hoover, 2003; Pascual et al., 2008). Bacteriocin L23 produced by *Lactobacillus fermentum* L23 (Pascual et al., 2008), plantaricin produced by *L. plantarum* (Sharma and Srivastava, 2014), and pentocin TV35b produced by *L. pentosus* (Okkers et al., 1999) appear

TABLE 1 | Summary of studies that examined the antifungal activity of probiotics against *Candida albicans*.

Reference	Test strains	Test design/Feature tested	Results
Clinical studies			
Ahola et al. (2002)	*L. rhamnosus GG/LS*	Intervention with cheese, Double-blinded placebo RCT	Reduction in the risk of a high level of *Candida*
Hatakka et al. (2007)	*L. lactis, L. helveticus, L. rhamnosus GG, P. freudenreichii*	Intervention of an elderly group with cheese for 16 weeks, Double-blinded randomized placebo trial (tested group, $n = 136$, control group, $n = 140$)	10% reduction of the high *Candida* count rate in the tested group (after 16-weeks intervention)
Dos Santos et al. (2009)	*L. casei. B. breve*	No control group, 26 individuals Intervention with a commercial probiotic drink for 20 days	Reduction of the *Candida* carrying rate, reduction of the sIgA level
Mendonça et al. (2012)	*L. casei, B. breve*	No control group, 42 individuals over 65 years of age Intervention with a commercial probiotic drink for 30 days	Decrement of *Candida* prevalence, increment of sIgA level
Sutula et al. (2013)	*L. casei*	No control group, 22 healthy individuals approximately 32 years of age Intervention with a commercial probiotic drink for 4 weeks	No reduction of the *Candida* CFU, reduction of the halitosis score, did not detect *L. casei* after tests
Ishikawa et al. (2015)	*L. rhamnosus, L. acidophilus, B. bifidum*	Double-blinded randomized trial (tested group, $n = 30$, control group, $n = 29$) Intervention with trial probiotic products for 5 weeks	Reduction of the *Candida* carrying rate in the tested group
Kraft-Bodi et al. (2015)	*L. reuteri*	Double-blinded placebo RCT, elderly individuals living in a nursing home (tested group, $n = 84$, control group, $n = 90$) Intervention with probiotic lozenges	Improved the *Candida* score
Animal studies			
Wagner et al. (1997)	*L. acidophilus, L. reuteri, L. casei, B. animalis*	Oral candidiasis model in immunodeficient bg/bg-nu/nu mice Estimated by the CFU and pathological examinations	Increased the life expectancy in the tested group
Elahi et al. (2005)	*L. acidophilus, L. fermentum*	*Candida* infection model using male DBA/2 mice (H-2d), 6–8 weeks of age Oral administration of probiotics	Reduction in the duration of *Candida* colonization in the tested group
Matsubara et al. (2012)	*L. acidophilus, L. rhamnosus*	DBA/2 murine oral *Candida* infection model. Control group was treated with nystatin, tested group was treated with probiotics	Reduction of the *Candida* level in the tested group compared with the control group
Zavisic et al. (2012)	*L. plantarum, L. casei*	Wister rats and NMRI Ham laboratory mice	Did not show an inhibition in *C. albicans* growth
Ishijima (2012)	*S. salivarius*	ICR mice, oral candidiasis model	Probiotics were not fungicidal, but inhibited *Candida* adhesion
***In vitro* test**			
Chung et al. (1989)	*L. reuteri*	MIC assay using partial purified reuterin	Reuterin, an anti- microbial substance with broad spectrum effects, led to the reduction of *C. albicans growth*
Koll et al. (2008)	*L. plantarum, L. paracasei, L. salivarius, L. rhamnosus*	Antimicrobial activity was detected using the antagonism method	Did not show an inhibition in *C. albicans* growth
Köhler et al. (2012)	*L. rhamnosus, L. reuteri*	Antimicrobial activity was detected using an overlay plate or co-culture assay. The genome-wide transcriptional profile of *C. albicans* was assayed with a cDNA microarray	*C. albicans* was antisepticized by inhibition of the metabolic activity under a low pH
Hasslof et al. (2010)	*L. plantarum, L. rhamnosus GG, L. paracasei, L. reuteri, L. acidophilus*	Agar overlay interference tests	*Candida* growth was reduced, however, the effect was generally weaker than for mutans streptococci
Jiang et al. (2014)	*L. rhamnosus GG, L. casei, L. reuteri, L. brevis, L. bulgaricus*	Estimated the inhibition effect by pH conditions and the combination of saccharides using EIR	Inhibition capacity differed in the probiotic strains, *L. rhamnosus* showed the strongest inhibition effects against *C. albicans*

(Continued)

TABLE 1 | Continued

Reference	Test strains	Test design/Feature tested	Results
Shokryazdan et al. (2014)	*L. acidophilus,* *L. buchneri,* *L. casei,* *L. fermentum*	Co-culture test with 12 pathogenic microorganisms	The active substance was organic acid
Kheradmand et al. (2014)	*L. johnsonii,* *L.plantarum*	After selenium treatment, the antimicrobial effects improved	The active substances were exometabolities or novel anti-*Candida* compounds
Kojima et al. (2015)	*L. fermentum,* *L. plantarum,* *L. paracasei* per 12 species (40 strains)	Co-culture and growth inhibition assays of *C. albicans* with *Lactobacilli* culture supernatant or saccharides	Three saccharides and five strains became candidates for pre- and probiotics, respectively

to be effective against the yeast form of *Candida*. Bacteriocins effective for hyphal forms of *Candida* have not yet been identified (Calderone and Fonzi, 2001; Douglas, 2003). Low molecular substances of lactobacilli, such as reuterin (Talarico et al., 1988), reutericyclin (Ganzle, 2000), and dyacetyl (Jay, 1982), have also been shown to be effective against the yeast forms of *Candida* (Chung et al., 1989).

PREBIOTICS

The term "prebiotics" was defined by Gibson and Roberfroid (1995) as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health." Studies of oral prebiotics are limited. Sugars and dietary fiber have been considered to be prebiotics for intestinal lactic acid bacteria (Gibson and Roberfroid, 1995). However, this is not the case for the oral environment, as the presence of sugars increases the risk of dental caries. The mutans group of streptococci metabolizes cariogenic sugars, such as glucose and sucrose, and produces organic acid and insoluble glucan factors that contribute to dental caries. On the other hand, sugar alcohols such as xylitol suppress the growth of *Streptococcus mutans*. Xylitol, a reduced derivative of xylose, converts to xylitol-5-phosphate inside *S. mutans* cells and inhibits glycolysis (Miyasawa-Hori et al., 2006). Similarly, arabinose, a member of the same aldopentose group as xylose, is not assimilated by *S. mutans* (Coykendall, 1977) and likely has a similar effect as xylitol. We recently demonstrated that xylitol, xylose, and arabinose inhibited the growth of *S. mutans*, but were utilized for the growth of most of the lactobacilli strains we tested (Kojima et al., 2015). Although xylitol is generally not assimilated by lactobacilli, a recent report showed that 36% of lactobacilli strains isolated from human oral cavities were able to metabolize xylitol (Almstahl et al., 2013). Meanwhile, our previous data on *Candida albicans* ATCC18804 showed decreased growth in the presence of three saccharides (xylitol, xylose, and arabinose) compared with glucose (Kojima et al., 2015). There are conflicting reports on the ability of *C. albicans* to assimilate xylitol and aldopentose. Mäkinen et al. (1975) and Maleszka and Schneider (1982) showed that *C. albicans* is not capable of proper growth in the presence of xylitol. Uittamo et al. (2011) suggested that xylitol metabolism of *Candida* might compete for the nicotinamide adenine dinucleotide (NADH) coenzyme, leading to the downregulation of alcohol dehydrogenase (ADH). Clinical trials of Turku sugar studies III and VIII showed significantly decreased colony counts and detection frequency of oral *Candida* in the xylitol intake group [Larmas et al. (1974, 1976)]. On the other hand, yeast is known to possess a pentose assimilation pathway that produces ethanol from arabinose and xylose by an enzymatic reaction (Chiang and Knight, 1960; Ônishi and Suzuki, 1966). Even if *Candida* is capable of slowly assimilating those three candidate sugars, the phenomenon of slower growth compared to that of probiotic bacteria may have a competitive inhibition on *Candida*. The presence of xylitol inhibits the adhesion of *Candida* to mucosal surfaces (Pizzo et al., 2000; Abu-Elteen, 2005). In an experimental murine model of gastrointestinal candidiasis, the colonization and invasion of *C. albicans* was significantly reduced in the group supplemented with xylitol compared to the group supplemented with glucose (Vargas et al., 1993).

SYNBIOTICS

The Noteworthy Features of Synbiotics Associated with the Oral Application

Gibson and Roberfroid (1995) proposed the use of probiotics and prebiotics fusion products or "synbiotics" for the intestinal tract microbiota (Panigrahi et al., 2008). However, the use of synbiotics for the oral microbiota has not been well studied (Kojima et al., 2015). It is important to understand the limitations associated with the oral application of synbiotics. Probiotic bacteria are not able to easily colonize adult oral cavities (Lazarevic et al., 2010; Tiihonen et al., 2010). Therefore, it appears that synbiotics are more effective for oral applications than probiotics alone. One must, however, consider the risk of dental caries while applying lactic acid bacteria in the oral cavity. Lactobacilli have long been considered to be one of the cariogenic bacteria present in dental plaque (Glass, 1952). Currently, there are two concepts on the association of lactobacilli with dental caries. Lactobacilli comprise a very small proportion of normal oral microbiota and are primarily present on the tongue dorsum, rather than in dental plaque (van Houte et al., 1972). However, they are hardly detected in the oral cavity of caries-free individuals (Yang et al., 2010).

The lactobacilli count in the saliva is an indicator of the dental caries activity as lactobacilli penetrate porous tooth surfaces in early caries lesions or adhere to type I collagen exposed in the carious portion of the tooth (Caufield et al., 2015). As the salivary lactobacilli count correlates with the amount and frequency of carbohydrate (sugar) intake (Jay, 1947; Becks, 1950), the presence of lactobacilli is a reliable indicator for the dental caries activity (Crossner, 1981). Therefore, if one can maintain good oral hygiene, oral probiotic therapy with lactobacilli alone may not contribute to the development of dental caries. In addition, if appropriate prebiotics are administered simultaneously, then synbiotic therapy may suppress the development of oral candidiasis.

The Different Immune Responses Associated with Synbiotics in the Intestinal Tracts or Oral Cavities

The important considerations for synbiotic therapy of the intestine and oral cavity are the host immune component and reactions. While activation of a substantial host immune response can be expected in the intestine, a similar phenomenon is not expected in the oral cavity as it is not an organ of mucosa-associated lymphoid tissues (MALT). In the intestine, probiotic bacteria are incorporated into M cells in Peyer's patches (PP), which is a major component of gut-associated lymphoid tissues (GALT), and digested to form active antigens. Macrophages and dendritic cells in PP phagocytize probiotic bacteria and are activated to produce several cytokines, which stimulate T-cell and B-cell functions (Matsuzaki et al., 2007). Moreover, daily supplementation of lactobacilli as part of a normal diet increased the number and activity of natural killer cells in healthy elderly individuals (Tiihonen et al., 2010). Thus, synbiotics in the intestinal tract can be expected to activate both innate immunity and acquired immunity of cell-mediated and humoral immunity. Conversely, the oral cavity it is not an immune organ and phenomenon such as direct antigen presentation to adaptive immune cells does not occur. Nevertheless, some probiotic clinical trials and animal studies using oral candidiasis models have reported an increase of sIgA against *Candida,* leading to the suppression of *Candida* in the oral cavity (Wagner et al., 1997; Elahi et al., 2005; Mendonça et al., 2012). It is well known that secretion of sIgA at the salivary gland is through differentiated plasma cells from B cells stimulated at MALT. According to the results of clinical and animal studies described above, oral synbiotics appear to transition into intestinal synbiotics, as the oral cavity is connected to the intestine. Children who were oral lactobacilli carriers were found to have similar lactobacilli in their feces (Caufield et al., 2015). Hence, it appears that the intestinal colonization of lactobacilli is transmitted though the oral cavity, which may provide simultaneous synbiotic activity at the oral cavity and the intestine.

BIOGENICS

Previous studies have highlighted the limitation of colonization and fixation of non-nature probiotic bacteria in the intestinal tracts of human bodies (Haenel, 1960; Mitsuoka and Kaneuchi, 1977). This scenario is also relevant for the probiotic application in oral cavities, particularly when considering the associated risk of oral probiotics and dental caries. In order to address foregoing concerns, the concept of "biogenics" has been suggested as a solution (Mitsuoka, 2000). Biogenics is defined as

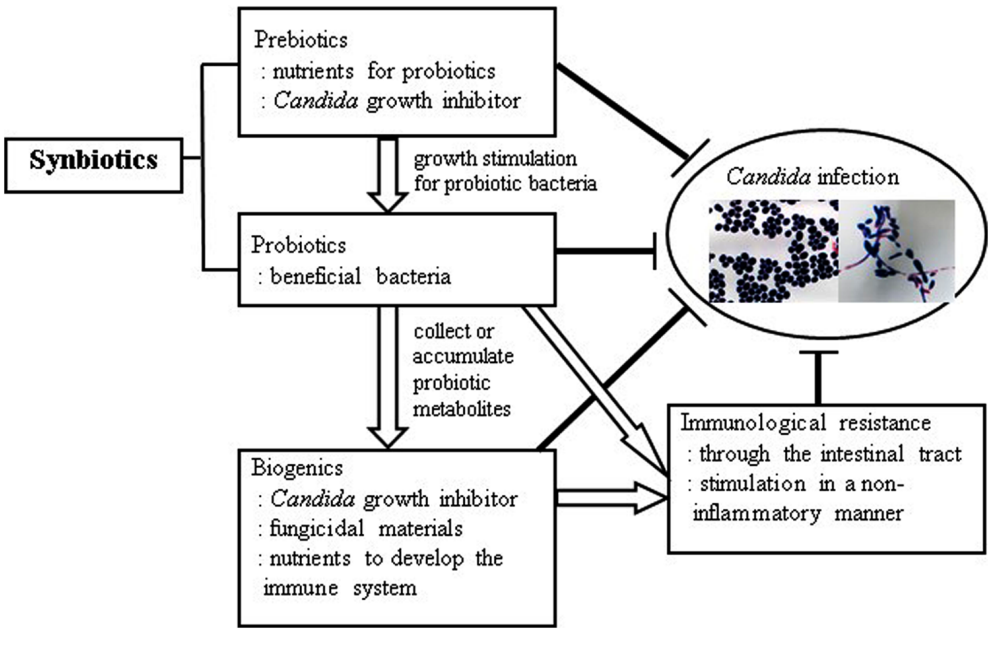

FIGURE 1 | Anti-*Candida* effects with synchronized prebiotics, probiotics, biogenics, and immunological resistance.

"food ingredients which beneficially affect the host by directly immunostimulating or suppressing mutagenesis, tumorigenesis, peroxidation, hypercholesterolemia, or intestinal putrefaction" (Mitsuoka, 2000). Hence, previous studies have suggested the administration of non-viable probiotic bacteria to obtain some "probiotic" effects. It was reported that the consumption of pasteurized fermented milk elongated the lifespan of mice (Arai et al., 1980; Takano et al., 1985). A significant reduction of the Ehrlich ascites tumor growth in mice was also reported (Takano et al., 1985). In addition, it was shown that heat-inactivated *Enterococcus faecalis* (Terada et al., 2004) or *L. gasseri* (Sawada et al., 2016) retained a beneficial regulatory function in the gut. Moreover, Nakamura et al. (1995) identified an angiotensin I-converting enzyme (ACE) inhibitor in a Japanese sterilized milk beverage fermented by *L. helveticus* and *Saccharomyces cerevisiae*. The active substance was lactotripeptides metabolically generated in the fermentation pathway. Follow-up studies were able to determine the bioactive metabolites of probiotic bacteria in addition to the antimicrobial substances, such as bacteriocin (Ross et al., 2010; O'Shea et al., 2012), and other beneficial active substances, such as conjugated linoleic acid (CLA; Hayes et al., 2006; Ross et al., 2010; O'Shea et al., 2012), protein or peptides (Möller et al., 2008; Bogsan et al., 2013), and polyphenols (Dharmaraj, 2010; Monagas et al., 2010). Taking all these observations into account, the new concept, biogenics, which makes use of the bioactive metabolites as foods or medicine, was recently advocated (Mitsuoka, 2000, 2014). The biogenics effect is independent of the colonization and viability of probiotic bacteria. Hence, biogenics is the direct delivery of an isolated and purified active ingredient of probiotics to the local environment. This strategy may also be used as an antifungal therapy. It may be possible to purify the active ingredients of probiotic bacteria that demonstrate antifungal activity for use in the biogenics process. However, this idea requires further study before clinical use.

CONCLUSION

Taking the abovementioned studies into consideration, it is conceivable that an innovative combination of prebiotics, probiotics, synbiotics, and biogenics instrumental in devising a successful, novel antifungal regime in the future (**Figure 1**). More comprehensive investigations on the mechanism of synbiotics and biogenics are needed for this purpose. Hence, more studies are warranted to examine the bioactive metabolites of probiotic bacteria that induce favorable immunological outcomes and suppress *Candida* infection in the human oral cavity.

AUTHOR CONTRIBUTIONS

TO made the description plan of this review article, and carried out the manuscript writing and figure charting. YK, CS, and NM read carefully and made arrangements on the manuscript according their discussions.

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Microbial Biotransformation to Obtain New Antifungals

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Antifungal drugs belong to few chemical groups and such low diversity limits the therapeutic choices. The urgent need of innovative options has pushed researchers to search new bioactive molecules. Literature regarding the last 15 years reveals that different research groups have used different approaches to achieve such goal. However, the discovery of molecules with different mechanisms of action still demands considerable time and efforts. This review was conceived to present how Pharmaceutical Biotechnology might contribute to the discovery of molecules with antifungal properties by microbial biotransformation procedures. Authors present some aspects of (1) microbial biotransformation of herbal medicines and food; (2) possibility of major and minor molecular amendments in existing molecules by biocatalysis; (3) methodological improvements in processes involving whole cells and immobilized enzymes; (4) potential of endophytic fungi to produce antimicrobials by bioconversions; and (5) *in silico* research driving to the improvement of molecules. All these issues belong to a new conception of transformation procedures, so-called "green chemistry," which aims the highest possible efficiency with reduced production of waste and the smallest environmental impact.

Keywords: microbial biotransformation, biocatalysis, bioconversion, metabolism, antifungals

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INTRODUCTION

The search for new molecules has forced the pharmaceutical industry to modernize its synthetic processes. Such modernization has occurred with the adoption of new techniques, such as miniaturization, nanotechnology, microdosing, chemometrics, and high-throughput analysis (Koh et al., 2003). Another aspect of modernization, so-called "Green Chemistry," requires new synthetic processes with the highest possible efficiency, resulting in reduced production of waste and the smallest environmental impact (Tucker, 2006).

More than 20,000 molecules with antibiotic activity that are produced by microorganisms have been described since the discovery of penicillin by Sir Alexander Fleming; however, only a small fraction of them are clinically useful due to their toxicity. Since the 1980s, a decline in the discovery of new molecules has been observed (Murphy, 2012).

The antifungal agents available on the market act on different targets such as ergosterol synthesis, chitin synthesis, glucan synthesis, nucleic acid synthesis, protein synthesis, microtubule synthesis, or as inhibitors of squalene epoxidase or ergosterol disruptors (Kathiravan et al., 2012). However, although there is substantial variability in the mechanisms of action and despite the technical advances, the development of new antifungal drugs persists considering the co-evolution of resistance mechanisms. Between 2006 and 2010, only one antifungal was approved for use, a natural

echinocandin that was chemically changed by a semi-synthetic route (Chen et al., 2011). In the last 30 years, among the 28 new naturally occurring molecules, only three semi-synthetic molecules that underwent chemical changes have been authorized for clinical use (Newman and Cragg, 2012).

It is estimated that 25% of the world's population presents some episode of superficial mycosis and the mortality rate associated with invasive fungal infections frequently exceeds 50%, even with the available antifungal medications. This amount corresponds to approximately 1.5 million deaths annually (Brown et al., 2012). Even under prophylactic use with antifungals, some changes in epidemiological features have been reported in more aggressive mucormicoses in patients using voriconazole, as well as the rampant development of resistance to azole by *Aspergillus* spp. (Perfect et al., 2014).

Echinocandins and allylamines are more modern drug classes approved, but they date back to the 1970s and 1980s, respectively (Odds, 2003). Echinocandins have been used as the treatment of choice for systemic candidoses and are effective for strains resistant to azoles (Zimbeck et al., 2010; Eschenauer et al., 2014); however, a mutation in the *FKS* gene increases the resistance of *Candida* spp. to echinocandins (Zimbeck et al., 2010; Beyda et al., 2012). A multicenter study demonstrated that *C. glabrata* and *C. krusei* have lost their susceptibility to caspofungin (24 and 52%, respectively) and that other common *Candida* species are rapidly losing their susceptibility to echinocandins (Zimbeck et al., 2010).

Even assuming great advances in synthetic chemistry, biotransformation (or biocatalysis) remains the most cost-effective path to discover new pharmaceuticals (Zaks and Dodds, 1997). Special attention should be given to the significant number of drugs produced by microorganisms or by interactions with the host from which they were isolated. Both cases contribute to the idea that biotransformation processes shall expand significantly in the future (Newman and Cragg, 2012). Nevertheless, there are some questions regarding biotransformation that have to be addressed: (1) How can structural changes occur in a way to make the processes more time- and cost-efficient? (2) How can the biological activity of products be enhanced by optimizing the pharmacokinetic/pharmacodynamic (PK/PD) properties and safety? (3) How may a supplier guarantee large-scale drug production under good quality practices? (Bauer and Brönstrup, 2014).

With the appropriation of concepts from White Biotechnology and Green Chemistry, this review aims to assess the technological advances in the development of microbial biotransformation products with antifungal activity.

MICROBIAL METABOLISM VS. MICROBIAL BIOTRANSFORMATION

Natural products compose more than 2/3 of antibiotics used in the medical/dental/veterinary practice (Schmitz et al., 2013). Thus, it is not an erroneous statement to assume that active substances from plants or those isolated from microorganisms are the simplest way to search for new molecules. If they have antifungal potential, some criteria must be observed (Barrett, 2002): (1) Do they present novel mechanisms of action or any useful known mechanisms? (2) Is it possible to obtain clinical proof with good biological activity? (3) Is it possible to change the molecule to make it a tolerable drug? A good example of a bioactive molecule obtained from plant extracts is eugenol, which is extracted from *Eugenia caryophillis* (Indian clove). It is a phenylpropanoid that presents considerable fungicidal activity *in vitro* against *C. albicans*, and, unlike fluconazole, is also effective against *C. krusei* and *C. glabrata* (Ahmad et al., 2010).

In addition to plants, microorganisms have provided some bioactive molecules with remarkable antimicrobial activity, especially in the last two decades.

For fungal infections, the glycopeptide occidiofungin A is produced by *Burkholderia contaminans* MS14 and presents great antifungal activity against pathogens of plants and animals (Lu et al., 2009). Its mechanism of action has not been elucidated, but it is assumed that it differs from the known classes and can bypass fungal resistance problems (Tan et al., 2012). Benanomicin A and benanomicin B are fermentatively produced by the cultivation of *Actinomadura spadix* MH193-16F4 and are broad-spectrum antimicrobials against various fungi including endemic and opportunistic pathogens (Kumagai et al., 2008).

Although there remains a myriad of naturally occurring secondary metabolites to be evaluated and discovered, microbial biotransformation has emerged as an important tool for obtaining novel structural analogs or to improve the pharmacokinetic parameters of other substances (Parshikov et al., 2000; Borges et al., 2008; Baydoun et al., 2014).

It is important to emphasize that metabolism and biotransformation are distinct systems of molecular processing. Microbial metabolism is composed of two major processes; primary metabolism, which is responsible for cellular function, and secondary metabolism, which uses pre-existing metabolic pathways to produce substances from endogenous intermediates to allow better adaptation of the organism to the environment (Keller et al., 2005; Brakhage, 2013). Primary metabolism consists of reactions associated with energy generating, biomass production, and essential cell components. Events such as glycolysis, oxidative phosphorylation, and the Calvin-Benson cycle (in algae and photosynthesizing bacteria) are examples of typical sets of reactions of primary metabolism.

In contrast, secondary metabolism involves important events for adaptation to environmental conditions. In general, it generates low molar mass metabolites that are not essential for growth but that offer some advantages and may sometimes have medical/veterinary/agricultural/industrial importance. They include antibiotics, pigments, anti-tumor agents, etc. They have unusual structures and are normally synthesized during the late phase of cell growth (Ruiz et al., 2010).

Regarding to antibiotics, it is estimated that actinomycetes are responsible for 70–80% of all molecules produced by secondary metabolism. Different species belonging to the genus *Streptomyces* produces important antibiotics as chloramphenicol, streptomycin, macrolides, and rifampicin, among others (Raja and Prabakarana, 2011). A recent review regarding to this theme presented new perspectives about the optimization in the

production of actinomycetes-derived antibiotics (Antoraz et al., 2015).

Biotransformation, sometimes inaccurately called "xenobiotic metabolism," is responsible for minor structural modifications in exogenous substances by enzyme systems that lead to the formation of molecules with relatively greater polarity (Asha and Vidyavathi, 2009; Pervaiz et al., 2013). Phenomena such as stereoselective hydroxylation, epoxidation, and oxidation are common reactions attributed to biotransformation processes and have been reported to occur in fungi (Farooq et al., 2002; Choudhary et al., 2005, 2007, 2009, 2010; Al-Aboudi et al., 2009). It is a mechanism that microorganisms developed to adapt to environmental changes and it is useful in a wide range of biotechnological processes (Crešnar and Petric, 2011).

One of the most remarkable features of biotransformation reactions is the maintenance of the original carbon skeleton after obtaining the products. During metabolism, the carbon atoms are transferred to other molecules with different chemical functions (**Figure 1**).

Biotransformation reactions may involve various events such as the formation of stable intermediates, which may be devoid of toxic or pharmacologic activity. Sometimes, short-lived reactants may also be generated. Further, biotransformation reactions can result in chemically stable compounds with desired pharmacological activity (Fura, 2006).

The use of microbial biotransformation is part of a new movement named White Biotechnology, which is an emerging field of modern biotechnology that serves industry. It employs living cells (animals, plants, algae, filamentous fungi, yeast, actinomycetes, and bacteria), as well as enzymes produced by these cells during the generation of products of interest.

Wisely, Venisetty and Ciddi (2003) presented nine practical advantages in the use of microbial systems as models for drug metabolism:

(1) The low-cost and facility to maintain stock cultures of microorganisms;
(2) Procedures with large number of strains are simple repetitive processes;
(3) The concentrations of parental molecules used (generally ranging from 0.2 to 0.5 g/L) are much higher than those employed in other cell or tissue models;

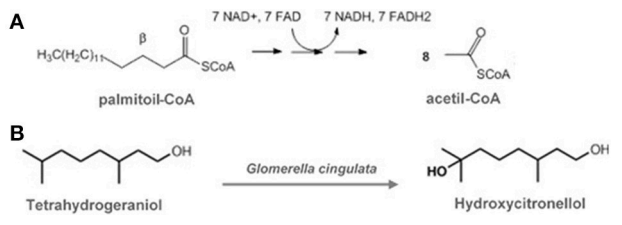

FIGURE 1 | (A) Typical multi-step metabolic reactions, with deep alterations on carbon skeleton (in this case, fatty acid oxidation). **(B)** Typical one-step biotransformation reaction, with minor punctual alterations on carbon skeleton (Adapted from: Hegazy et al., 2015).

(4) Novel products can be isolated with new or different activities;
(5) There is a possibility to predict the most favored metabolic reactions;
(6) The models can be scaled up easily for the preparation of metabolites for pharmacological and toxicological evaluation;
(7) These models can be utilized in synthetic reactions where tedious steps are involved;
(8) In most cases, relatively mild incubation conditions are used;
(9) The models can be useful in cases where regio- and stereo-specificity are involved, becoming molecular handling more easily achieved by biotransformation than by synthetic chemistry.

With regards to these last two statements, obtaining an antimicrobial may become critically laborious if only chemical procedures are employed, even for semi-synthetic compounds (Wild, 1994; Claes et al., 2013). In this context, microbial biotransformation becomes an attractive resource.

Living cells can be used in their original state (wild strains) or improved to work as "cell factories" to produce enzymes or consumer goods (Carballeira et al., 2009). Despite their potential, the number and diversity of applications is still modest when considering the wide availability of microorganisms, the large number of reactions that they can achieve and the fact that biotransformation reactions are considered economically and ecologically competitive (Borges et al., 2009).

BIOTRANSFORMATION BY WHOLE CELLS VS. IMMOBILIZED ENZYMES

It is insufficient to know the microbial biotransformation pathways to establish whether they are economically useful; rather, it is necessary to define reproducibility at a production scale. This assumption demands some concern regarding raw materials (the molecule to be biotransformed and the microorganism/enzyme responsible for the reaction), equipment (bioreactors), and the necessary technology to be employed in the purification of the products (downstream processing). In addition, it is imperative to take into account the lowest possible production of pollutants and the highest possible enantiomeric purity of the final product.

The criteria above are focuses of Green Chemistry that have allowed the construction of chiral chemical building blocks that lead to the development of enantiopure products obtained under mild reaction conditions at physiological pH and temperature, using water as reaction milieu and environmentally friendly catalysts (enzymes or cells). The obtained products are usually multifunctional molecules that exhibit highly chemo-regio-stereoselective activity (Borges et al., 2009; Muñoz Solano et al., 2012).

Immobilized enzymes are used for the conversion of molecules in many industrial fields, although they are preferably directed to simple catalytic processes. For the production of antimicrobials, the applicability of individualized enzymes in *stricto sensu* biotransformation processes is still incipient and

little explored (Banerjee, 1993a,b; Mujawar, 1999; Takimoto et al., 2004; Hormigo et al., 2010). Possibly, this may result from the functional complexity required, which is readily attainable by living organisms.

An important enzyme feature that has not been properly explored is their promiscuity, which is the ability of enzymes to catalyze different reactions with distinct catalytic mechanisms to create new pathways (Wu et al., 2010). If we obtained a new molecule with remarkable antifungal properties but with inappropriate characteristics for ADMET (administration, distribution, metabolism, excretion, and toxicity), it is possible to "improve" its structure by enzyme promiscuity. For this, *in silico* technologies favor technical development processes, predicting PK/PD characteristics and increasing the use of immobilized enzymes in the synthesis of pharmaceuticals.

Some complex processes of anti-proliferation production via enzymatic biotransformation are already known. A good example is the biosynthesis of griseofulvin from malonyl-CoA using purified enzymes (Cacho et al., 2013). Several phases including aldol condensation, cyclization, halogenation, and oxidation are enzyme-mediated steps that can be reproduced without the need of a living organism.

Penicillin G acylase (PGA) is one of the most relevant and widely used biocatalysts for the industrial production of β-lactam semisynthetic antibiotics (Srirangan et al., 2013). Such an enzyme may be bulk produced as heterologous PGA in competent strains such as *Escherichia coli* ATCC® 11105™ (Erarslan et al., 1990) or *Bacillus badius* PGS10 (Rajendran et al., 2011).

Considering the processes using whole cells, it is assumed that fungi are the organisms most commonly used to obtain natural metabolic products and for biotransformation reactions (Borges et al., 2009), even for the procurement of antifungal drugs; however, microorganisms from other kingdoms may also conduct dedicated biotransformation processes in order to obtain antifungal molecules (**Table 1**).

The use of whole cells is advantageous once they present all the needed enzymes and cofactors in adequate concentrations and energy status. These favorable conditions may modulate the activity of multienzymatic complexes and contribute to increase conversion rates (Restaino et al., 2014).

The obtaining of biotransformation products may be conducted using co-cultivation of two or more distinct entities. It has been proposed by Wu et al. (2015), who reported the increased obtaining of bioactive molecules during the co-cultivation of *Streptomyces coelicolor* A3(2)M145 (actinomycete) and *Aspergillus niger* N402 (fungus). According to the authors, "growth in microbial communities or interactions between different microorganisms is the next logical step in the search for new molecules."

Another interesting approach involves the possibility of heterologous expression of biotransformation-related cytochrome P450 enzymes from actinomycetes in bacteria to obtain new antimycotic derivates (Kumagai et al., 2008). It becomes useful for biotransformation procedures in large scale producing plants where large bioreactors are employed.

It has been reported that pyrethrosin, a germacrane sesquiterpene lactone commonly found in *Chrysanthemum cinerariaefolium* Visiani (Asteraceae), can be converted to several new molecules by *Cunninghamella elegans* NRRL1392 and *Rhizopus nigricans* NRRL1477 (Galal, 2001). Such filamentous fungi are able to completely deplete pyrethrosin, transforming it into five more polar metabolites that are very active against *Cryptococcus neoformans* ($IC_{50} = 25–35.0\ \mu g.mL^{-1}$) and *Candida albicans* ($IC_{50} = 10–30\ \mu g.mL^{-1}$).

The search for new antifungal molecules via microbial biotransformation involves not only purified precursor molecules, but may encompass complex substrates that can be bioconverted into extracts rich in active substances.

Biotransformation products of cabbage-crude extracts (*Brassica oleracea* var. *capitata*) processed by *Pseudomonas syringe* pv. T1 showed promising inhibitory activity for several *Candida* spp., with values close to those obtained for amphotericin B (Bajpai et al., 2011). *Pectobacterium atrosepticum* Pepto-A, a Gram-negative plant pathogen, can also be produced from cabbage extracts to yield anti-*Candida* compounds (Bajpai et al., 2012a).

Another *Pectobacterium* species, *P. carotovorum* subsp. *carotovorum* 21, also presented the ability to biotransform compounds found in cabbage (Bajpai et al., 2010a) and in tomatoes (*Solanun esculentum*) to generate others with remarkable activity against *Candida* spp. (Bajpai et al., 2012b,c).

Biotransformation has been successfully utilized as a tool to generate pharmaceutical compounds from natural products. Through this process, ethyl p-methoxycinnamate (EPMC) was extracted from *Kaempferia galanga* (a Malaysian plant) and was transformed using *Aspergilus niger* to ethyl p-hydroxycinnamate (EPHC) (Omar et al., 2014). Looking at antimicrobial activities, EPHC has a potential inhibitory effect against *C. albicans* that is better than the effect by EPMC. This highlights the possibility of increasing antifungal value to other known antifungal molecules.

Studies in this area shall be directed toward the diversification of substrates combined with the numerous species of biotransforming microorganisms. This can thus result in the expansion of the antifungal library. In a recent review, a number of molecules with antimicrobial activity derived from monoterpenes have been described after biotransformation processes by various microorganisms (Bhatti et al., 2014).

ENDOPHYTIC FUNGI PRODUCE ANTIMICROBIALS BY BIOTRANSFORMATION

Despite the substantial worldwide diversity, the discovery of new families of bioactive molecules is surprisingly declining, driving the need to bioprospect new sources (Joseph and Priya, 2011). Following such a thought, a group with promising potential for new discoveries in the biotransformation area is that of endophytic fungi. In recent years, endophytic fungi have garnered great interest.

They are organisms that can grow either intra or extracellularly in the tissues of higher plants in a clear mutualistic relationship without causing any symptoms. Evidence shows that they are a rich source of bioactive natural products. Active

TABLE 1 | Antifungal molecules obtained by microbial biotransformation.

Antifungal chemical	Biotransformer microorganism	Parental chemical	Antifungal activity	Citation
bEPA	*Pseudomonas aeruginosa* NRRL-B-18602	Eicosapentaenoic acid	*Botrytis cinerea*	Bajpai et al., 2008
			Colletotrichum capsici	Bajpai and Kang, 2007
bDHA		Docosahexaenoic acid	*Fusarium oxysporum*	Bajpai et al., 2009b
bEFA		Hydroxifatty acids: ricinoleic acid, linoleic acid, eicosadienoic acid, etc	*Fusarium solani* *Phytophthora capsici* *Rhizoctonia solani* *Sclerotinia sclerotiorum*	Bajpai et al., 2006
2,3-dihydrotrichostatin A	*Streptomyces venezuelae* YJ028	Trichostatin A	*Saccharomyces cerevisiae*	Park et al., 2011
Ethyl p-hydroxycinnamate	*Aspergillus niger*	Ethyl p-methoxycinnamate	*Candida albicans*	Omar et al., 2014
9-keto-(-)-vasicine	*Aspergillus braziliensis* ATCC®16404™ *Penicillium notatum* ATCC®36740™ *Rhizopus arrizus* ATCC®10260™ *Trametes versicolor* ATCC®20869™	(-)-vasicine	*Candida albicans*	Gopkumar and Mugeraya, 2010
5-p-menthene-1,2-diol	*Alternaria alternata* NRRL20593 *Aspergillus alliaceus* NRRL317 *Aspergillus flavus* *Botrytis cinerea* AHU9424 *Devosia riboflavina* NRRL-B-784 *Fusarium culmorum* *Fusarium heterosporium* DSM62719 *Fusarium solani* ATCC®1284™ *Kluyveromyces lactis* NRRL-Y-8279 *Neurospora crassa* N23 and N24 *Phanerochaete chrysosporium* ATCC®24725™ *Saccharomyces cerevisae* ATCC®9763™ *Yarrowia lipolitica* NRRL-Y-B423	α-phellandrene	*Candida* spp.	İşcan et al., 2012
Oxylipins	*Pseudomonas aeruginosa* 42A2	Hydroxifatty acids: ricinoleic acid, linoleic acid, oleic acid, palmitic acid, etc.	*Verticillium dhaliae* *Macrophonia phaesolina* *Arthroderma uncinatum* *Trycophyton mentagrophytes*	Martin-Arjol et al., 2010
Biotransformed galbonilides I and II	*Streptomyces halstedii* ATCC®55964™	Galbonolides A and B	*Candida albicans* *Cryptococcus neoformans*	Shafiee et al., 1999

metabolites of endophytics show positive actions as antibiotics, immunosuppressives, anti-helminthics, antioxidants, and anticancer drugs (Pimentel et al., 2011).

There is a noticeable increase in the rate of resistance to antimicrobials, which is, at least in part, related to the insufficient number of effective molecules and the small amount of new antimicrobial agents in development, probably due to unfavorable investment returns. In this context, the endophytic fungi present themselves as an attractive alternative to modify the current paradigm (Molina et al., 2012).

However, although a promising field, some problems cannot be ignored in studies with endophytes. Special attention is recommended in face of:

(1) The high nonspecific toxicity of some antimicrobials already obtained;
(2) The fact that fungi tend to not produce toxic substances against themselves, resulting in antimicrobial compounds with moderate antifungal activity without potential as a medicament or pesticide;
(3) The difficulty in large scale production of certain antimicrobial compounds in artificial culture media;
(4) The biosynthesis and regulation of production of antimicrobials from endophytes are partially or totally unknown (Yu et al., 2010).

It is important to mention that many of the products related to endophytics are derived from their secondary metabolism,

and do not necessarily have a biotransformation background. In some cases, it is not clear whether the final product is the result of secondary metabolism or biotransformation. In spite of this last finding, it is common sense amongst investigators devoted to biotransformation that these fungi present vast potential (Pimentel et al., 2011).

Table 2 presents some molecules with antifungal activity that are produced exclusively by endophytic fungi via secondary metabolism. As such, organisms that usually live in harsh conditions in the presence of numerous natural compounds are expected to be exceptional biotransforming entities (Shibuya et al., 2003, 2005; Agusta et al., 2005; Fu et al., 2011; Maehara et al., 2011; Huang et al., 2015; Khoyratty et al., 2015). Although endophytic fungi are promising organisms for biotransformation processes, their ability and potential to produce antimicrobial molecules remain unexplored.

CANDIDATE MOLECULES FOR BIOTRANSFORMATION

One point of concern when evaluating the feasibility of producing antifungals via microbial biotransformation is the choice of parental molecules to be modified. Such chemicals must not be toxic or inhibitory to the biotransforming organism. However, this can be circumvented if the concentrations during fermentation processes remain inferior to those considered as inhibitory.

Accumulated data has revealed that some classes of molecules seem to be more ready to undergo biotransformation and generate antifungals.

Some unsaturated fatty acids have shown interesting results. Bajpai et al. (2009a, 2010b) have conducted extensive reviews regarding this matter and reported that *Pseudomonas aeruginosa* NRRL-B-18602 PR3 produced mono-, di-, and tri-hydroxy fatty acid derivatives from unsaturated fatty acids with recognized antifungal properties. This bacterium can convert oleic acid to 7,10-dihydroxy-8(E)-octadecenoic acid, an anticandidal compound (Hou and Forman, 2000).

Other unsaturated fatty acids can also undergo oxidation in the presence of bacteria (*Pseudomonas* sp. 42A2 or *Bacillus megaterium* ALA2 NRRL-B-21660) or plants (*Colocassia antiquorum*) to produce mono-, di-, and tri-hydroxy fatty acids with antifungal potential such as 15,18-dihydroxy-14,17-epoxy-5(Z),8(Z),11(Z)-eicosatrienoic acid, 17,20-dihydroxy-16, 19-epoxy-4(Z),7(Z),10(Z),13(Z)-docosatetraenoic, 9,12,13-trihydroxy-(E)-octadecenoic acid, 12,13,16-trihydroxy-9(Z)-octadecenoic acid, and 12,13,17-trihydroxy-9(Z)-octadecenoic acid (Masui et al., 1989; De Andrés et al., 1994; Hou, 1996; Hou et al., 1997; Hosokawa et al., 2003a,b).

Based on the mechanism proposed for (Z)-9-heptadecenoic acid (Avis and Bélanger, 2001), the most probable antifungal mechanism of action must involve the disruption or disintegration of the plasma membrane caused by a hydrostatic turgor pressure within the cell resulting in the release of intracellular electrolytes and proteins (Carballeira, 2008).

Another class of molecules with a promising outlook are sterols. This class of substances has been evaluated in relation to their ability to be biotransformed for many years (Mahato and Mukherjee, 1984; Mahato and Garai, 1997; Holland, 1999; Malaviya and Gomes, 2008; Bhatti and Khera, 2012; Donova and Egorova, 2012). Surprisingly, there are few investigations on the production of antifungals, despite the fact that many compounds such as 24-amino-lanosterol, 24-amino-cholesterol, and 24-amino-cholesterol-N-sulfate possess potent antifungal activities against *Candida* spp., *C. neoformans*, and *Trichophyton mentagrophytes* (Chung et al., 1998).

Preliminary studies have shown that steroids and steroidal lactones biotransformed by *Cunninghamella* spp. produce metabolites with leishmanicidal activity (Choudhary et al., 2006; Baydoun et al., 2014). It is reasonable that such metabolites act on the synthetic pathway of ergosterol in *Leishmania* spp. We speculate that these putative mechanisms of action may be extrapolated to fungi, which should encourage investigators to drive their efforts toward this problem.

Alkaloids may also be converted into antifungal compounds. As part of an extensive program which aimed the discovery and development of antimicrobials from higher plants, Orabi and colleagues conducted a series of experiments in order to obtain antifungals from sampangine, an alkaloid found in the West African tree *Cleistophathis patens* (Annonaceae) (Orabi et al., 1999). Their results showed that *Beauveria bassiana* ATCC®7159™, *Doratomyces microsporus* ATCC®16225™, and *Filobasidiella neoformans* ATCC®10226™ produced the 4′-O-methyl-β-glucopyranose conjugate, while *Absidia glauca* ATCC®22752™, *Cunninghamella elegans* ATCC®9245™, *Cunninghamella* sp. NRRL5695, and *Rhizopus arrhizus* ATCC®11145™ produced the β-glucopyranose conjugate. Both metabolites presented significant *in vitro* activity against *C. neoformans,* but were inactive against *C. albicans* (Orabi et al., 1999).

The same group published interesting results about the biotransformation of the synthetic antifungal alkaloid benzosampangine (Orabi et al., 2000). They showed that *Absidia glauca* ATCC®22752™, *Cunninghamella blakesleeana* ATCC®8688a™, *Cunninghamella* sp. NRRL5695, *Fusarium solani* f. sp. *cucurbitae* CSIH#C-5, and *Rhizopogon* species ATCC®36060™ each produced a β-glucopyranose conjugate of benzosampangine. Such a substance possesses good *in vitro* antifungal activity against *C. albicans*, *Aspergillus fumigatus* (MIC = 0.39 $\mu g.mL^{-1}$; amphotericin B: 0.78 $\mu g.mL^{-1}$ and 0.39 $\mu g.mL^{-1}$, respectively), and *C. neoformans* (MIC = 1.56 $\mu g.mL^{-1}$; amphotercin B, 0.39 $\mu g.mL^{-1}$). The authors emphasized that microbial biotransformation is reliable and produces significant quantities of metabolites. In addition, they showed that alkaloids could be converted into conjugate metabolites with increased antifungal activity.

IN SILICO PREDICTIVE IMPROVEMENT OF BIOPROCESSABLE MOLECULES

In silico is the term used to define experimentation carried out in computers. In turn, *in silico* pharmacology is a large growing area that helps to develop molecular arrangements using dedicated software to capture, analyze and integrate biological and medical

TABLE 2 | Antifungal molecules obtained from secondary metabolism of endophytic fungi.

Antifungal chemical	Endophytic fungi	Origin	Antifungal activity	Citation
Camptothecin Periconicin A and B Phomol	*Colletotrichum* sp.	*Artemisia annua*	Various human and plant pathogens	Guo et al., 2008
Pyrrocidines A and B	*Acremonium zeae* NRRL13540	*Zea mays*	*Aspergillus flavus* *Fusarium verticillioides*	
Sordaricin	*Xylaria* sp. PSU-D14	*Garcinia dulcis*	*Candida albicans*	Pongcharoen et al., 2008
Lactone multiplolides A and B	*Xylaria multiplex* BCC 1111	Unidentified Thai tree	*Candida albicans*	Boonphong et al., 2001
7-amino-4-methylcoumarin	*Xylaria* sp. YX-28	*Gynkgo biloba*	*Aspergillus niger* *Candida albicans* *Penicillium. expansum*	Liu et al., 2008
Griseofulvin	*Xylaria* sp. F0010	*Abies holophylla*	*Blumeria graminis* f. sp. *hordei* *Corticium sasaki* *Magnaporthe grisea* *Puccinia recondite*	Park et al., 2005
Chaetomugilin A and D	*Chaetomium globosum*	*Ginkgo biloba*	*Mucor miehei*	Qin et al., 2009
Cytosporone B and C	*Phomopsis* sp. ZSU-H76	*Excoecaria agallocha*	*Candida albicans* *Fusarium oxysporum*	Huang et al., 2008
(-)-Mycorrhizin A (+)-Cryptosporiopsin	*Pezicula* spp.	Various German trees	*Euratium repens* *Mycatypha micraspara* *Ustilaga vialacea*	Schulz et al., 1995
Pestalachlorides A, B, and C	*Pestalotiopsis adusta* (L416)	Unidentified Chinese tree	*Fusarium culmorum* *Gibberella zeae* *Verticillium albo-atrum*	Li et al., 2008
Emodin Hypericin	*Thielavia subthermophila*	*Hypericum perforatum*	*Aspergillus niger* *Candida albicans*	Kusari et al., 2008, 2009; Kusari and Spiteller, 2011
Brefeldin A	*Cladosporium* sp.	*Quercus variabilis*	*Aspergillus niger* *Candida albicans* *Epidermophyton floccosum* *Microsporum canis* *Trichophyton rubrum*	Wang et al., 2007
Cytochalasin D 2-hexyl-3-methyl-butanodioic acid	*Xylaria* sp	*Palicourea* marcgravii	*Cladosporium cladosporioides*	Cafêu et al., 2005
Ethyl 2,4-dihydroxy-5,6-dimethylbenzoate Phomopsilactone	*Phomopsis cassia*	*Cassia spectabilis*	*Cladosporium sphaerospermum*	Silva et al., 2005
Asperfumoid Fumigaclavine C Fumitremorgin C Helvolic acid Physcion	*Aspergillus fumigatus* CY018	*Cynodon dactylon*	*Candida albicans*	Liu et al., 2004

(Continued)

TABLE 2 | Continued

Antifungal chemical	Endophytic fungi	Origin	Antifungal activity	Citation
2,6-diOH-2-methyl-7-(prop-1E-enyl)-1-benzofuran-3(2H)-one Ergosterol peroxide	*Verticillium* sp.	*Rehmannia glutinosa*	*Fusarium* sp. *Rhizoctonia* sp. *Septoria* sp.	You et al., 2009

data (Ekins et al., 2007). The use of *in silico* techniques allows the prediction of the pharmacokinetic aspects of absorption, distribution, biotransformation and excretion of new substances. It can be assumed that *in silico* projections of new molecules allow the prioritization of chemicals to be tested, identifying hazard and risk assessment (Kulkarni et al., 2005). The computer-assisted simulations based on pharmacological and biological data reduce time and costs during the screening of new substances once they cease to categorize undesirable molecules with improper characteristics during the early stages of discovery.

In addition, it is possible to evaluate the coupling of molecules to their possible targets. Using databases of 3D molecular structures, it is possible to anticipate connections between new molecules and possible binding sites. Numerous substances have been evaluated by this method, and it was possible to propose important features of some of them as the mechanism of action of antifungal piranocoumarins and antibacterial xanthone derivatives (Do et al., 2015).

CaCYP51 is a sterol 14-α demethylase that binds the CYP51 substrates lanosterol and eburicol in *C. albicans*. *In silico* techniques based on the molecular structure of CaCYP51 have allowed the development of new azoles by replacing the side chains provided in the simulation. The new azoles showed excellent antifungal activity *in vitro* with broad spectrum (Che et al., 2009). The development of new molecules *in silico* largely depends on the molecular recognition of possible couplings between the drug and microorganisms. Therefore, once elucidated, the mechanism of action of an antifungal obtained by biotransformation will allow the simulation of molecules with improved pharmacokinetic characteristics and may accelerate the development of potential drugs (Rask-Andersen et al., 2011).

PERSPECTIVES

The prospection of new molecules, principally from endophytic fungi, is a vast field of study with huge potential for growth. However, consistent efforts are necessary to achieve results. Professionals from the fields of biotechnology, pharmacology, computer sciences, engineering and some other related areas have to work closely together to explore possible binding sites for new and existing molecules. The use of *in silico* analyses anticipate investigations in a timesaving manner and reduce the demands for inputs and even animals used in the initial tests. These features drive future research toward the development of new and feasible technologies within the Green Chemistry guidelines connected to the principle of the 3Rs (replacement, reducing, refinement) for the use of experimental animals (Russell and Burch, 1959).

A reasonable research design for the prospecting of new antifungal drugs under the above stated conditions involves the following:

(1) The *in silico* analysis of existing molecules for possible new couplings to different targets;
(2) The *in silico* identification of necessary changes in the precursor molecule for effective coupling on targets;
(3) In the absence of new discoveries, the biotransformation of various candidate substrates for obtaining new molecules;
(4) The recognition of which organisms or isolated enzymes may modify structural precursor molecules to yield enantiomerically pure antifungal molecules based on the theory of enzyme promiscuity;
(5) The *in silico* evaluation of toxicity for newly developed antifungal molecules;
(6) The process of scaling up with environmentally friendly inputs to test the economic viability.

Once the goals outlined above are achieved, the investigator is ready to conduct clinical trials to confirm the PK/PD properties, security, and all other steps necessary for commercialization of the drug.

It is important to notice that with the advent of *in silico* technologies, laboratory tests tend to reduce their margin of error, and therefore, save both environmental and financial resources. However, they primarily accelerate new discoveries to minimize the impact of developed resistance to existing drugs.

Therefore, it is necessary to sequence and deposit the genomes of microorganisms with biotransformation capacity in databases. In addition, further study on the molecular structure of possible targets in pathogenic fungi is mandatory. This will provide subsidies to find coupling regions for the developed molecules.

Currently, we have high cost and low return as factors related to the decline in the development of new antimicrobial drugs. There is no other way to achieve such a market if the costs of research are not reduced and production is enabled in a sustainable way.

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Screening of Pharmacologically Active Small Molecule Compounds Identifies Antifungal Agents Against *Candida* Biofilms

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Candida species have emerged as important and common opportunistic human pathogens, particularly in immunocompromised individuals. The current antifungal therapies either have toxic side effects or are insufficiently effect. The aim of this study is develop new small-molecule antifungal compounds by library screening methods using *Candida albicans*, and to evaluate their antifungal effects on *Candida* biofilms and cytotoxic effects on human cells. Wild-type *C. albicans* strain SC5314 was used in library screening. To identify antifungal compounds, we screened a small-molecule library of 1,280 pharmacologically active compounds (LOPAC^{1280TM}) using an antifungal susceptibility test (AST). To investigate the antifungal effects of the hit compounds, ASTs were conducted using *Candida* strains in various growth modes, including biofilms. We tested the cytotoxicity of the hit compounds using human gingival fibroblast (hGF) cells to evaluate their clinical safety. Only 35 compounds were identified by screening, which inhibited the metabolic activity of *C. albicans* by >50%. Of these, 26 compounds had fungistatic effects and nine compounds had fungicidal effects on *C. albicans*. Five compounds, BAY11-7082, BAY11-7085, sanguinarine chloride hydrate, ellipticine and CV-3988, had strong fungicidal effects and could inhibit the metabolic activity of *Candida* biofilms. However, BAY11-7082, BAY11-7085, sanguinarine chloride hydrate and ellipticine were cytotoxic to hGF cells at low concentrations. CV-3988 showed no cytotoxicity at a fungicidal concentration. Four of the compounds identified, BAY11-7082, BAY11-7085, sanguinarine chloride hydrate and ellipticine, had toxic effects on *Candida* strains and hGF cells. In contrast, CV-3988 had fungicidal effects on *Candida* strains, but low cytotoxic effects on hGF cells. Therefore, this screening reveals agent, CV-3988 that was previously unknown to be antifungal agent, which could be a novel therapies for superficial mucosal candidiasis.

Keywords: drug discovery, antifungal drug, biofilm, Small molecules, *Candida albicans*

INTRODUCTION

Candida species have emerged as important and common opportunistic human pathogens, particularly in immunocompromised individuals, such as patients with HIV/AIDS, patients with cancer undergoing chemotherapy, organ transplant recipients receiving immunosuppressive drugs and patients with advanced diabetes (Richardson, 2005; Aperis et al., 2006). *Candida* sp. are responsible for a spectrum of diseases, which range from local mucosal infections to life-threatening invasive systemic candidiasis (Wisplinghoff et al., 2004).

A key feature of the virulence of *Candida* sp. is their ability to adhere to surfaces, before developing into distinct surface-attached communities called biofilms. Biofilms may develop on biological and inert surfaces, such as intravascular catheters, stents, shunts, prostheses and implants (Raad, 1998; Ramage et al., 2006). *Candida* biofilms are intrinsically more resistant to commercially available antifungal agents than their planktonic counterparts (Hawser and Douglas, 1995; Chandra et al., 2001; LaFleur et al., 2006; Seneviratne et al., 2008). Thus, the biofilms that form on medical device can resist the host immune defenses and antifungal treatments, thereby causing chronic infections and failure of implanted medical devices (Ramage et al., 2005). The increasing number of immunocompromised patients and advances in medical technology has led to an increase in biofilm-related infectious diseases, where *Candida albicans* is the major fungal pathogen. Recently, the frequency of these candidiasis caused by the non *C. albicans* species of *Candida*, such as *C. glabrata, C. parapsilosis, C. dubliniensis,* and *C. tropicalis*, has increased due to the indiscriminate use of antifungal drugs (Cuellar-Cruz et al., 2012; Pfaller, 2012).

In addition, *C. glabrata, C. parapsilosis,* and *C. krusei* exhibit intrinsic resistance to most azole-based antifungal drugs (Lee et al., 2009a; Kothavade et al., 2010; Pfaller et al., 2011) and the emergence of acquired drug resistance to most commercial antifungals has been reported.(Sanglard and Odds, 2002; Pfaller et al., 2010). Despite the urgent requirement for efficient antifungal therapies of systemic infections, the available antifungal drugs, such as novel polyene formulations, new azoles and echinocandins, are few and expensive and have side effects (Rex et al., 2000; Francois et al., 2005; Cornely et al., 2007; Pasqualotto and Denning, 2008). Furthermore, common non-life-threatening superficial infections, such as recurrent vulvovaginal candidiasis, impose significant restrictions on patients and result in a reduced quality of life. Thus, it is necessary to develop new antifungal agents that are effective against *Candida* biofilms. These agents should overwhelm biofilm-related candidiasis and lead to more effective antifungal treatments.

In recent studies, library screening methods have been used to identify new antifungal agents, which have focused on growth retardation or killing the pathogens (LaFleur et al., 2011; Siles et al., 2013; Stylianou et al., 2014). This type of screening method can identify candidate antifungal agents from large numbers of small-molecule compounds. Small-molecule compounds have many advantages, such as simple synthesis, high chemical stability and low costs compared with organic compounds. Therefore, the aim of the present study was to develop new small-molecule antifungal compounds by library screening methods using *C. albicans*. Moreover, we evaluated the antifungal effects of the small molecules detected by the library screening method using *Candida* biofilms as well as their cytotoxic effects on human cells.

MATERIALS AND METHODS

Drugs and Fungal Strains

The *in vitro* susceptibility of well-characterized wild-type *C. albicans* strain SC5314, which was provided by Prof. N.A.R. Gow (University of Aberdeen, Aberdeen, UK) was tested against 1280 compounds from the Library of Pharmacologically Active Compounds (LOPAC^{1280TM}, Sigma–Aldrich, USA). The screen was performed with *C. albicans* SC5314, and hits were further confirmed with the type strains *C. dubliniensis* MYA 577, *C. glabrata* ATCC 2001, *C. kusei* ATCC 6258, *C. palapsilosis* ATCC 22019, and *C. tropicalis* ATCC13803.

High-Throughput Screening (HTS) with Antifungal Susceptibility Tests (ASTs)

High-Throughput Screening was conducted using ASTs, according to the standard Clinical and Laboratory Standard Institute (CLSI) method (Watamoto et al., 2009). Inocula from 24-h yeast cultures on Sabouraud's dextrose agar (SDA) (Gibco, UK) were adjusted to a turbidity equivalent to a 0.5 McFarland standard at 520 nm using a spectrophotometer. The suspension was diluted further with RPMI 1640 medium (Gibco, UK) to yield an inoculum concentration of 0.5×10^3 to 2.5×10^3 cells/mL. *C. albicans* was incubated with small-molecule compounds (10 μM) from LOPAC^{1280TM}, which total volume was 150 μL, in 96-well plates at 37°C for 24 h to evaluate the antifungal effects. After incubation, the viability of the fungal cells was determined using the CellTiter-Glo luminescent cell viability kit (Promega, USA). The CellTiter-Glo reagent (150 μL) was added to the medium and incubated for 15 min at room temperature with shaking at 900 rpm. The luminescent signals were detected using a luminometer (GloMax Discover System, Promega, USA). The resulting signal intensity corresponds to ATP amounts and thus to the number of viable microbial cells upon drug exposure (Stylianou et al., 2014). In all 96-well plates, 100 and 0% growth controls were included as microbes plus dimethyl sulfoxide (0.1%) and microbes plus amphotericin B (100 μM), respectively. All assays were performed at least as two biological replicates in triplicate. The ATP level of *C. albicans* cells, which corresponded to the cell metabolic activity and viability, was

calculated for each compound using the following equation (**Figure 1A**).

$$\text{Percentage inhibition} = 100 \times 1 - \left\{ \frac{(\text{experimental} - \text{positive control average})}{(\text{negative control average} - \text{positive control average})} \right\}$$

Wells were scored as hits if the percentage inhibition was >50%. Hit compounds were evaluated further to assess their antifungal effects.

ASTs of Hit Compound in Various Growth Modes Against *Candida* Strains

To investigate the antifungal effects of the hit compounds, ASTs were conducted using broth microdilution assays with high cell densities of the planktonic mode, adhesion phase and biofilm mode against *Candida* strains (*C. albicans, C. dubliniensis*, *C. glabrata*, *C. kusei*, *C. palapsilosis,* and *C. tropicalis*). First, high density cell (1×10^7 cells/mL) suspensions were added to the RPMI medium containing each hit compound (10–1000 μM) in 96-well plates and incubated at 37°C for 24 h. Next, the 50% minimum inhibitory concentrations (MICs) of high-density *Candida* planktonic cultures were determined using the CellTiter-Glo luminescent cell viability kit, as described above. The antifungal effects of the hit compounds were also evaluated in the adhesion phase and the biofilm mode, in the same manner as the planktonic mode. *Candida* biofilms were produced as described previously (Jin et al., 2004). In brief, *Candida* cells were grown on SDA at 37°C for 18 h. A loopful of the yeast culture was then inoculated into yeast nitrogen base (YNB) (Difco, USA) medium supplemented with 50 mM glucose. After overnight broth culture in a rotary shaker at 75 rpm, the cells were washed twice with 20 mL of PBS (pH 7.2, 0.1 M). The yeast cells were re-suspended in YNB supplemented with 100 mM glucose and adjusted to an optical density of 0.38 (1×10^7 cells/mL) at 520 nm. This standardized cell suspension was used immediately to form biofilms in the wells of 96-well polystyrene culture plates (Iwaki, Tokyo, Japan). First, the cells were incubated for 90 min at 37°C in a shaker at 75 rpm to allow yeast adherence to the well surface (adhesion phase), before the medium was aspirated and each well was washed once with PBS to remove non-adherent cells. YNB containing 100 mM glucose was then pipetted into each well and the plate was incubated at 37°C in a shaker at 75 rpm for 24 h. Non-adherent cells were removed by pipetting and the biofilms were washed twice with PBS. Following this biofilm growth phase, microscopic examination of the cultures was performed to exclude contamination. These ASTs were repeated on three different occasions.

Cytotoxicity

Primary human gingival fibroblast (hGF) cultures were established from discarded healthy gingival tissues after surgery with the informed consent of the donors (Nikawa et al., 2006). In brief, the gingival tissue specimens were treated overnight with 0.025% trypsin and 0.02% EDTA at 4°C. After trypsin neutralization, the lamina propria mucosae were separated from the epithelial layer and minced into pieces in a plastic tissue culture dish, and then maintained in Dulbecco's modified Eagle medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin and 250 ng/mL amphotericin B (Nacalai Tesque, Kyoto, Japan). After the fibroblasts had migrated out of the tissue, the tissues were removed and the cells were cultured until they reached confluence. The cells were then seeded onto 96-well tissue culture plates (500 cells per well) and the culture medium was exchanged with fresh growth medium containing the hit compounds (0.98–1000 μM). The cells were cultured continuously and the culture medium containing the hit compounds was renewed every other day. The number of cells was evaluated using the WST-1 cell counting assay (Dojindo Laboratories, Kumamoto, Japan), as described previously (Hamada et al., 2007). The highest concentration of each compound that caused greater than 50% reduction in the number of cell compare to that of compound free control cell was reported as the cytotoxic concentration. All the experiments were performed using three samples for each condition in triplicate.

RESULTS

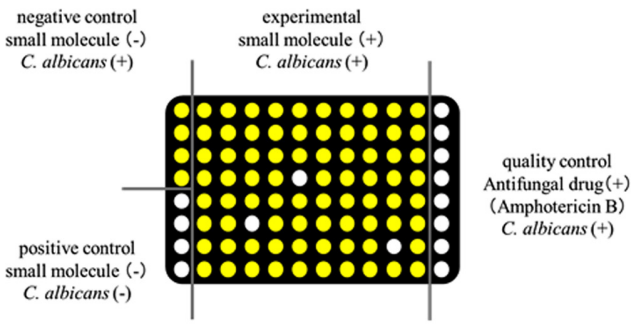
High-Throughput Screening (HTS) Results

We screened 1280 compounds using antifungal susceptibility tests (ASTs) in 96-well plates to identify antifungal agents. Only 35 compounds were identified, which inhibited the metabolic activity of *C. albicans* by >50%. Thus, the overall hit rate for HTS was approximately 3.9%. Among the hit compounds, 26 compounds had fungistatic effects and nine compounds had fungicidal effects on *C. albicans* (**Figure 1B**). Five compounds, BAY11-7082, BAY11-7085, sanguinarine chloride hydrate, ellipticine and CV-3988, had strong fungicidal effects and inhibited the metabolic activity of *C. albicans* by >90% (**Figure 1B**). The structures of these five compounds are shown in **Figure 1C**. The antifungal effects of these five compounds were evaluated using *Candida* strains (*C. albicans, C. dubliniensis*, *C. glabrata*, *C. kusei*, *C. palapsilosis,* and *C. tropicalis*) in high density planktonic, adhesion and biofilm modes.

ASTs of Hit Compounds Using *Candida* Strains in Various Growth Modes

The HTS results showed that *C. albicans* was susceptible to all the hit compounds when a low inoculum size (1×10^3 cells/mL) was used, according to the CLSI methodology (MIC < 1 μM). When the cell density increased to 1×10^7 cells/mL, *Candida* strains were slightly resistant to four of the compounds, but not sanguinarine chloride hydrate. However, all five compounds inhibited the metabolic activity of *Candida* strains at <31.3 μM and they had fungicidal effects on the high cell density planktonic

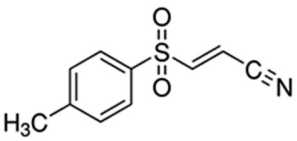
A



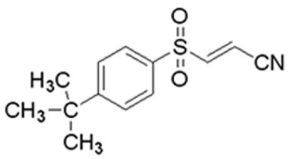
B

hit compound	percent inhibition
Bay 11-7085	100
Chelerythrine chloride	82.4
Dequalinium chloride hydrate	71.5
Ellipticine	100
Indatraline hydrochloride	85.2
Palmitoyl-DL-carnitine chloride	76.0
Bay 11-7082	100
Sanguinarine chloride hydrate	100
CV-3988	93.3

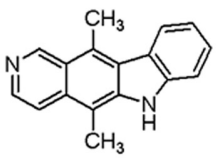
C



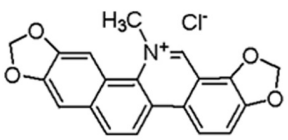
Bay 11-7082 (MW: 207.3)
An inhibitor of IκB-α phosphorylation by cytokines



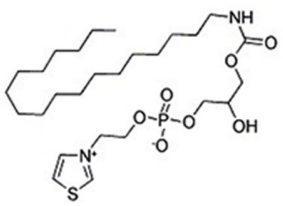
Bay 11-7085 (MW: 249.3)
Repress ICAM-1, VCAM-1, E-selectin, IL-6 and IL-8 activated by NF-κB



Ellipticine (MW: 246.3)
Ellipticine is an antitumor alkaloid isolated from *Ochrosia* spp. It inhibits cytochrome P450 (CYP1A1) and DNA topoisomerase II activities



Sanguinarine chloride hydrate (MW: 367.8)
Natural product that has antibacterial, antiinflammatory and antioxidant effect. Anti-proliferation and apoptosis promoting effect against some cancer cells.



CV-3988 (MW: 592.8)
Competitive PAF receptor antagonist. Inhibits PAF-induced human platelet aggregation (3-30 μM) and bronchoconstriction in the guinea pig.

FIGURE 1 | Identification of small-molecule compounds that inhibited the metabolic activity of *Candida albicans* using high-throughput screening (HTS). (A) Schematic showing the HTS procedure. White circles: low ATP level and no metabolic activity in *C. albicans*. Yellow circles: high ATP level and high metabolic activity in *C. albicans*. **(B)** Compounds that inhibited the metabolic activity of *C. albicans*. **(C)** Structures of the five compounds that inhibited the metabolic activity of *C. albicans* by >90%. MW, molecular weight.

mode (**Table 1**). As a control, amphotericin B inhibited the metabolic activity of *C. albicans* at <3.9 μM.

The drug susceptibility of adhesion phase *Candida* strains to the five compounds was higher than that of the high density planktonic cultures (**Table 2**). In particular, sanguinarine chloride hydrate was effective against adhesion phase and it could inhibit the metabolic activity at <15.6 μM. Bay 11-7082 and Bay 11-7085 were also effective against the adhesion phase and could inhibit

TABLE 1 | Minimum inhibitory concentrations (MICs) of five candidate compounds against planktonic mode of *Candida* strains.

	C. albicans	*C. glabrata*	*C. dubliniensis*	*C. tropicalis*	*C. kusei*	*C. palapsilosis*
Bay11-7082	7.8	7.8	3.9	7.8	3.9	3.9
Bay11-7085	3.9	3.9	3.9	3.9	3.9	3.9
Sanguinarine	<1	<1	<1	<1	<1	<1
Ellipticine	7.8	7.8	3.9	15.6	7.8	7.8
CV-3988	7.8	7.8	31.3	31.3	15.6	15.6
AMB	3.9					

(μM)

Sanguinarine, sanguinarine chloride hydrate; AMB, amphotericin B; MICs, minimal concentration of compound resulting in >50% growth inhibition. MICs were determined by ATP measurement after 24 h of incubation. The data were analyzed and evaluated from 3 biological replicate in triplicate (n = 3).

TABLE 2 | Minimum inhibitory concentrations of five candidate compounds against adhesion phase of *Candida* strains.

	C. albicans	*C. glabrata*	*C. dubliniensis*	*C. tropicalis*	*C. kusei*	*C. palapsilosis*
Bay11-7082	31.3	31.3	15.6	31.3	15.6	15.6
Bay11-7085	31.3	31.3	15.6	31.3	15.6	7.8
Sanguinarine	15.6	15.6	15.6	7.8	7.8	7.8
Ellipticine	62.5	62.5	125	250	250	125
CV-3988	62.5	62.5	125	62.5	125	125
AMB	15.6					

(μM)

Sanguinarine, sanguinarine chloride hydrate; AMB, amphotericin B; MICs, minimal concentration of compound resulting in >50% growth inhibition. MICs were determined by ATP measurement after 24 h of incubation. The data were analyzed and evaluated from 3 biological replicate in triplicate (n = 3).

the metabolic activity at <31.3 μM. As a control, amphotericin B inhibited the metabolic activity of *C. albicans* adhesion phase at 15.6 μM.

Most *Candida* biofilms were more resistant to the five compounds than other growth mode. Especially, *C. tropicalis* biofilm was most resistant to the five compounds in all growth modes (**Table 3**). Bay 11-7082, Bay 11-7085, Ellipticine and CV-3988 could inhibit the metabolic activity of *Candida* biofilms at <62.5, 62.5, 500, and 125 μM, respectively. Sanguinarine chloride hydrate was the most effective antifungal agent in this study and it could inhibit the metabolic activity of *Candida* strains at <31.3 μM. As a control, amphotericin B inhibited the metabolic activity of *C. albicans* biofilm at 62.5 μM.

Cytotoxicity

In addition to pharmacologically active compounds, small-molecule libraries often contain toxic molecules that do not make good drug candidates. To evaluate the safety for clinical use, we tested the cytotoxic effects of the hit compounds using human cell cultures. We used hGF cells because of their ubiquitous nature and their widespread use in cytotoxicity testing (Egusa et al., 2009; LaFleur et al., 2011). The hGF cells were grown in 96-well plates and exposed to increasing doses (two-fold increments) of each hit compound for 4 days. The hGF metabolic activity was measured every other day and used as an indicator of cell viability. After 4 days, Bay 11-7082, Bay 11-7085, ellipticine, sanguinarine chloride hydrate and CV-3988 inhibited cell proliferation no more than 50%, namely, did not kill cells at less than 7.81, 7.81, 1.95, 0.73, and 250 μM, respectively (**Table 4**).

DISCUSSION

Candida species are the main fungal pathogen that causes infections in humans, ranging from superficial mucosal infection to systemic mycoses (Navarro-Garcia et al., 2001). *Candida* infections are intractable and recurrent diseases, which have increased due to the rise in the number of immunocompromised host populations (Beck-Sague and Jarvis, 1993; Wisplinghoff et al., 2004). Drug-resistant *Candida* strains have also increased dramatically because of the increased use of antifungal agents. Thus, the development of novel antifungal drugs and treatment strategies are essential for combating *Candida* infections. High-throughput screening (HTS) is an effective method for identifying candidate novel antifungal drugs. It is important to apply adequate screening methods to small-molecule compound libraries because appropriate selection procedures are the key to successful screening. In this study, LOPAC^{1280TM} was used as the small-molecule library, which contained pharmacologically active compounds and all the compounds were commercially available. Thus, the main effects of these small molecules on human cells are already known and described in database of manufacture. Therefore, it may be easier to apply these compounds in clinical practice with fewer unexpected drug side effects.

In general, polyenes, azoles, allylamines, morpholines, antimetabolites, and echinocandins are the six major antifungal drug categories to manage fungal infections (Khan and Jain, 2000; Ruhnke et al., 2008). Most of these antifungal drugs

TABLE 3 | Minimum inhibitory concentrations of five candidate compounds against biofilm mode of *Candida* strains.

	C. albicans	*C. glabrata*	*C. dubliniensis*	*C. tropicalis*	*C. kusei*	*C. palapsilosis*
Bay11-7082	31.3	62.5	31.3	62.5	62.5	15.6
Bay11-7085	31.3	62.5	62.5	62.5	62.5	15.6
Sanguinarine	15.6	15.6	15.6	31.3	31.3	7.8
Ellipticine	125	62.5	500	250	250	250
CV-3988	125	125	125	125	125	125
AMB	62.5					

(μM)

Sanguinarine, sanguinarine chloride hydrate; AMB, amphotericin B; MICs, minimal concentration of compound resulting in >50% growth inhibition. MICs were determined by ATP measurement after 24 h of incubation. The data were analyzed and evaluated from 3 biological replicate in triplicate (n = 3).

TABLE 4 | Cytotoxic concentrations of five candidate compounds on human gingival fibroblasts.

	Cytotoxic concentration
Bay11-7082	7.81
Bay11-7085	7.81
Ellipticine	1.95
Sanguinarine chloride hydrate	0.73
CV-3988	250

(μM)

Cytotoxic concentration, maximal concentration of compound resulting in >50% the number of cell reduction compares to compound free control. The data were analyzed and evaluated from 3 biological replicate in triplicate (n = 3).

have fungistatic or fungicidal effects on exponentially growing planktonic cells, but *Candida* cells are resistant to these drugs after biofilm formation (Watamoto et al., 2009). Interestingly, we found that five small-molecule compounds (BAY11-7082, BAY11-7085, sanguinarine chloride hydrate, ellipticine and CV-3988) were antifungal drug candidates with inhibitory effects on various *Candida* biofilms at concentrations below 500 μM.

BAY11-7082 and BAY 11-7085 is known to be an inhibitor of nuclear factor κB (NF-κB) activation by the blockade of inhibitor κB (IκB) phosphorylation, which is a trigger of apoptosis (Pierce et al., 1997; Guzman and Jordan, 2005; Chopra et al., 2008; Lee et al., 2009b; Zanotto-Filho et al., 2010). Bay 11-7082 triggers cell membrane scrambling and cell shrinkage (Lang et al., 2008). BAY 11-7085 has been shown to activate c-jun N-terminal kinase and p38 mitogen-activated protein kinase (MAPK) (Pierce et al., 1997). BAY 11-7085 inhibits cell proliferation by inducing apoptosis and G0/G1 arrest of the cell cycle in human cells (Bockelmann et al., 2005). These actions have anti-inflammatory, anticancer and slight hemolytic effects (Ghashghaeinia et al., 2011).

Sanguinarine chloride hydrate is a phytoalexin and has been reported to suppress activation of the transcription factor NF-κB (Chaturvedi et al., 1997) and to modulate the functions of various enzymes, such as MAPK phosphatase-1 (Vogt et al., 2005), protein kinase C (Gopalakrishna et al., 1995) and phosphoinositide-dependent protein kinase 1 (Vrba et al., 2008). These actions of Sanguinarine have antimicrobial, antioxidant, anti-inflammatory, hemolytic and cytotoxic effects (Lenfeld et al., 1981; Godowski, 1989; Malikova et al., 2006; Babu et al., 2008; Matkar et al., 2008; Jang et al., 2009).

Ellipticine, an alkaloid isolated from Apocyanaceae plants, has been reported to mediate primarily DNA damage such as DNA intercalation (Auclair, 1987), inhibition of topoisomerase II (Auclair, 1987; Stiborova et al., 2006), inhibition of casein kinase 2 (Prudent et al., 2010) and the formation of covalent DNA adducts by cytochrome P450s and peroxidases (Stiborova et al., 2011). These actions of Ellipticine has anti-tumor, cytotoxic, hemolytic and mutagenic activities (Lee, 1976; Rouesse et al., 1985). Therefore, the known cell proliferation inhibitory effects of these four small-molecules agree with the findings of the present study. Furthermore, the antifungal and cytotoxic effects of these small molecules on *Candida* strains may involve the same mechanism because *Candida* strains are eukaryotes and possesses the same targets. Thus, these small molecules are toxic to human cells and *Candida* strains, and inappropriate for clinical use corroborated by the relatively low cytotoxic concentration on hGF.

On the other hand, platelet-activating factor (PAF), which is released almost immediately in response to inflammatory stimuli (Im et al., 1997) by various inflammatory cells, is a potent lipid messenger involved in cellular activation, fertilization, intracellular signaling, apoptosis and diverse inflammatory reactions (Braquet et al., 1987; Shukla, 1992; Buttke and Sandstrom, 1995; Fukuda and Breuel, 1996). CV-3988 (Terashita et al., 1983; Terashita et al., 1987) is a structural analog of PAF, which has been shown to specifically inhibit the *in vitro* and *in vivo* activities of PAF (Sultana et al., 1999) by competitive binding with the PAF receptor (PAF-R) (Terashita et al., 1983; Summers and Albert, 1995; Negro Alvarez et al., 1997). Therefore, CV-3988 is an antagonist of PAF-R, which inhibits the functions of leukocytes, including platelet aggregation, inflammation and anaphylaxis. We showed for the first time that CV-3988 had a fungicidal effect on various *Candida* biofilms and low cytotoxity effect on hGF cells. In past study, CV-3988 had slight hemolytic effect and can safely be administered to human (Arnout et al., 1988). These results demonstrate that CV-3988 has a novel and specific fungicidal effect on *Candida* strains and may become initial drug choice for the treatment of candidiasis. Furthermore, *Candida* sp. are common microbes in the oral cavity and vagina and causes mucotitis in immunocompromised and healthy hosts. Mouthwashes and ointments containing antifungal agents are

primary treatment for oral and vaginal candidiasis. Therefore, CV-3988 may be suitable for use on oral mucosal surfaces to combat *Candida* biofilm infections such as thrush and denture-related stomatitis. Although CV-3988 may facilitate novel treatment strategies to combat *Candida* infections, further studies about fungicidal mechanism and pharmacokinetics are required before it can be applied in clinical practice.

CONCLUSION

We identified five small-molecule compounds (BAY11-7082, BAY11-7085, sanguinarine chloride hydrate, ellipticine and CV-3988) as novel antifungal drug candidates using HTS methods. BAY11-7082, BAY11-7085, sanguinarine chloride hydrate and ellipticine were toxic to *Candida* strains as well as hGF cells. In contrast, CV-3988 had fungicidal effects on *Candida* strains, but low cytotoxic effects on hGF cells. Therefore, in future, mouthwashes and ointments containing CV-3988 may be used as a novel treatment for superficial mucosal candidiasis.

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REVIEW
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Potential Use of Phenolic Acids as Anti-*Candida* Agents: A Review

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There has been a sharp rise in the occurrence of *Candida* infections and associated mortality over the last few years, due to the growing body of immunocompromised population. Limited number of currently available antifungal agents, undesirable side effects and toxicity, as well as emergence of resistant strains pose a considerable clinical challenge for the treatment of candidiasis. Therefore, molecules that derived from natural sources exhibiting considerable antifungal properties are a promising source for the development of novel anti-candidal therapy. Phenolic compounds isolated from natural sources possess antifungal properties of interest. Particularly, phenolic acids have shown promising *in vitro* and *in vivo* activity against *Candida* species. However, studies on their mechanism of action alone or in synergism with known antifungals are still scarce. This review attempts to discuss the potential use, proposed mechanisms of action and limitations of the phenolic acids in anti-candidal therapy.

Keywords: *Candida*, phenolic acids, phenolic compounds, antifungal effect, synergism

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INTRODUCTION

Candida species are a major group of fungal pathogens in humans, particularly among immunocompromised and hospitalized patients (Cuellar-Cruz et al., 2012). *Candida albicans* inhabits various body surfaces like oral cavity, gastrointestinal tract, vagina, and skin of the healthy individuals as a commensal organism (Kleinegger et al., 1996; Huffnagle and Noverr, 2013). Host-related factors can predispose the transformation of harmless *Candida* into an opportunistic pathogen, causing infection or candidiasis in superficial mucous surfaces which can progress into invasive mycoses (Nett and Andes, 2006). Foregoing factors include, but not limited to immuno-suppression, prolonged treatment with wide-spectrum antibiotics and chronic diseases (Kullberg and Arendrup, 2015; Polke et al., 2015). The epidemiology of invasive candidiasis varies geographically (Morgan, 2005; Pfaller et al., 2011). It significantly increases the period of hospitalization, economic burden and mortality, especially in ICU patients or those under chemotherapy or with a history of abdominal surgery (Falagas et al., 2006; Berdal et al., 2014; Drgona et al., 2014).

Only few classes of antifungals such as polyenes, azoles, echinocandins, allylamines, and flucytosine are available for the treatment of *Candida* infections (Sanglard et al., 2009). However, there are various undesirable properties, most importantly the dose-related toxicity in aforementioned antifungals (Chandrasekar, 2011). Ideally, an antifungal should have null or reduced toxicity toward human cells (Wong et al., 2014). For instance, amphotericin B is a polyene

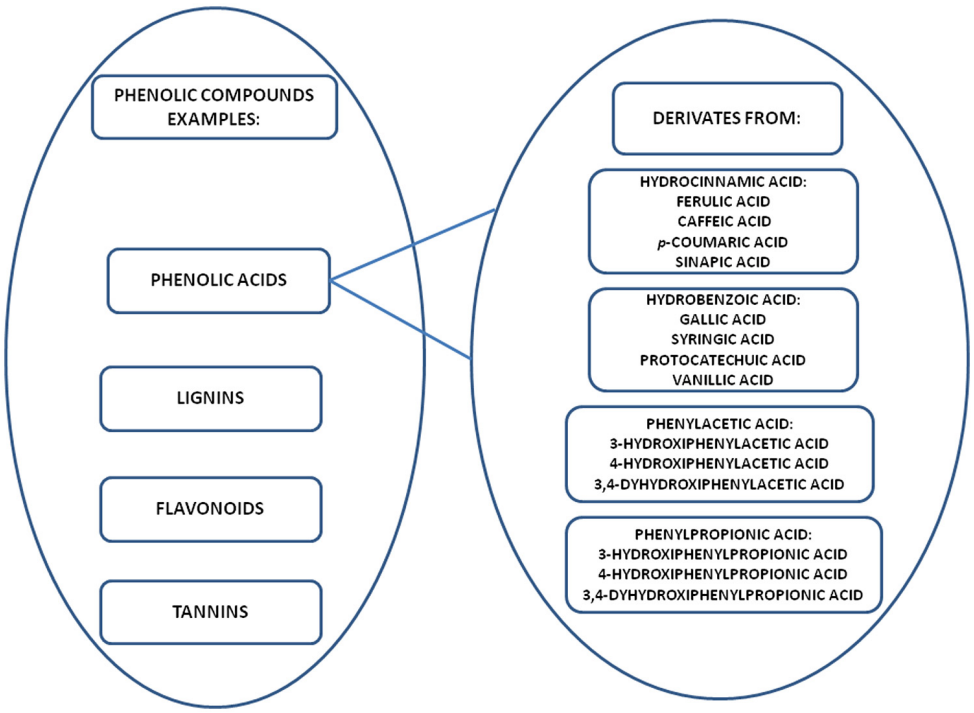

FIGURE 1 | Examples of phenolic compounds and main derivates classified as phenolic acids.

available for systemic administration, but its use has been limited due to its systemic side effects such as nephrotoxicity (Odds et al., 2003). Azole antifungals have some side effects associated with gastrointestinal, hepatic, and endocrinologic disorders and interfere with oxidative drug metabolism in the liver (Joly et al., 1992).

In addition, rising drug resistance is an inevitable problem. In particular, *Candida glabrata* and *Candida krusei* show intrinsic resistance to fluconazole, the drug of choice for AIDS patients (Kanafani and Perfect, 2008; Siikala et al., 2010; Rautemaa and Ramage, 2011). Drug resistance has already been reported for recently introduced echinocandin antifungal agents (Hakki et al., 2006; Ben-Ami et al., 2011; Clancy and Nguyen, 2011; Seneviratne et al., 2011). Moreover, biofilm mode of *Candida* is known to be highly resistant to antifungal agents (Chandra et al., 2005; Niimi et al., 2010). Therefore, it is necessary to discover new antifungal agents or safer alternatives to improve the efficacy of treatment against *Candida* infections. In this regard, antifungal agents based on natural resources, such as phenolic compounds may be an alternative strategy to negate the rising antifungal drug resistance (Negri et al., 2014). This review attempts to critically analyze the possible use of phenolic acids as a therapeutic strategy against *Candida* infections.

Phenolic compounds are widely found in plant foods (fruits, cereal grains, legumes, and vegetables) and beverages (tea, coffee, fruits juices, and cocoa). The most common phenolic compounds are phenolic acids (cinnamic and benzoic acids), flavonoids, proanthocyanidins, coumarins, stilbenes, lignans, and lignins (**Figure 1**; Cowan, 1999; Chirinos et al., 2009; Khoddami et al., 2013). The anti-*Candida* properties of phenolic compounds that have been widely reported in the literature include inactivation of enzyme production (Evensen and Braun, 2009) and anti-biofilm effect (Evensen and Braun, 2009; Shahzad et al., 2014).

Phenolic acids are derivatives of hydrocinnamic, hydrobenzoic, phenylacetic, and phenylpropionic acids (**Figures 1** and **2**; Pereira et al., 2009; Cueva et al., 2010). Phenolic acids commonly exist as esters, glycosides or amides in nature, but not in their free form. The determining factor for characterization of phenolic acids is the number and the location of hydroxyl groups on the aromatic ring. Some natural sources are rich in phenolic acids and shown to possess a promising action against *Candida* (**Table 1**). In this review, we discuss the anti-candidal activity of the phenolic acid compounds, possible mechanism of actions and future directions.

ANTIFUNGAL ACTIVITY OF PHENOLIC ACIDS AGAINST *Candida* SPECIES

Natural extracts containing phenolic acids have demonstrated antifungal activity against *Candida* species (**Table 1**). Phenolic acid derivatives isolated from these sources such as gallic, caffeic, cinnamic, benzoic, protocatechuic, and phenylacetic acids also have antifungal activity (**Table 2**). However, the antifungal effect of the natural extracts may vary due to the differences in the quantity and the type of phenolic acid. In addition, the solvents used for extraction may also affect the antifungal effect. Moreover, other compounds present in natural extracts may

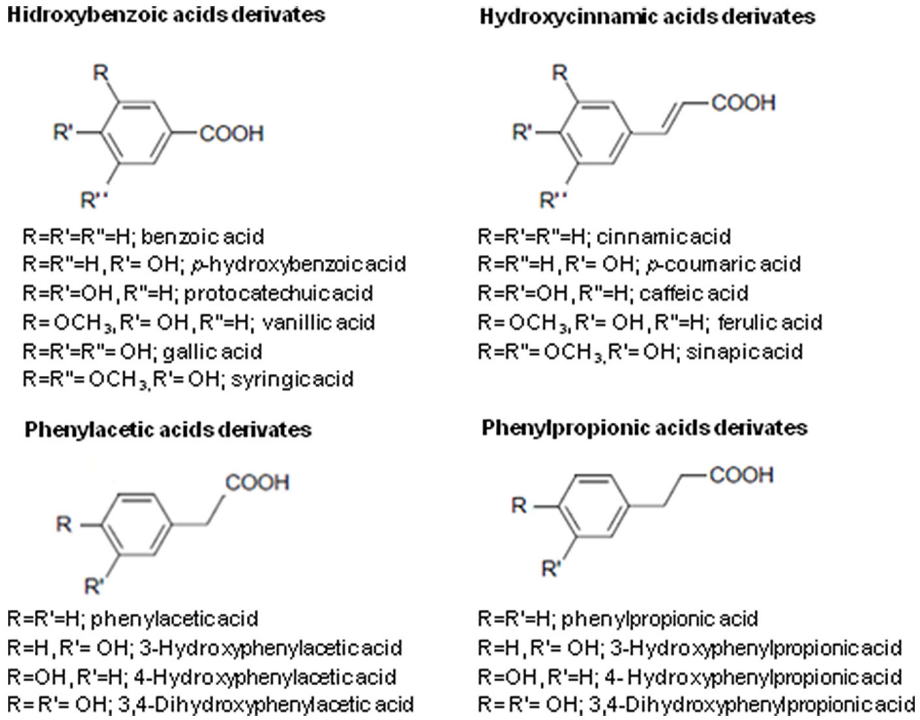

FIGURE 2 | General chemical structures of the phenolic acids [based on Pereira et al. (2009) and Cueva et al. (2010)].

act synergistically with phenolic acids to enhance the overall antifungal effect (Pereira et al., 2007; Nowak et al., 2014). Therefore, phenolic acids derived from different natural sources have highly variable MIC values against *Candida* (**Table 2**). Hence, a clear understanding of the composition of phenolic acids present in the natural extract is important to assess its potential as an antifungal agent (Salvador et al., 2004; Rangkadilok et al., 2012).

The main *Candida* virulence factors are exoenzymes production, biofilm formation, adherence, and dimorphism (Vuong et al., 2004; Netea et al., 2008; Williams et al., 2011). Few studies have demonstrated the influence of phenolic acids against these factors. Anti-biofilm effect of phenolic acids against *Candida* sp. was reported (Wang et al., 2009; Alves et al., 2014; De Vita et al., 2014). However, the studies used only reference samples or did not cite the tested strain (**Table 2**). The anti-biofilm effect of these molecules should be carried out with clinical isolates *in vitro* and *in vivo*, since the ultimate goal of using these molecules is to treat candidiasis and a wider range of strains could provide more reliable results. Besides that, it also has found an influence of caffeic acid derivate against the *Candida* dimorphism (Sung and Lee, 2010).

However, several studies described effect on *Candida* virulence factors of some others phenolic molecules. For instance, bisbibenzyl stimulates the synthesis of farnesol, an inhibitor of hyphae formation, via upregulation of *Dpp3* gene (Zhang et al., 2011). Hence, bisbibenzyl may reduce *C. albicans* hyphal formation and affect biofilm formation. Moreover, anti-hyphae effect in *C. albicans* was also found following the treatment with epigallocatechin-gallate (Han, 2007), licochalcone A, gladribin (Messier and Grenier, 2011), and thymol (Braga et al., 2007). Additionally, eugenol reduces germ tube formation in *C. albicans* (Pinto et al., 2009). Beyond that, several studies have shown anti-biofilm (Messier et al., 2011; Alves et al., 2014; Rane et al., 2014; Shahzad et al., 2014) and anti-adhesive (Feldman et al., 2012; Rane et al., 2014; Shahzad et al., 2014) activities of phenolics against C*andida*.

The number of studies on other phenolic molecules on *Candida* virulence factors with interesting results inspires a carefully investigation of phenolic acids influence on these factors.

MECHANISM OF ACTION, BIOLOGICAL PATHWAYS, AND SYNERGISM WITH ANTIFUNGAL AGENTS OF PHENOLIC ACIDS AGAINST *Candida*

In order to obtain some insights on the antifungal activity of phenolic acids, herein we compare the existing data along the lines of mechanism of action, synergy with known antifungal agents and others biological pathways (**Figure 3**).

Mechanisms of Action and Biological Pathways

Phenolic acids such as ferulic and gallic acids are known to affect the cell membrane of Gram-positive and Gram-negative

TABLE 1 | Phenolic acids derived from plants extracts showing activity against *Candida* sp.

Plant	Phenolic acids found	Type of extract	Microorganism	MIC value μg/ml	MBC value μg/ml	Reference
Buchenavia tomentosa	Gallic acid	Aqueous	*C. albicans* ATCC 18804 *C. tropicalis* ATCC 13803 *C. krusei* ATCC 6258 *C. glabrata* ATCC *C. parapsilosis* ATCC 22019 *C. dubliniensis* NCPF 3108	200–12500	6500 *C. krusei* (ATCC 6258)	Teodoro et al., 2015
Rosa rugosa	Protocatechuic, gallic, and *p*-coumaric acids	Methanolic	*C. albicans* ATCC 10231 *C. parapsilosis* ATCC 22019	156	1250	Nowak et al., 2014
Teucrium arduini L.	Ferulic acid	Ethanolic	*C. albicans* ATCC 10231	4000	NR	Kremer et al., 2013
Potentilla sp.	Caffeic acid and ferulic acid	Acetonic and methaolic	*C. albicans* ATCC 10231	780–1560	NR	Wang et al., 2013
Dimocarpus longan Lour	Gallic acid	Spray-dried or Freeze-dried water	*C. krusei* ATCC 10231 *C. parapsilosis* ATCC 22019 *C. albicans* ATCC 90028 and clinical strains	500–4000	NR	Rangkadilok et al., 2012
Ligusticum mutellina L.	Gallic, *p*-OH-benzoic, caffeic, *p*-coumaric, and ferulic acids	Methanolic	*C. albicans* ATCC 10231 *C. parapsilosis* ATCC 22019	1250	2500	Sieniawska et al., 2013
Limonium avei	Caffeic, *m*-coumaric, *p*-coumaric, ferulic, isovanillic, *p*-methoxybenzoic, protocatechuic, sinapinic, and vanillic acids	Ethanolic	*C. albicans* ATCC 10231	4000	>4000	Nostro et al., 2012
Kitaibelia vitifolia	*p*-hydroxybenzoic, caffeic, syringic, *p*-coumaric, and ferulic acids	Ethanolic	*C. albicans* ATCC 10231	15.62	NR	Maskovic et al., 2011
Tamarix gallica L.	Gallic, synnapic, *p*-hydroxybenzoic, syringic, vanillic, *p*-coumaric, ferrulic, *trans*-2-hydroxycinnamic and *trans*-cinnamic acids	Hydromethanolic	*C. kefyr, C. holmii, C. albicans, C. sake, C. glabrata*	2000	NR	Ksouri et al., 2009
Cirsium sp.	Caffeic, *p*-coumaric, ferulic, *p*-hidroxybenzoic, protocatechuic vanillic, and gallic acids	Aqueous	*C. albicans* ATCC 10231	780–1560	6250 to >50000	Nazaruk et al., 2008
Olea europaea L.	Caffeic acid	Aqueous	*C. albicans* CECT 1394	5000*	NR	Pereira et al., 2007
Anogeissus latifolia	Gallic acid	Hydroalcoholic after maceration with ether	*Candida albicans* (MTCC 183)	7.28 μg/ml	NR	Govindarajan et al., 2006
Berry (Cloudberry Raspberry, Strawberry)	Hydroxycinnamic acids	Acetonic 70%	*Candida albicans* NCPF 3179	1000 μg/ml	NR	Nohynek et al., 2006

*NR, not reported; *IC_{25}.*

bacteria leading to a change in cell surface hydrophobicity and charge, ultimately causing leakage of cytoplasmic content (Borges et al., 2013). A similar effect has been suggested for the caffeic acid derivative on *Candida* cytoplasmatic membrane (Sung and Lee, 2010). Furthermore, a possible effect on the *C. albicans* cell wall has been shown for caffeic acid derivatives which may interfere with 1,3-β-glucan synthase (Ma et al., 2010).

It is noteworthy that polyene antifungals also cause pouring of cellular contents through direct binding to ergosterol, distorting the membrane function. Also, azole antifungal agents inhibit biosynthesis of ergosterol (Vanden Bossche et al., 2004). No study on the effect of phenolic acid on the ergosterol composition or biosynthesis could be detected.

Mode of action of several others phenolic compounds provide some clues to deduce the mechanism of phenolic acids. For instance, isoquercetin (Yun et al., 2015), curcumin (Lee and Lee, 2014), and lariciresinol (Pinto et al., 2009) can damage the *C. albicans* cell membrane. On the other hand, eugenol and methyleugenol cause considerable reduction in the ergosterol biosynthesis in *Candida* and subsequently affecting the cell membrane (Ahmad et al., 2010b). Similar effect has been observed with epigallocatechin-3-gallate (Navarro-Martinez et al., 2006), thymol and carvacrol (Ahmad et al., 2011). Besides,

TABLE 2 | Evidences from literature regarding anti-*Candida* effect of phenolic acids.

Molecule	Anti-*Candida* effect	Result found	Reference
Gallic acid	Planktonic cells of *C. albicans* (ATCC 18804), *C. krusei* (ATCC 6258), *C. parapsilosis* (ATCC 22019), *C. dubliniensis* (NCPF 3108), and *C. glabrata* (ATCC 90030)	MIC (μg/ml) respectively: 10000, 10000, 10000, 10000, 8	Teodoro et al., 2015
	Planktonic cells and biofilm of *C. albicans* (ATCC 90028), *C. glabrata* (ATCC 2001), *C. parapsilosis* (ATCC 22019), and *C. tropicalis* (ATCC 750)	MIC (μg/ml) planktonic: <156 μg/ml MIC (μg/ml) biofilm respectively: 5000, 1250, 625, 625	Alves et al., 2014
	Planktonic cells (plate diffusion)	MIC (mg cm^{-3}): 2.5	Manayi et al., 2013
	Planktonic cells of *C. albicans* (ATCC 10231) and *C. tropicalis* (ATCC 750)	MIC and MFC (μg/ml) respectively: 200, 200, 200, 100	Gehrke et al., 2013
	Planktonic cells of *C. albicans* (ATCC 90028) and 5 clinical strains, *C. krusei* (ATCC 6258), and *C. parapsilosis* (ATCC 20019)	MIC (μg/ml) respectively: 4000,4000,8000,4000, 16000, 16000, 8000, 4000	Rangkadilok et al., 2012
	Planktonic cells of *C. albicans* (ATCC 10231) and *C. parapsilosis* (ATCC 22019)	MIC (μg/ml) respectively: 8, 16	Ozcelik et al., 2011
	Planktonic cells of *C. albicans* (ATCC 90028), *C. krusei* (ATCC 6258), and *C. parapsilosis* (ATCC 22019)	MIC (μg/ml): 100	Liu et al., 2009
	Biofilm of *C. albicans* (not cited strain)	MIC (μg/ml): 1000	Wang et al., 2009
	Planktonic cells of *C. albicans* (MTCC 183)	MIC (μg/ml): 1.78	Govindarajan et al., 2006
	Planktonic cells of *C. albicans* (not cited strain)	Halo: 12 mm (100 μg on a sterile filter paper disk with 6 mm diameter)	Fogliani et al., 2005
Caffeic acid	Planktonic cells of *C. albicans* and inhibition of isocitrate lyase activity assay	MIC (μg/ml): 1000; inhibition of 91,5% of the isocitrate lyase enzyme activity	Cheah et al., 2014
	Planktonic cells and biofilm of *C. albicans* (ATCC 10231)	MIC (μg/ml): planktonic: 128; pre-formed, 4 and 24 h biofilm: 256	De Vita et al., 2014
	Planktonic cells of *C. albicans* (ATCC 10231) and *C. parapsilosis* (ATCC 22019)	MIC (μg/ml) respectively: 8, 16	Ozcelik et al., 2011
Protocatechuic acid	Planktonic cells of *C. albicans* (LMP709U)	MIC and MFC (μg/ml) respectively: 156, 312	Kuete et al., 2009
	Planktonic cells of *C. albicans* (10231) and *C. tropicalis* (ATCC 7349)	MIC (μg/ml) respectively: 500, 400	Pretto et al., 2004
Phenylacetic acid	Planktonic cells (plate diffusion) of *C. albicans* (clinical strains)	Halo: 8–10.5 mm (20 μl of a 2000 ng/ml phenylacetic acid water solution on sterile filter paper disk with 6 mm diameter)	Mendonca Ade et al., 2009
Cinnamic acid	Immunoregulatory effect on monocytes activation against *C. albicans* (SC 5314)	Significant reduce of *C. albicans* counts in 50 and 100 μg/ml	Conti et al., 2013
	Planktonic cells of *C. albicans* (ATCC 90028, ATCC 10231, PYCC 3436T) *C. parapsilosis* (ATCC 22019, PYCC 2545), *C. glabrata* (PYCC 2418T) *C. tropicalis* (PYCC 3097T), *C. krusei* (PYCC 3341), *C. lusitaniae* PYCC 2705T and synergism with antifungals	IC 50 (mmol l^{-1}): 0.09 to 0.74; none synergism found	Faria et al., 2011
Benzoic acid	Planktonic cells of *C. albicans* (ATCC 90028, ATCC 10231, PYCC 3436T) *C. parapsilosis* (ATCC 22019, PYCC 2545), *C. glabrata* (PYCC 2418T) *C. tropicalis* (PYCC 3097T), *C. krusei* (PYCC 3341), *C. lusitaniae* PYCC 2705T and synergism with antifungals	IC 50 (mmol l^{-1}): 0.05–0.73 Synergism found to *C. albicans* with amphotericin and itraconazole	Faria et al., 2011

cardanol demonstrated chitin-binding ability in *C. albicans* cell wall (Mahata et al., 2014).

Few studies have found about others biological pathways of phenolic acids against *Candida*. Exemplifying, an *in vitro* immunoregulatory effect on monocytes against *C. albicans* by cinnamic acid (Conti et al., 2013) and a inhibition of *C. albicans* isocitrate lyase enzyme activity after treatment with caffeic acid (Cheah et al., 2014) was reported. However, several studies have suggested that the other biological pathways and cellular targets of others phenolic compounds may be different from that of existing antifungal agents. Some phenolic compounds have shown to induce apoptotic mechanisms in *Candida*, thereby contributing to their antifungal activity (Zore et al., 2011). For instance, eugenol inhibits the cell cycle at G1, S, and G2-M phases in *C. albicans* and consequently induces apoptosis. Another phenolic compound, curcumin also induces apoptosis in *C. albicans*, by increasing the reactive oxygen species (ROS) and induction of *CaMCA1* gene expression (Cao et al., 2009). On the contrary, baicalein increases ROS causing perturbation in mitochondrial homeostasis in *C. krusei* without inducing apoptosis (Kang et al., 2010). Methyl chavicol seemed to induce aptotosis in *C. albicans* although the exact pathway is still not clear (Khan et al., 2014). Blocking effect of thymol, carvacrol (Ahmad et al., 2013) and baicalein (Huang et al., 2008) on the

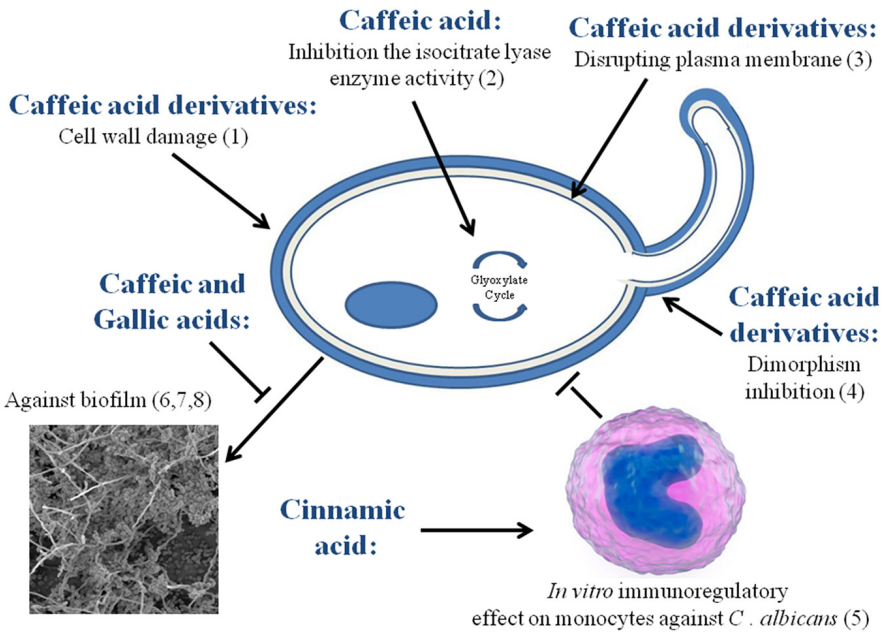

FIGURE 3 | Described mechanisms of action and biological pathways of some phenolic acids against *Candida*. (1) Ma et al. (2010); (2) Cheah et al. (2014); (3,4) Sung and Lee (2010); (5) Conti et al. (2013); (6) Alves et al. (2014); (7) De Vita et al. (2014); (8) Wang et al. (2009).

drug transporter pumps in *Candida* has been demonstrated using rhodamine 6G dye. Inhibition of efflux transporters results in accumulation of antifungal compounds inside the cell making *Candida* highly susceptible to the antifungal agent (Huang et al., 2008). These helpful anti-*Candida* biological pathways observed for phenolic molecules, mainly on the drug transporters pumps may contribute to elucidate the possible effects of phenolic acids against *Candida*.

Another aspect to be considered is that previous studies reported that some *Candida* species were able to metabolize phenolic acids (Middelhoven et al., 1992; Middelhoven, 1993). *C. parapsilosis* was able to grow in the presence of some phenolic acids after 3 days of cultivation. On the other hand, *C. tropicalis* was unable to grow in the presence of phenolic acids even after 14 days of cultivation (Middelhoven, 1993). These evidences should be better investigated in the future. Further studies are warranted to obtain a deeper understanding of the mechanism of action and others biological pathways of phenolic acids on *Candida* cells.

Synergism with Existing Antifungal Agents

Apart from rising antifungal resistance, there are other important limitations in the existing antifungal agents, such as inadequate spectrum of activity, poor bioavailability, small tolerance index, interactions with other drugs, inadequate pharmacokinetic profile, and considerable toxic effects (Lewis and Graybill, 2008; Pfaller et al., 2010). Although phytochemicals remain an important source for the discovery of new antifungal agents, micro-plate based *in vitro* screening assays have not shown higher effectiveness of plant extracts when compared to the existing antifungal agents with higher efficacy (Newman and Cragg, 2012). Hence, in general, plant extracts with higher minimum inhibitory concentrations (MICs) such as 1000 μg/ml are considered ineffective (Morales et al., 2008).

Therefore, some studies have explored the possibility of synergistic activity of phenolic acids and existing antifungal agents in order to maximize the antifungal effect. It is a good strategy to study the synergistic effect when MIC values of phenolic acids against *Candida* are highly variable (Rauha et al., 2000; Kalinowska et al., 2014). Synergistic effect of benzoic acid with amphotericin B and itraconazole against *C. albicans* has been reported in literature (Faria et al., 2011; **Table 3**). However, mechanism of this synergistic effect of phenolic acids and conventional antifungal agents is poorly understood. Therefore, it is important to examine similar synergistic effects shown by others phenolic compounds and conventional antifungal agents in order to obtain some insight.

A promising synergism between phenolic compounds and fluconazole against resistant strains of *Candida tropicalis* was described recently (da Silva et al., 2014). Several other studies have also demonstrated a significant synergism between other known antifungals and phenolic compounds against *C. albicans* (**Table 3**). Some studies suggested that the synergism is due to the induction of apoptosis by an increase in the production of ROS. Hence, it was found that amphotericin B together with baicalein or curcumin increases the production of ROS (Sharma et al., 2010; Fu et al., 2011). A similar effect has been observed with fluconazole and curcumin (Sharma et al., 2010).

TABLE 3 | Synergism of phenolic compounds with traditional antifungals in their action against *Candida albicans*.

Compound	Fluconazole	Amphotericin B	Itraconazole	Others
2,5 Dihydroxybenzaldehyde	—	Faria et al., 2011	Faria et al., 2011	—
Baicalein	Huang et al., 2008†	Fu et al., 2011	—	—
Benzoic acid**	—	Faria et al., 2011	Faria et al., 2011	—
Benzyl benzoate	Zore et al., 2011†	—	—	—
Butylated hydroxyanisole	Simonetti et al., 2002†	Andrews et al., 1977*; Beggs et al., 1978*		Simonetti et al., 2003†
Carvacrol	Ahmad et al., 2013‡	—	—	—
Cinnamaldehyde	Khan and Ahmad, 2012	—	—	—
Curcumin I	Sharma et al., 2010‡	Sharma et al., 2010	Sharma et al., 2010‡	Sharma et al., 2010‡
Epigallocatechin-gallate	Hirasawa and Takada, 2004‡	Hirasawa and Takada, 2004‡; Han, 2007	Navarro-Martinez et al., 2006	Navarro-Martinez et al., 2006
Eugenol	Ahmad et al., 2010a‡; Zore et al., 2011; Khan and Ahmad, 2012***	—	—	—
Glabridin	Liu et al., 2014	—	—	Messier and Grenier, 2011
Honokiol	Jin et al., 2010†	—	—	—
Licochalcone A	—	—	—	Messier and Grenier, 2011
Methyleugenol	Ahmad et al., 2010a‡	—	—	—
Punicalagin	Endo et al., 2010†	—	—	—
Propyl gallate	D'Auria et al., 2001†	Andrews et al., 1977; Beggs et al., 1978*	D'Auria et al., 2001†	Strippoli et al., 2000†
Thymol	Guo et al., 2009‡; Faria et al., 2011; Ahmad et al., 2013‡	Guo et al., 2009; Faria et al., 2011	Faria et al., 2011	—

*Ineffectiveness antifungal effect of phenolic alone; **phenolic acid; ***performed on biofilm formation; †resistant strain; ‡resistant and susceptible strains.

Another hypothesis for the aforementioned synergism is the association between folic acid cycle and ergosterol biosynthesis pathways of *C. albicans*. Hence, epigallocatechin-gallate, a phenolic compound was demonstrated to have a synergistic antifungal effect on *Candida* when combined with itraconazole or ketoconazole (Navarro-Martinez et al., 2006). Azoles directly inhibit the ergosterol biosynthesis while epigallocatechin-gallate has an antifolatic effect that indirectly affects the ergosterol biosynthesis. Epigallocatechin-gallate causes a depletion of the enzyme S-adenosylmethionine which in turn affects the enzyme Sterol C24 methyltransferase. Hence, lower production of C24 methyltransferase negatively affects the ergosterol biosynthesis. Direct and indirect effects on ergosterol biosynthesis explain the synergism between epigallocatechin-gallate and azoles (Navarro-Martinez et al., 2006).

Another study has shown that phenolic compounds such as thymol and carvacrol significantly decrease the expression levels of virulence genes *CDR1* and *MDR1* in fluconazole-resistant *C. albicans* (Ahmad et al., 2013). An *in vivo* study on systemic candidiasis in mice demonstrated that following the treatment with honokiol and fluconazole, the survival rate was 100% while a monotherapy showed only a survival rate of 80% to fluconazole and 20% to honokiol, respectively. Furthermore, the synergism of these two compounds led to a notable reduction in *C. albicans* counts in mouse kidneys compared with the fluconazole treatment alone (Jin et al., 2010). Similarly, mice treated with epigallocatechin-gallate and amphotericin B survived approximately 24 and 30 days longer when compared to the groups treated only with epigallocatechin-gallate or amphotericin B, respectively (Han, 2007). Considering the foregoing evidence obtained for other phenolic compounds, it is likely that potential of synergism exists between known antifungal agents and phenolic acids and this possibility needs to be examined in future.

Safety of the Phenolic Acids *In vitro* and *In vivo*

An 'ideal' antifungal agent for *Candida* infections should not have side effects or toxicity (Chapman et al., 2008; Wong et al., 2014). However, in reality, all the antifungals currently in use have some side effects on gastrointestinal tract, liver and kidney (Wingard et al., 1999; Bates et al., 2001). Therefore, practically one would expect to have some dose-related side effects from any new antifungal agent. It is imperative to understand this limitation in order to appreciate promising qualities of the drug under investigation. DNA-damaging effect of phenolic acids has been observed in p53R cell lines treated with gallic acid (Hossain et al., 2014). Moreover, *in vivo* hepatotoxicity was observed in rats when given a diet supplemented with more than 200 mg/kg/day of gallic acid (Galati et al., 2006). In addition, hematological disorders, as well as liver and kidney weight increase were observed in rats fed with 0.6–5% of gallic acid daily for 13 weeks (Niho et al., 2001).

A potential carcinogenicity was observed on the fore-stomach of rats when fed with a powdered diet containing 0.4% of caffeic acid for up to 28 weeks (Hirose et al., 1998). The clastogenic power of caffeic and cinnamic acids have been described *in vitro* (Maistro et al., 2011). Subcronic administration of protocatechuic acid (0.1% in drinking water) for 60 days has shown a possible liver and kidney toxicity in mice (Nakamura et al., 2001).

Sodium benzoate and sodium phenylacetate have been used in the treatment of acute hyperammonaemia and are derived from benzoic acid and phenylacetic acid respectively. Inappropriate doses of both substances may cause plasma acidosis, hypotension, cerebral edema and other neurotoxical effects, sometimes even death of patients (Kaufman, 1989; Praphanphoj et al., 2000). Phenylacetic acid can also affect the osteoblastic functions *in vitro* and increase cell proliferation in the alveolar region (Kaufmann et al., 2005; Yano et al., 2007). Sodium and potassium benzoates could be clastogenic, mutagenic and cytotoxic to human lymphocytes *in vitro* (Zengin et al., 2011). Therefore, is imperative to examine the dose-related toxicity of phenolic acids in a series of comprehensive *in vitro*, *in vivo* and clinical studies before administration as an antifungal agent.

CONCLUSION

Phenolic acids demonstrate considerable antifungal properties against *Candida*. Previous studies have shown phenolic acid compounds possess considerable anti-adhesion, anti-biofilm effects, and inhibitory activity on morphogenesis and exoenzyme production of *Candida* species. However, hitherto no clear mechanism of action of phenolic acids on *Candida* cells and virulence factors has been described compared to the existing antifungal agents. Interestingly, there is substantial evidence of the synergistic effect of phenolic acids and existing antifungal agents which may become a promising anti-candidal strategy. However, more studies are in demand for a conclusive statement regarding their role. Therefore, we propose that more comprehensive studies are mandatory to obtain evidence regarding the suitability of the use of phenolic acids as a successful antifungal agent in future.

AUTHOR CONTRIBUTIONS

GT conceived, designed, did the literature review, provided and wrote the manuscript. KE assisted in the preparation, design, final review, and co-wrote the manuscript. CK-I and CS conceived, designed, assisted in the literature and final review, and co-wrote the manuscript.

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REVIEW
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Clinical Appearance of Oral Candida Infection and Therapeutic Strategies

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Candida species present both as commensals and opportunistic pathogens of the oral cavity. For decades, it has enthralled the clinicians to investigate its pathogenicity and to improvise newer therapeutic regimens based on the updated molecular research. Candida is readily isolated from the oral cavity, but simple carriage does not predictably result in development of an infection. Whether it remains as a commensal, or transmutes into a pathogen, is usually determined by pre-existing or associated variations in the host immune system. The candida infections may range from non-life threatening superficial mucocutaneous disorders to invasive disseminated disease involving multiple organs. In fact, with the increase in number of AIDS cases, there is a resurgence of less common forms of oral candida infections. The treatment after confirmation of the diagnosis should include recognizing and eliminating the underlying causes such as ill-fitting oral appliances, history of medications (antibiotics, corticosteroids, etc.), immunological and endocrine disorders, nutritional deficiency states and prolonged hospitalization. Treatment with appropriate topical antifungal agents such as amphotericin, nystatin, or miconazole usually resolves the symptoms of superficial infection. Occasionally, administration of systemic antifungal agents may be necessary in immunocompromised patients, the selection of which should be based upon history of recent azole exposure, a history of intolerance to an antifungal agent, the dominant *Candida* species and current susceptibility data.

Keywords: antifungal therapy, *Candida*, NCAC species, oral candidosis, opportunistic infections

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INTRODUCTION

The malady of thrush or candidiasis has been known to occur in people for over 2000 years. As mentioned by the famous Greek physician, Hippocrates in his findings, it commonly presents as superficial infections of the oral and vaginal mucosa. However, it was not until the mid-1800s that the documented research on pathogenesis of candidiasis were instigated. The principal yeast pathogen, *Candida albicans*, itself, was identified in the nineteenth century. In the early 1900s, *C. albicans* was found in the oral cavity of 54% of 2–6 weeks old and 46% of 1 year old infants and in 39% of 1–6 years old children, nonetheless several of them were rather healthy (Barnett, 2008). It was only later, that the subsequent studies revealed the normal oral carriage of *C. albicans* is 2.0–69.1% among the healthy adult population, depending upon the sampled population and technique (Scully et al., 1994).

In recent years, noteworthy escalation in pathogenic state of this commensal has been observed, as reflected in the increased incidence of the common and infrequent forms of candidiasis

(Williams and Lewis, 2011). The probable explanations include changes in the practice of medicine like introduction of broad-spectrum antibiotics, immunosuppressive agents, transplantations, indwelling catheters, etc., and morbid conditions such as diabetes, severe malnutrition in children and AIDS (Lalla et al., 2013). Oral candidiasis is a significant source of morbidity, as it can cause chronic pain or discomfort upon mastication, limiting nutrition intake in the elderly or immunodeficient patients. There are multiple clinical presentations of oropharyngeal and esophageal candidiasis caused by *C. albicans*, either alone or in mixed infection (Sherman et al., 2002). Thus, with the above outlook, the present review comprehends the varied clinical manifestations and the current treatment strategies for this opportunistic pathogen.

EPIDEMIOLOGY

Oral candidosis is frequent in the extremes of age (Akpan and Morgan, 2002). Approximately 5–7% of infants develop oral candidiasis. Its prevalence in AIDS patients is estimated to be 9–31% and close to 20% in the cancer patients (Lalla et al., 2013). The oral carriage of candida organisms is reported to be 30–45% in the general healthy adult population (Akpan and Morgan, 2002). The incidence of *C. albicans* in healthy and various health conditions is depicted in **Table 1**. The additional important species isolated from clinical infections include, *C. glabrata, C. guillierimondii, C. krusei, C. lusitaniae, C. parapsilosis, C. pseudotropicalis, C. stellatoidea,* and *C. tropicalis* (Crist et al., 1996). In recent years higher incidences of the above mentioned non- *C. albicans Candida* (NCAC) species have been also reported (Williams and Lewis, 2011).

Systemic candidiasis is less frequent but carries a mortality rate of 71–79%. The annual incidence of bloodstream infection (BSI) associated with candida ranges from 6–23/100,000 to 2.53–11/100,000 individuals in USA and European countries, respectively. Overall NCAC species have shown an increasing trend as causative pathogens in BSIs with a 10–11% increment over a 6.5−year period in a global report. In addition to *C. albicans,* the common NCAC species involved in BSIs include *C. parapsilosis* (premature neonates and catheterized patients); *C. glabrata* (elderly patients); *C. tropicalis* (hematological malignancies); and *C. krusei* (Richardson, 2005).

TABLE 1 | Oral carriage of *Calbicans albicans* in various subjects (Akpan and Morgan, 2002).

Subjects	Oral carriage of *C. albicans*
Neonates	45%
Healthy children	45–65%
Healthy adults	30–45%
Removable denture wearers	50–65%
Long term facilities	65–88%
Acute leukemia undergoing chemotherapy	90% (approximately)
HIV patients	95% (approximately)

FACTORS PREDISPOSING FOR ORAL CANDIDIASIS (TABLE 2)

Local Factors

Saliva

Salivary gland dysfunction predisposes to oral candidiasis. Constituents of saliva such as histidine-rich polypeptides, lactoferrin, lysozyme, and sialoperoxidase inhibit the overgrowth of candida. Hence, conditions affecting the quantity and quality of salivary secretions may lead to an increased risk of oral candidosis (Scully et al., 1994; Turner and Ship, 2007).

Dental Prostheses

Dental prostheses creates a favorable microenvironment for the candida organisms to thrive. Approximately 65% of complete denture wearers are predisposed to candida infection. The possible explanations include enhanced adherence of candida to the acrylic, ill-fitted appliances, decreased saliva flow under the denture surfaces or inadequate hygiene (Ashman and Farah, 2005; Martori et al., 2014).

Topical Medications

Another important local factor increasing the risk of oral candidosis could be use of topical or inhalational corticosteroids and overzealous use of antimicrobial mouthwashes. They temporarily suppress the local immunity and cause alterations in the oral flora (Scully et al., 1994; Jainkittivong et al., 2007).

Smoking

Some studies suggest that smoking alone or in combination with other factors, significantly affects the oral candida carriage while few studies propose otherwise (Soysa and Ellepola, 2005; Barnett, 2008; Munshi et al., 2015). The precise mechanism is not established but various theories have been postulated. The possible explanations facilitating candida colonization include localized epithelial alterations caused by smoking (Arendorf and Walker, 1980); smoking in association with denture friction altering the mucosal surface (Arendorf and Walker, 1987); nutritional products obtained through enzymatic breakdown

TABLE 2 | Factors predisposing for oral candidiasis (Rautemaa and Ramage, 2011).

Local factors	Systemic factors
• Impaired local defense mechanisms	• Impaired systemic defense mechanisms
• Decreased saliva production	• Primary or secondary immunodeficiency
• Smoking	• Immunosuppressive medications
• Atrophic oral mucosa	• Endocrine disorders- Diabetes
• Mucosal diseases (Oral lichen planus)	• Malnutrition
• Topical medications – corticoids	• Malignancies
• Decreased blood supply (radiotherapy)	• Congenital conditions
• Poor oral hygiene	• Broad spectrum antibiotic therapy
• Dental prostheses	
• Altered or immature oral flora	

of aromatic hydrocarbons contained in cigarette smoke (Hsia et al., 1981; Krogh et al., 1987); suppression of local immunity and reduction in gingival exudate; elevation of glycosylated hemoglobin levels and lastly tobacco smoke increasing the adrenaline levels in blood, indirectly affecting the blood glucose levels.

Diet

Unbalanced dietary intake of refined sugars, carbohydrates and dairy products (containing high content of lactose) might serve as growth enhancers by reducing the pH levels and hence favoring the candida organisms to thrive (Martins et al., 2014).

Systemic Factors

Age

Extremes of age may predispose to candidiasis due to immature or weakened immunity (Weerasuriya and Snape, 2008).

Nutritional Status

Among the nutritional deficiency states, iron has been the most common deficient essential micronutrient implicated in the colonization of candida. Deficiency of iron diminishes the fungistatic action of transferrin and other iron-dependant enzymes. In addition, other nutrients frequently deficit in chronic candidiasis includes essential fatty acids, folic acid, vitamins A and B6, magnesium, selenium, and zinc (Paillaud et al., 2004; Martins et al., 2014).

Systemic Drugs

Prolonged use of systemic drugs like broad-spectrum antibiotics, immune-suppressants and drugs with xerostomic side-effects, alter the local oral flora or disrupt mucosal surface or reduce the salivary flow, creating a favorable environment for candida to grow (Martins et al., 2014). Escalation in candida organisms has also been reported in patients undergoing radiation therapy to the head and neck region.

Endocrine Disorders

Various reports reveal that oral and invasive candidiasis are more prevalent in patients with endocrine dysfunctions such as diabetes and Cushing's syndrome (Graham and Tucker, 1984; Bakker et al., 1998; Sashikumar and Kannan, 2010).

Immune Disorders

Immunodeficiency conditions such as AIDS and severe combined immunodeficiency syndrome (SCID) are also predisposing factors for candidiasis (Anil and Challacombe, 1997; Owotade and Patel, 2014).

Malignancies

The host defense mechanisms are compromised by chemotherapy and radiotherapy administered for the treatment of malignant conditions. The prevalence of oral candidiasis for all cancer treatments, according to a systematic review, was reported to be 7.5% pre-treatment, 39.1% during treatment and 32.6% post-cancer therapy. The prevalence of oral candidiasis during head and neck radiation therapy and chemotherapy was observed to be 37.4 and 38%, respectively. The colonization by *C. albicans* was reported to be 46.2%. The prevalence of NCAC species were *C. tropicalis* (16.6%), *C. glabrata* (5.5%), and *C. krusei* (3%) (Scheffel et al., 2010).

Congenital Conditions

Lastly, individuals affected by congenital conditions associated with defective immune system such as Di George's syndrome, hereditary myeloperoxidase deficiency and Chediak–Higashi syndrome are commonly predisposed to candida infections (Ashman and Farah, 2005).

FORMS OF ORAL CANDIDA INFECTIONS (TABLE 3)

Primary Oral Candidiasis

Primary Triad

Pseudomembranous candidiasis

This form of candidiasis classically presents as acute infection, though the term chronic pseudomembranous candidiasis has been used to denote chronic recurrence cases. It is commonly seen in extremes of age, immunocompromised patients especially in AIDS, diabetics, patients on corticosteroids, prolonged broad-spectrum antibiotic therapy, hematological, and other malignancies (**Figure 1**). On the oral surfaces, the superficial component presents as white to whitish-yellow creamy confluent plaques resembling milk curds or cottage cheese. These plaques consist of desquamated epithelial cells, tangled aggregates of fungal hyphae, fibrin, and necrotic material (Lalla et al., 2013). The superficial pseudo-membrane can be removed by wiping gently, leaving behind an underlying erythematous and occasionally bleeding surface (Ashman and Farah, 2005; Farah et al., 2010). The oral surfaces frequently involved include labial and buccal mucosa, tongue, hard and soft palate and oropharynx.

TABLE 3 | Classification of oral candidosis (Axell et al., 1997).

Primary oral candidosis	Secondary oral candidosis
Acute forms	**Oral manifestations of systemic mucocutaneous candidosis**
Pseudomembranous	Thymic aplasia
Erythematous	Candidosis endocrinopathy syndrome
Chronic forms	
Hyperplastic (nodular or plaque-like)	
Erythematous	
Pseudomembranous	
Candida-associated lesions	
Denture stomatitis	
Angular cheilitis	
Median rhomboid glossitis	
Keratinized primary lesions with candidal super infection	
Leukoplakia	
Lichen planus	
Lupus erythematosus	

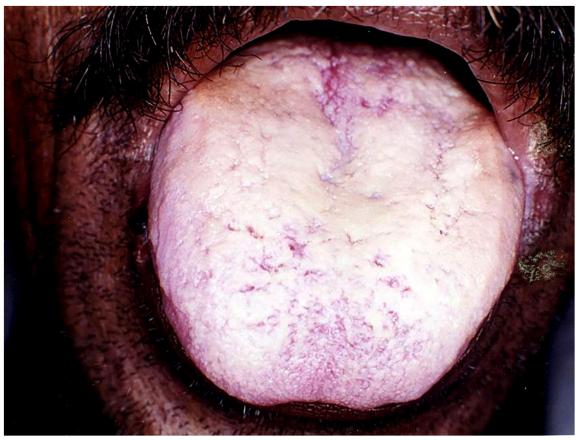

FIGURE 1 | Pseudomembranous candidiasis of the tongue. The copyright of the images is owned by Prof. Anil and a written consent was obtained for the **Figures 1–6**.

The involvement of both oral and oesophageal mucosa is prevalent in AIDS patients. The symptoms of the acute form are rather mild and the patients may complain only of slight tingling sensation or foul taste, whereas, the chronic forms may involve the oesophageal mucosa leading to dysphagia and chest pains. Few lesions mimicking pseudomembranous candidiasis could be white coated tongue, thermal and chemical burns, lichenoid reactions, leukoplakia, secondary syphilis and diphtheria (Lalla et al., 2013).

Erythematous candidiasis

Erythematous candidiasis is relatively rare and manifests as both acute and chronic forms (Ashman and Farah, 2005). Previously known as 'antibiotic sore mouth,' due to its association with prolonged use of broad-spectrum antibiotics (Farah et al., 2010). The chronic form is usually seen in HIV patients involving the dorsum of the tongue and the palate and occasionally the buccal mucosa (**Figure 2**). Clinically, it manifests as painful localized erythematous area. It is the only form of candidiasis associated with pain. The lesions are seen on the dorsum of the tongue typically presenting as depapillated areas. Palatal lesions are more common in HIV patients. Differential diagnosis may include mucositis, denture stomatitis, erythema migrans, thermal burns, erythroplakia, and anemia (Dodd et al., 1991).

Hyperplastic candidiasis

The hyperplastic candidiasis mainly presents as chronic form. It has been commonly referred previously by several authors as 'candidal leukoplakia.' Clinically, it may manifest as one of the two variants; homogeneous adherent white plaque-like or erythematous multiple nodular/speckled type (Holmstrup and Bessermann, 1983; Sanketh et al., 2015). The lesions usually occur bilaterally in the commissural region of the buccal mucosa and less frequently on the lateral border of the tongue and palate (**Figure 3**). Unlike the pseudomembranous type, hyperplastic candidiasis lesions are non-scrapable. There appears to be a positive association with smoking and in addition may present

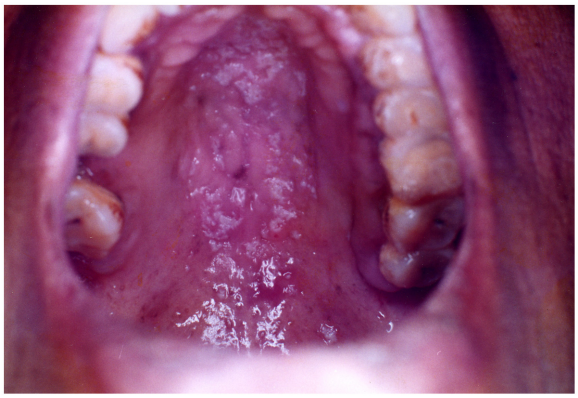

FIGURE 2 | Erythematous candidiasis of the palate.

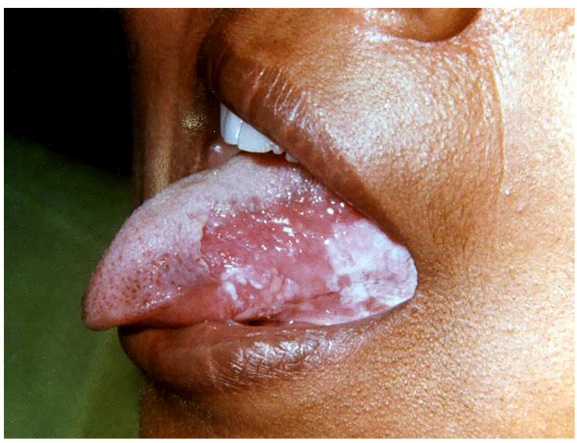

FIGURE 3 | Hyperplastic candidiasis at the lateral border of the tongue.

with varying degrees of dysplasia (Williams and Lewis, 2011). A confirmed association between Candida and oral cancer is yet to be recognized, although *in vitro* studies have shown that the candida organisms can generate carcinogenic nitrosamine (Farah et al., 2010; Sanketh et al., 2015). A small percentage of cases occur in association with iron and folate deficiencies and with defective cell-mediated immunity. Differential diagnosis may include leukoplakia, lichen planus, angular cheilitis and squamous cell carcinoma.

Candida-associated Lesions

Denture stomatitis

It is also known as "chronic atrophic candidiasis." As the name indicates, it is chronic inflammation of the mucosa typically restricted to the denture-bearing area, seen in association with candidiasis (Lund et al., 2010). It is seen in almost 50–65% of the denture wearers (Ashman and Farah, 2005; Williams and Lewis, 2011). Clinically, the lesions may be seen as pinpoint hyperaemia, diffuse erythematous or granular/papillary type. It occurs frequently along with angular cheilitis and median rhomboid glossitis. The lesions are usually asymptomatic, though

occasionally patients may complain of burning sensation or soreness. It commonly affects the palate although mandibular mucosa may also be affected (**Figure 4**). The associated etiological factors include poor oral hygiene practice, nocturnal denture wear, ill-fitting prostheses and limited flow of saliva (Farah et al., 2010; Williams and Lewis, 2011).

Angular cheilitis

This form of candidiasis usually manifests as erythematous or ulcerated fissures, typically affecting unilaterally or bilaterally the commissures of the lip (Samaranayake et al., 1995; Sharon and Fazel, 2010). Angular cheilitis often represents an opportunistic infection of fungi and/or bacteria, with multiple local and systemic predisposing factors involved in the initiation and persistence of the lesion (Park et al., 2011). The factors associated include old age and denture-wearers (due to reduced vertical dimension), vitamin B12 deficiency and iron deficiency anemia (Jenkins et al., 1977). Other causative organisms implicated are *Staphylococcus* and *Streptococcus* (Farah et al., 2010).

Median rhomboid glossitis

Median rhomboid glossitis appears as the central papillary atrophy of the tongue and is typically located around the midline of the dorsum of the tongue. It occurs as a well-demarcated, symmetric, depapillated area arising anterior to the circumvallate papillae (**Figure 5**). The surface of the lesion can be smooth or lobulated (Joseph and Savage, 2000). While most of the cases are asymptomatic, some patients complain of persistent pain, irritation, or pruritus (Lago-Mendez et al., 2005). The lesion is now believed to be a localized chronic infection by *C. albicans*. It is commonly seen in tobacco smokers and inhalation-steroid users (Aun et al., 2009; Williams and Lewis, 2011).

Linear gingival erythema

It was previously referred to as "HIV-gingivitis" since its typical occurrence was in HIV associated periodontal diseases (**Figure 6**). It manifests as linear erythematous band of 2–3 mm on the marginal gingiva along with petechial or diffuse erythematous lesions on the attached gingiva. The lesions may present with bleeding. In addition to *C. albicans*, *C. dubliniensis* has been reported as an emerging pathogen in this form of candidiasis (Williams and Lewis, 2011).

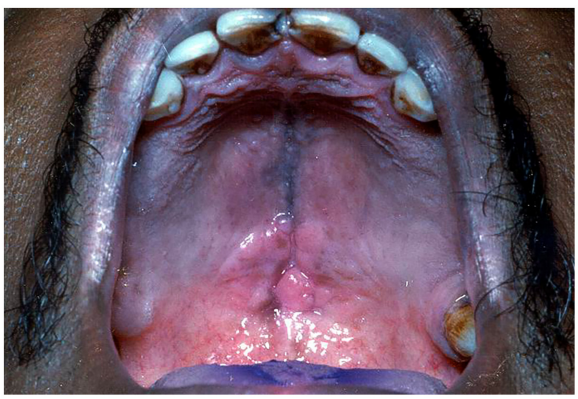

FIGURE 4 | Denture stomatitis of the palate.

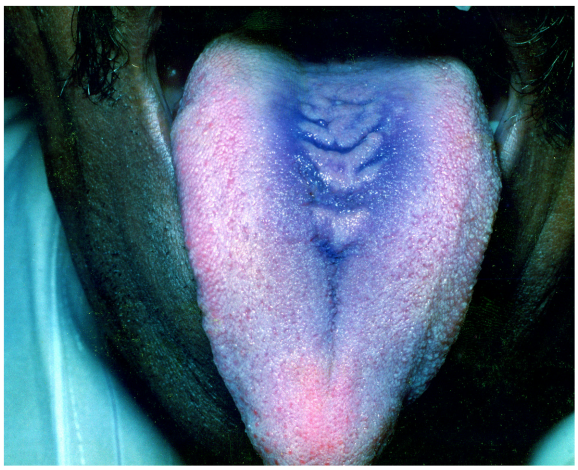

FIGURE 5 | Median Rhomboid glossitis-note the candidal overgrowth.

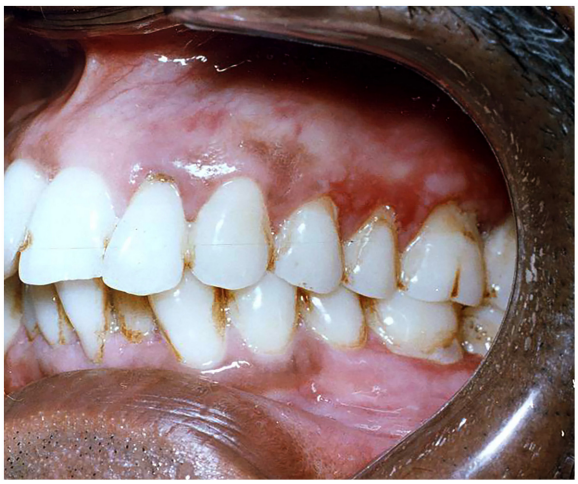

FIGURE 6 | Linear Gingival erythema in an HIV infected patient.

Secondary Oral Candidiasis

This group is characterized by chronic mucocutaneous candidiasis, which consists of heterogeneous disorders, presenting as persistent or recurrent superficial candida infections of the mouth, skin, nail beds, and occasionally producing granulomatous masses over the face and scalp. The primary clinical features include chronic oral, cutaneous and vulvovaginal candidiasis. Oral cavity involvement is reported in more than 90% cases and the lesions can occasionally spread into the larynx, pharynx or esophagus but further involvement is infrequent. It is associated with diverse immunodeficiency disorders such as, Di George syndrome, hyper-immunoglobulin E syndrome, Nezelof's syndrome MPO deficiency, SCID syndrome and endocrine disorders like Addison's disease and

hypoparathyroidism (Ashman and Farah, 2005; Farah et al., 2010; Williams and Lewis, 2011; Lalla et al., 2013).

ORAL CANDIDA INFECTION IN NEWBORNS

Oral candidiasis in neonates is reported to be 0.5–20%, depending upon the various studies (Yilmaz et al., 2011; Stecksen-Blicks et al., 2015). The most common form of candidiasis affecting this age group is the acute pseudomembranous candidiasis (Berdicevsky et al., 1984). *Candida* species isolated from these lesions include *C. albicans*, followed by *C. glabrata, C. tropicalis* and *C. krusei* (Tinoco-Araujo et al., 2013). Majority of the lesions are asymptomatic. They mainly present as white scrapable pseudomembranous lesions. The major predisposing factors were low birth weight, prolonged hospital stay and associated increased risk of exposure to environmental factors. The participation of dental surgeon is essential in early diagnosis of the oral signs and symptoms of this opportunistic infection in order to prevent disseminated candidiasis and subsequent mortality (2–20%; Sitheeque and Samaranayake, 2003). Treatment for superficial infection is topical administration of antifungals such as 1% clotrimazole solution thrice daily for 7 days. In case of invasive or disseminated candidiasis, systemic interventions are obligatory (Sitheeque and Samaranayake, 2003).

MANAGEMENT OF ORAL CANDIDOSIS

An effective management of oral candidiasis can be achieved by adhering to the following simple guidelines:

(1) Diagnosis through detailed medical and dental history, clinical manifestations confirmed with laboratory tests.
(2) Correction of predisposing factors where achievable.
(3) Maintenance of proper hygiene of the oral cavity and oral prostheses, if any.
(4) Selection of antifungal therapy based on severity of the infection and susceptibility of the *Candida* species prevalent in that patient.

Diagnosis of oral candidosis, includes identification of clinical signs and symptoms, presence of the candida organisms on direct examination of a smear from the lesion or biopsy examination showing hyphae in the epithelium, positive culture, and serological tests (Rossie and Guggenheimer, 1997; Ellepola and Morrison, 2005). Another concern with respect to the treatment, is the increase in NCAC species which are naturally resistant to some of the common antifungal drugs (**Table 4**). For example, in HIV positive cases there is reported increase in *C. glabrata*, followed by *C. krusei;* in insulin using diabetes mellitus patients' significant percentage of *C. dubliniensis* and *C. glabrata* was noted; also certain mucosal lesions, oral cancer and elderly hospitalized patients have shown increase in NCAC species carriage (Gutierrez et al., 2002).

ANTIFUNGAL AGENTS

Antifungal agents that are available for the treatment of candidosis fall into three main categories: the polyenes (nystatin and amphotericin B); the ergosterol biosynthesis inhibitors-the azoles (miconazole, clotrimazole, ketoconazole, itraconazole, and fluconazole), allylaminesthiocarbamates, and morpholines; and DNA analog 5-fluorocytosine, and newer agents such as caspofungins (Ghannoum and Rice, 1999; Pappas et al., 2009). The choice of antifungal treatment depends on the nature of the lesion and the immunological status of the patient. There are three main antifungal drug targets in *Candida*: the cell membrane, cell wall, and nucleic acids (**Figure 7**) (Cannon et al., 2007).

Superficial oral candidosis in generally healthy patients can be treated topically and oral candidosis in immunocompromised patients should be treated systemically as well as topically. Patients with persisting risk factors and relapsing candidosis should be treated with antifungals with the lowest risk of development or selection of resistant strains (Soysa et al., 2008; Rautemaa and Ramage, 2011). The commonly used antifungal agents in the management of OPC is listed in **Table 5**.

TOPICAL ANTIFUNGALS

Topical antifungals are usually the drug of choice for uncomplicated, localized candidiasis in patients with normal immune function. High levels can be achieved in the oral epithelium with topically administered antifungals. Polyenes are fungicidal drugs that act through direct binding to the ergosterol within the fungal cell membranes, inducing leakage

TABLE 4 | Susceptibility of *C. albicans* and common NCAC species (Gutierrez et al., 2002; Pappas et al., 2009).

Candida species	Fluconazole	Itraconazole	Amphotericin B	Echinocandin	Flucytosine
Candida albicans	S	S	S	S	S
Candida tropicalis	S	S	S	S	S
Candida glabrata	S-DD to R	S-DD to R	S-I	S	S
Candida krusei	R	S-DD to R	S to S-I	S	S-I to R
Candida dubliniensis	S to R	S to R	S	S	S

S, Susceptible; S-DD, Susceptible dose-dependent; S-I, Susceptible intermediate; R, Resistant.

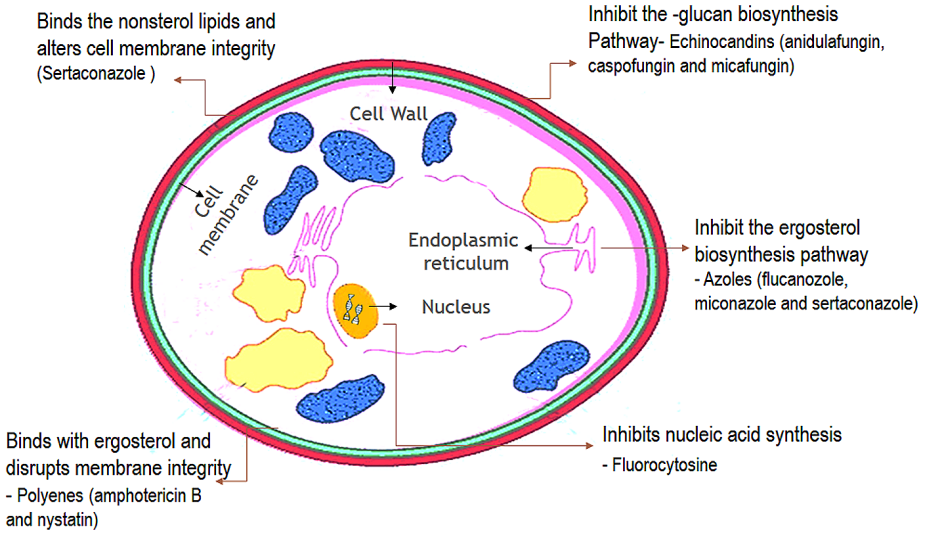

FIGURE 7 | Cellular targets of antifungal agents. (The antifungal agents target three cellular components of fungi. Azoles inhibit the synthesis of ergosterol in the endoplasmic reticulum of the fungal cell. Polyenes such as amphotericin B bind to ergosterol in the fungal membrane causing disruption of membrane structure and function. Flucytosine is converted within the fungal cell to 5-fluorouracil which inhibits DNA synthesis.)

TABLE 5 | Treatment of oropharyngeal candidiasis (OPC; Thompson et al., 2010).

Severity	Antifungal drug	Dosage/ Duration
First-line agents		
	Fluconazole (PO or IV)	100–200 mg/7–14 days
	Clotrimazole troches	10 mg five times/7–14 days
	Nystatin suspension (100,000 U/mL)	4–6 ml four times/7–14 days
	Nystatin pastilles (200,000 U each)	1–2 pastilles four times/7–14 days
Second-line agents		
	Itraconazole solution (PO)	200 mg/28 days
	Posaconazole (PO)	400 mg daily in divided doses
	Voriconazole (PO or IV)	200 mg twice daily
Agents used in refractory case of OPC		
	Caspofungin (IV)	70 mg loading dose followed by 50 mg daily
	Micafungin (IV)	100-150 mg daily
	Anidulafungin (IV)	100 mg loading dose followed by 50 mg daily
	Amphotericin B oral suspension	500 mg every 6 h
	Amphotericin B deoxycholate (IV)	0.3 mg/kg once

of cytoplasmic contents leading to the fungal cell death. Nystatin or amphotericin B solutions are used for 4 weeks. In recurrent cases the duration of treatment should be for at least 4–6 weeks.

Topically administered miconazole gel is also suitable for the treatment of uncomplicated infections in generally healthy patients (Bensadoun et al., 2008). It should also be used for 1 week after resolution of symptoms. The gel inhibits the action of fungal ergosterol synthesis; interacts with the cytochrome P450 enzyme 14-alpha demethylase; inhibits growth of pathogenic yeasts by altering cell membrane permeability. Repeated use of miconazole, however, may cause a risk of development of azole-resistant strains (Rautemaa et al., 2008).

SYSTEMIC ANTIFUNGALS

Systemic antifungals are usually indicated in cases of disseminated disease and/or in immunocompromised patients. Azoles are fungistatic drugs that inhibit the fungal enzyme lanosterol demethylase responsible for the synthesis of ergosterol. Among the azoles, fluconazole attains a higher concentration in the saliva making it principally the suitable drug for treating this oral infection. Fluconazole and itraconazole are administered orally and it gets secreted onto mucous membranes. The oral solution also has a topical effect (Pappas et al., 2004). The other antifungals, echinocandins, and flucytosine act through inhibition of D-glucan synthase and DNA/protein synthesis, respectively (Muir et al., 2009; Vandeputte et al., 2012).

Posaconazole, is available only as an oral solution and is used in immunocompromised patients and patients resistant to other drugs (Clark et al., 2015).

One of the risks while using fluconazole and other drugs of the azole group is the development of resistant strains (Siikala et al., 2010). For fluconazole-refractory disease, either itraconazole solution at a dosage of 200 mg daily or posaconazole suspension at a dosage of 400 mg twice daily for 3 days, then 400 mg daily for up to 28 days, are recommended. Voriconazole at a dosage of 200 mg twice daily or a 1-mL oral suspension of AmB-d, administered at a dosage of 100 mg/mL four times daily, are recommended when treatment with other agents has failed. Intravenous echinocandin or AmB-d at a dosage of 0.3 mg/kg daily can be used in treating patients with refractory disease (Vazquez, 2003).

ALTERNATIVE ANTI CANDIDAL AGENTS

Lastly, to mention a few natural anti-yeast substances which can be used as an alternative treatment. These agents with recognized activity against *C. albicans* includes berberine-containing plants; caprylic acid; grapefruit seed extract; garlic; probiotics; tea tree oil and enteric-coated volatile oil preparations containing cinnamon, ginger, oregano, peppermint and rosemary; propolis and thyme (Hofling et al., 2010; Valera et al., 2013). Agents capable of inhibiting microbial growth such as xylitol is known to inhibit microbial metabolism in the oral cavity. It is therefore incorporated in chewing gums and tablets as well as in health care products such as dentifrice and oral rinses. Although it has a limited effect on *Candida*, it could be beneficial in prevention of the mixed biofilm infection (Pizzo et al., 2000). The essential oil of *Melaleuca alternifolia*, also known as tea tree oil has been shown to be promising as a topical antifungal agent, with recent clinical data indicating efficacy in the treatment of oral candidiasis (Hammer et al., 2004; Sitheeque et al., 2009).

PREVENTION OF ORAL CANDIDOSIS

Good oral hygiene practices may help to prevent oral thrush in people with weakened immune systems. Careful mechanical cleaning of teeth and dentures with a toothbrush is the cornerstone of the prevention of candida infections. Oral decontamination using antifungal and antibacterial rinses is one of the approaches often used to manage oral mucositis (Fathilah et al., 2012). Chlorhexidine digluconate, and cetylpyridinium chloride are two antiseptics often incorporated in mouth rinses and used as prophylaxis for both chemotherapy and radiotherapy induced mucositis (Salim et al., 2013). People who use inhaled corticosteroids may be able to reduce the risk of developing thrush by washing out the mouth with water or mouthwash after using an inhaler (Soares et al., 2011). For susceptible denture wearers, it is advisable to remove the denture at night and soak in 0.2% Chlorhexidine solution or 15–30 min in white vinegar (diluted 1:20) or 0.1% hypochlorite solution (Kassaify et al., 2008). The elimination or at least regulation of the predisposing factors for candidiasis is essential. Failure to recognize this may only provide a temporary relief using antifungal therapy, but with inevitable relapse of the infection (Akpan and Morgan, 2002).

CONCLUSION

In the past few decades extensive clinical data has been recorded on oral candidiasis with respect to its advent with the various immunocompromised conditions. With the increasing incidence of NCAC species and the development of antifungal resistance, there is a persistent requirement in research for newer effective agents. One such prospect is development of vaccine against candida organisms. Various experimental strategies have been employed for developing such a vaccine, like attenuated live candida organisms, SAP gene family proteins, glycoconjugates (mannans and β-glucans) to mention a few, but clinical trials are still a distant vision.

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SNF3 as High Affinity Glucose Sensor and Its Function in Supporting the Viability of *Candida glabrata* under Glucose-Limited Environment

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Candida glabrata is an emerging human fungal pathogen that has efficacious nutrient sensing and responsiveness ability. It can be seen through its ability to thrive in diverse range of nutrient limited-human anatomical sites. Therefore, nutrient sensing particularly glucose sensing is thought to be crucial in contributing to the development and fitness of the pathogen. This study aimed to elucidate the role of *SNF3* (Sucrose Non Fermenting 3) as a glucose sensor and its possible role in contributing to the fitness and survivability of *C. glabrata* in glucose-limited environment. The *SNF3* knockout strain was constructed and subjected to different glucose concentrations to evaluate its growth, biofilm formation, amphotericin B susceptibility, *ex vivo* survivability and effects on the transcriptional profiling of the sugar receptor repressor (SRR) pathway-related genes. The *CgSNF3*Δ strain showed a retarded growth in low glucose environments (0.01 and 0.1%) in both fermentation and respiration-preferred conditions but grew well in high glucose concentration environments (1 and 2%). It was also found to be more susceptible to amphotericin B in low glucose environment (0.1%) and macrophage engulfment but showed no difference in the biofilm formation capability. The deletion of *SNF3* also resulted in the down-regulation of about half of hexose transporters genes (four out of nine). Overall, the deletion of *SNF3* causes significant reduction in the ability of *C. glabrata* to sense limited surrounding glucose and consequently disrupts its competency to transport and perform the uptake of this critical nutrient. This study highlighted the role of *SNF3* as a high affinity glucose sensor and its role in aiding the survivability of *C. glabrata* particularly in glucose limited environment.

Keywords: ***Candida glabrata*, glucose sensor, *SNF3*, glucose-limited environment, hexose transporter**

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INTRODUCTION

Glucose is commonly known as an important carbon source and energy for many organisms. Several studies have attempted to establish the linkage between glucose availability and physiological response of *Candida* species, including the biofilm formation, oxidative stress, and antifungal resistance (Rodaki et al., 2009; Uppuluri et al., 2010; Ene et al., 2012;

Ng et al., 2015b). The regulatory effect by glucose found in these studies is suggestive of the importance of glucose sensing and uptake mechanism in contributing to the fitness of *Candida* species. Brown et al. (2006) have demonstrated that the loss of Hgt4, a high affinity glucose sensor resulted in a less virulent *C. albicans* type that failed to grow in low glucose and fermentation-preferred environments. In addition, the loss of Hgt4 also affects the ability of *C. albicans* to perform the yeast-hyphal morphological switch and therefore compromises its pathogenicity in mouse model of disseminated candidiasis (Brown et al., 2006). Apart from that, the ability to transport glucose by *Cryptococcus neoformans* is also diminished with the loss of Hxs1, a high affinity glucose sensor-like protein (Liu et al., 2013). The diminished glucose uptake activity leads to an attenuated strain of *C. neoformans* in which the strain demonstrated a delay in lethal infection in mice model. However, little is known about the role of high affinity glucose sensor in the emerging human fungal pathogen, *Candida glabrata*.

The ability of *C. glabrata* to thrive in several glucose-limited anatomical sites of host such as vaginal and blood (Ehrström et al., 2006) is suggestive of its sensitivity toward the low glucose availability in the niches with its superior glucose sensing ability. The primary mechanism for *Saccharomyces cerevisiae* to sense and transport surrounding glucose is through *SNF3-RGT2* mediated sugar receptor repressor (SRR) pathway (Rolland et al., 2002; Santangelo, 2006; Gancedo, 2008). This pathway employs two glucose sensors with different affinity toward glucose: *SNF3* (high affinity) and *RGT2* (low affinity). They are located in the cell membrane and modulate the expression of the hexose transporters (*HXT*s) for the uptake of glucose through the interplay of transcription regulators (*RGT1* and *MIG1*) and downstream component of SRR: *YCK1* and *YCK2* (casein kinase), *GRR1* (Glucose Repression Resistant), *STD1* (repressor of *RGT1*; summarized and illustrated in **Figure 9**; Schmidt et al., 1999; Kim and Johnston, 2006). The homologs of these key genes were found in the *C. glabrata* genome. The phylogenetic analysis conducted demonstrates the shared neighborhood between Cg*SNF3* (sequence ID: CAGL0J09020g) and Sc*SNF3* (Palma et al., 2009; Ng et al., 2015a). In addition, the key feature of glucose sensor as found in Sc*SNF3*, for example the unusual long C-terminal segment amino acids and the signature Özcan motif were also found in the Cg*SNF3* (Palma et al., 2009; Ng et al., 2015a). Nevertheless, these studies have only managed to highlight the phylogenetic relatedness between Sc*SNF3* and Cg*SNF3*, but the actual physiological role of *SNF3* in *C. glabrata* is remains unknown. In respect to the importance of glucose in cell physiology, the disruption in SRR pathway may have negative impact on the fitness of *C. glabrata*. Therefore, this study aimed to explore the possible role of *SNF3* in supporting the growth and fitness of *C. glabrata* under low glucose concentration environment. Its growth profile, biofilm formation, antifungal susceptibility, and capability to withstand phagocytosis of macrophage were assessed. In addition, its role in regulating the expression of the SRR pathway-related genes, which includes the hexose transporters (*HXT*s), was also deciphered.

MATERIALS AND METHODS

Yeast Strain and Media Preparation

C. glabrata BG14 (gift from Brendan Cormack, John Hopkins University; Cormack and Falkow, 1999) and its parental strain *C. glabrata* BG2 (gift from Paul Fidel, Louisiana State University Health Sciences Center) were used in this study (**Table 1**). Three types of media were utilized: standard YPD (Becton, Dickinson and Company, USA; 20 g of peptone, 20 g of glucose, 10 g of yeast extract), synthetic minimal glucose medium, SD [0.67% of yeast nitrogen base (Becton, Dickinson and Company, USA) + glucose (Fisher Scientific, USA)] and synthetic complete media with uridine dropout [0.17% yeast nitrogen base without ammonium sulfate and amino acid (Becton, Dickinson and Company, USA) + 0.5% ammonium sulfate (Sigma Aldrich, USA) + 2% glucose (Fisher Scientific, USA) + complete supplement mixture with uridine dropout (ForMedium, UK)] (Sherman, 2002). All strains were maintained at 37°C in YPD, unless otherwise indicated.

Strain Construction

For the construction of the *C. glabrata SNF3*Δ strain, the *C. glabrata* BG14 was transformed to Ura3 + by replacing the *SNF3* open reading frame (ORF) with *SNF3*::*URA3* disruption cassette. *SNF3*::*URA3* disruption cassette that consists of "upstream *SNF3*-*URA3*-downstream *SNF3*" was amplified by PCR (primers TS_SNF3_F and TS_SNF3_R; **Table 2**) and purified through Expin™ Combo GP purification kit (GeneAll, Korea). The purified cassette was then transformed into *C. glabrata* BG14 as described in Cormack and Falkow (1999). Transformants were selected on synthetic complete media with uridine dropout and insertion was confirmed by diagnostic PCR (primers CHK_F_1 CHK_R_1 and CHK_F_2 CHK_R_2; **Table 2**) for the absence of *SNF3* and presence of *URA3* at the correct locus. In order to eliminate the possible effect from secondary mutation of mutant constructed, three independently constructed *SNF3*Δ mutants were analyzed, and treated as three biological replicates in the subsequent assays (Odds et al., 2006).

Growth Profiling and Growth Rate Calculation

The capability of *SNF3*Δ strain and parental wild type BG2 to grow in different levels of surrounding glucose was assessed using a modified procedure as described in Brown et al. (2006) and

TABLE 1 | *Candida glabrata* strains used in this study.

C. glabrata strains	Genotype	References
BG2	Wild type	Cormack and Falkow, 1999
BG14	Ura3Δ (−85 + 932):: Tn903NeoR	Cormack and Falkow, 1999
SNF3Δ_a *SNF3Δ_b* *SNF3Δ_c*	Derived from BG14, *SNF3*::*URA3*	This study

TABLE 2 | List of primers used in this study.

Target gene name	Direction	Sequence 5′–3′	Expected amplification size
RGT2 (Restores Glucose Transport 2)	Forward	CGTTTGTGGGACTTTTCGTT	203 bp
(CAGL0I03872)	Reverse	TGAAATCCATGGAGCAATGA	
GRR1 (Glucose Repression-Resistance 1)	Forward	TTGTCGAGCTTACAGGCAGA	149 bp
(CAGL0M09130)	Reverse	CCCTCCAATCTTTGGTTTCC	
STD1 (Suppressor of Tbp Deletion 1)	Forward	GAGTGCCCCACCAGAATATG	146 bp
(CAGL0L10043)	Reverse	AGGACTGCGAGCTGTGACTT	
YCK1 (Yeast Casein Kinase 1)	Forward	CGCTGACAATGCTAACCAGA	150 bp
(CAGL0G06138)	Reverse	TGAACACGTTGTCCTTGCAT	
YCK2 (Yeast Casein Kinase 1)	Forward	TCGGAGAGACTATGGACGGTA	149 bp
(CAGL0J05940)	Reverse	GGCAGCGTTTCTGTTCCTAT	
RGT1 (Restores Glucose Transport 1)	Forward	CCAACTCAAAGGATGGAGGA	194 bp
(CAGL0L01903)	Reverse	TATCGTTGGCGTCATTTTGA	
MIG1 (Multicopy Inhibitor of GAL gene expression 1)	Forward	CCGGGATGTGTCAAGAGATT	212 bp
(CAGL0A01628)	Reverse	CGTTTCGTCTTCCTCCTCAG	
HXT1 (Hexose Transporter 1)	Forward	AAACCAAGTCGGCAAGAATG	224 bp
(CAGL0A01804)	Reverse	ATTCAGTTCCGTCAGGATGC	
HXT3 (Hexose Transporter 3)	Forward	TGACCTTCGTTCCAGAATCC	165 bp
(CAGL0A0231)	Reverse	TACCAGCGGCATTAGCTTCT	
HXT5 (Hexose Transporter 5)	Forward	TATGTTTCGCATGGGCATTA	153 bp
(CAGL0A01826)	Reverse	CCAAAAGGACGATTGGAGAA	
HXT4 (Hexose Transporter 4)	Forward	TCCTGGGGTGAATTGTTCTC	228 bp
(CAGL0A01782)	Reverse	GCCAAATCTACCGACCAAGA	
HXT6/7 (Hexose Transporter 6/7)	Forward	GCTTCGGTCGTCGTAAATGT	195 bp
(CAGL0A00737/A02233)	Reverse	GAGTTGGTGCCCAAGTTGTT	
HXT6/7 (Hexose Transporter 6/7)	Forward	GGTCAAGACCAACCATCCTCC	182 bp
(CAGL0A02211)	Reverse	CCCCAGATCCAGTTGGAAGC	
HXT2/10 (Hexose Transporter 2/10)	Forward	AAGCTGGAAGGCGAAGATTT	146 bp
(CAGL0I00286)	Reverse	TCCCAACCAAAGACAAAACC	
HXT2/10 (Hexose Transporter 2/10)	Forward	TGCCGAAACCTACCCACTAC	147 bp
(CAGL0D02662/D02640)	Reverse	CAGCCCATGAAGACGTAACC	
HXT14 (Hexose Transporter 14)	Forward	TACGCCAGCACACTAAAGCA	153 bp
(CAGL0M04103)	Reverse	TTGCAGAGGACACAATCGTC	
UBC13 (Ubiquitin-Conjugating 13) (CAGL0G08063)	Forward	TGCCCGAGGACTACCCTATG	100 bp
	Reverse	AGCACGTCCAGGCAGATACG	
ACT1 (ACTin 1)	Forward	TTGCCACACGCTATTTTGAG	225 bp
	Reverse	ACCATCTGGCAATTCGTAGG	
TS_SNF3	Forward	CATGGCTGGAACTAGGCGCTTATTGACGGGTATTGGAGACTTAGGAT AGAGGAAGATTTTGGCATAGGATGTCCAGTGCCTCATATTTAC	–
	Reverse	GCTGCGTCTGATCGTTGTCGTTTTGTGAGTACCCTGTATTTTG GCTGGTATAGGTATTACTCTTCAGTTTCCTATTCTTTTCAAGTAAGC	–
CHK_F_1	Forward	AGCAGAGGACTCCCTCAATG	–
CHK_R_1	Reverse	TTTCAGCAACTTGGAAGCAA	–
CHK_F_2	Forward	GATACAGGAACAACAGCGAG	–
CHK_R_2	Reverse	CCATGAGCGTTGGTGATATC	–

Rodaki et al. (2009). *C. glabrata* cells were grown overnight in YPD medium at 37°C and washed with phosphate buffered saline (PBS, pH 7.4) for three times before re-inoculated into 50 mL fresh SD (OD_{600} = 0.1) with prepared glucose concentrations of 0.01 (extremely low), 0.1 (low), 0.2 (moderate), 1 (high), and 2% (extremely high), respectively. The cells were allowed to grow at 37°C, 200 rpm in shaking incubator (shaking). Another set of cells were incubated in the same manner but were left to be static. These two conditions served as respiration-preferred (shaking) and fermentation-preferred (static) atmosphere, respectively (Brown et al., 2006). The cells were harvested hourly for 10 h and the optical density of cells (OD_{600}) for each hour were recorded

and proceeded with the growth rate calculation as shown in Equation (1) (Widdel, 2010).

$$\mu = \frac{2.303\left(\lg OD_2 - \lg OD_1\right)}{t_2 - t_1} \quad (1)$$

where,

μ = growth rate, h^{-1}

OD_1 = optical density obtained from the log phase of growth curve

OD_2 = two times of OD_1 obtained from the log phase of growth curve

t_1 = time of OD_1 obtained

t_2 = time of OD_2 obtained.

Biofilm Formation

The biofilm formation assay were performed with minor modification as described in Pierce et al. (2008) by replacing RPMI1640 medium with SD (0.01 and 0.1% glucose). Overnight grown *C. glabrata* cells were harvested and washed prior to re-inoculation into the defined SD (OD_{600} = 0.1). A volume of 100 µl of cell suspension was added to microtitre plate (U-shaped, tissue culture treated) (Becton, Dickinson and Company, USA). The microtitre plate was covered with lid and sealed with parafilm, followed by incubation for 24 h at 37°C. The media was aspirated and the plate was washed three times using 200 µl of PBS, pH 7.4. The plate was placed in an inverted position on a blotting paper to remove residual PBS. The biofilm activity was quantified via XTT 2,3-Bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide reduction assay. A freshly prepared 100 µL mixture of 0.5 g/L XTT (Sigma Aldrich, USA) and 10 mM menadione (Sigma Aldrich, USA) (10000:1, v/v) was added to the washed-biofilm in the microtitre plate. The plate was wrapped with aluminum foil and incubated in dark at 37°C for 3 h. A volume of 80 µL of the supernatant was transferred to a new microtitre plate and the plate was read by using microtitre plate reader (Bio-Tek, USA) at wavelength of 490 nm.

Amphotericin B Susceptibility Assay

The inhibitory concentration of *C. glabrata* BG2 against amphotericin B was determined using the method as described in NCCLS (CLSI) M27-A2 by replacing the RPMI1460 with 0.1% glucose SD. The inhibitory concentration obtained was applied in the modified method from Rodaki et al. (2009) to elucidate the possible role of *SNF3* in contributing to the anti-amphotericin B susceptibility. Briefly, overnight grown *C. glabrata* cells were harvested and washed prior to re-inoculation into the 0.1% glucose SD (OD_{600} = 0.1) and regrown to OD_{600} = 0.5. Cell suspension was added to 1.5 mL centrifuge tube (Axygen, USA) and microtitre plate (U-shaped, tissue culture-treated) (Becton, Dickinson and Company, USA) together with defined concentration of amphotericin B. The centrifuge tube and microtitre plate was covered and sealed with parafilm, followed by incubation for 24 h at 37°C. CFU (colony-forming unit) was determined from cell suspension in centrifuge tube for the calculation of survival percentage. In addition, the plate was read by using microtitre plate reader at wavelength of 600 nm to examine the cell density for the confirmation of *C. glabrata* viability. The survivability percentage of *C. glabrata* was calculated by applying the formula as below:

$$\text{Survival percentage} = \frac{\text{CFU of stressed sample}}{\text{CFU of unstressed control}} \times 100\% \quad (2)$$

Candida-Macrophage Co-culture Assay

The capability of both *C. glabrata* strains to withstand engulfment of macrophage was analyzed as described by Kaur et al. (2006) and Collette et al. (2014) with minor modification. Murine macrophage cells, RAW264.7 (gift from Daud Ahmad Israf Ali, Universiti Putra Malaysia) were maintained and incubated in Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies, USA), supplemented with 10% Fetal Bovine Serum (FBS; Life Technologies, USA) at 37°C/5% CO_2. Prior to the co-culture step, 5×10^5 of RAW264.7 cells were seeded into 6-well plate (Becton, Dickinson and Company, USA) for 24 h at 37°C/5% CO_2. After incubation, the washed cell was counted for the determination of cells number. For the preparation of *C. glabrata* cells, overnight grown *C. glabrata* cells were washed and regrown in fresh YPD (OD_{600} = 0.5). Harvested mid-log phase cells were washed and re-inoculated in fresh DMEM + 10% FBS for desired cell density to match the ratio of 1:1 (effector: target) prior to the co-culturing with RAW264.7 cells prepared. The mixed culture of *C. glabrata* and RAW264.7 was incubated at 37°C/5% CO_2. In order to measure the growth of macrophage engulfed-yeast, non-engulfed yeast cell were washed away with DMEM after 2 h of incubation. The lysates of infected macrophages were scrapped and collected from wells at two time points (2 and 24 h) in ice-cold deionized water and plated on YPD agar. CFUs were determined after incubation of 24 h at 37°C and the growth ratio of engulfed cells were determined by applying the formula below:

$$\text{Growth ratio} = \frac{\text{CFU of 24 h sample}}{\text{CFU of 2 h control}} \times 100\% \quad (3)$$

RNA Extraction

Overnight-cultured *C. glabrata* cells in YPD medium were washed and regrown in fresh YPD (OD_{600} = 0.1) to mid-log phase (OD_{600} = 0.5). The mid-log phase cells were collected, washed and re-suspended in SD (0.01% glucose) and allowed to grow at 37°C for 2 h. The collected cell was washed and RNA extraction was performed based on the described protocol in Yeast Current Protocols in Molecular Biology (Collart and Oliviero, 1993). Verification of the RNA integrity and quality were performed by visualization on 1% Tris-acetate-EDTA gel and NanoPhotometer® (Implen, Germany). RNAs were treated with Maxima H minus first strand cDNA synthesis kit with dsDNase (Thermo Scientific, USA) as described in kit manual with minor modification where RNAs were reverse transcribed with the mixture of Oligo(dT) 18 and random hexamer for the generation of full length transcripts (Resuehr and Spiess, 2003). The RNAs were also reverse transcribed with reaction suspension lacking reverse transcriptase (Non Reverse Transcriptase, NRT)

and without RNA template (Non Template Control, NTC) as controls, respectively. The RNA isolation and subsequent cDNA synthesis were performed in three biological independent experiments.

Quantitative Real-time PCR (qRT-PCR)

Two reference genes were employed as internal controls namely the *ACT1* and *UBC13* (Li et al., 2012) for a more reliable and accurate normalization output. All PCR primers (**Table 2**) were designed to amplify target genes based on the gene sequences sourced from the *Candida* Genome Database (http://www.candidagenome.org/). The PCR efficiency using each set of primers of the respective genes was determined in two independent experiments by running a series of five-fold dilution of *C. glabrata* DNA in MiniOpticon™ Real time PCR (Bio-Rad, USA) machine. The amplification efficiency for each respective gene was determined to be between 90 and 110%. For the expression analysis of the genes, all samples were performed in technical triplicate. The total volume of each reaction was 20 µl where it contained the cDNA template, 500–600 nM primers, 2X SensiFAST SYBR No-ROX (SYBR green) master mix (Bioline, UK) and type-1 ultrapure water (Milipore, USA). The reagents mixture was placed in low-profile white strip tube (Life Technologies, USA) and allowed to amplify in two-step cycling PCR amplification (polymerase activation: 95°C for 2 min, 40 cycles of denaturation: 95°C for 5 s and annealing/extension: 60°C for 30 s). Melting curve analysis was performed to ensure no non-specific PCR products were generated. A NRT and NTC were included for each gene during the qRT-PCR analysis. For post-experimental expression analysis, normalized expression ratios were calculated based on the mathematical equation developed by Pfaffl (2001). Wild type BG2 was chosen as the reference strain (baseline) when interpreting the result for the transcript profiling. Normalized expression ratio calculated was presented in logarithms based ($\log_{10}$).

Statistical Analysis

Statistical analyses were performed using SPSS Statistics (Version 17.0) software. All the experiments were performed at least three times and the data presented are mean of all experiments performed. Error bars represent standard error of the mean (SEM). Statistical significance was assessed by unpaired *t*-test to compare control (wild type) and sample (mutant). The relative expression software tool (REST©) version 2009 (Pfaffl et al., 2002) was employed to test the statistical significance in qRT-PCR analysis.

RESULTS

Loss of *SNF3* Resulted in the Failure of *C. glabrata* to Thrive in Low Glucose Concentration Environments

The inabilities of *SNF3*Δ to grow in low glucose environments were demonstrated in both shaking and static condition (**Figures 1**–**3**). After 10 h of incubation, the growth rate of *SNF3*Δ strain was significantly reduced (p-value < 0.05) in 0.01 and 0.1% glucose environment for respiration preferred-condition (shaking) and in 0.01, 0.1, and 0.2% glucose environment for fermentation preferred-condition (static). However, deletion of *SNF3* did not weaken the growth of *SNF3*Δ strain in higher glucose environment (1 and 2%; **Figures 1**–**3**). These observations highlighted the role of *SNF3* in sustaining the growth of *C. glabrata*, particularly in low glucose

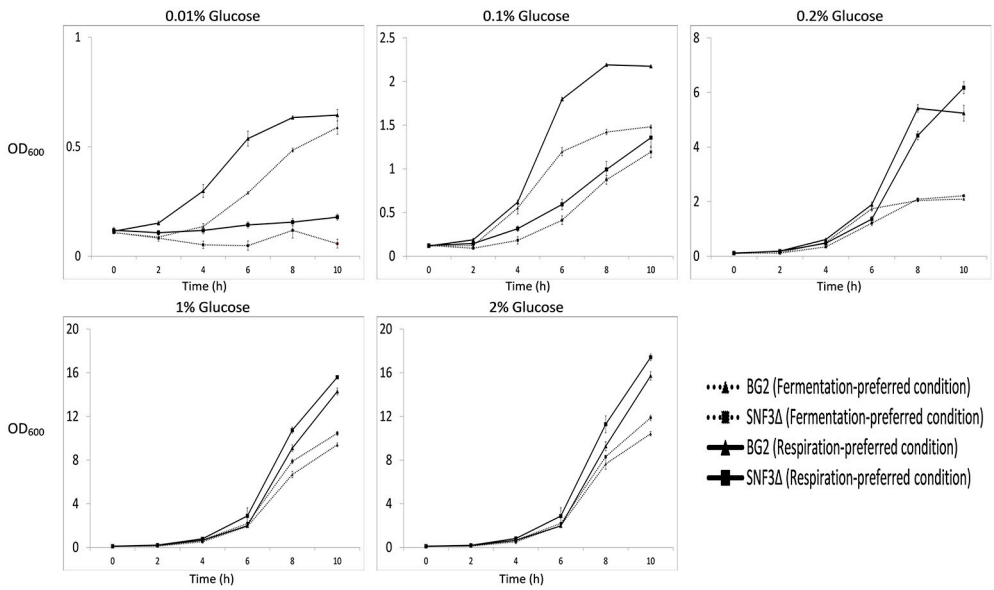

FIGURE 1 | Growth profile of *Candida glabrata* BG2 and *SNF3*Δ in five difference glucose concentrations tested for both fermentation and respiration-preferred condition.

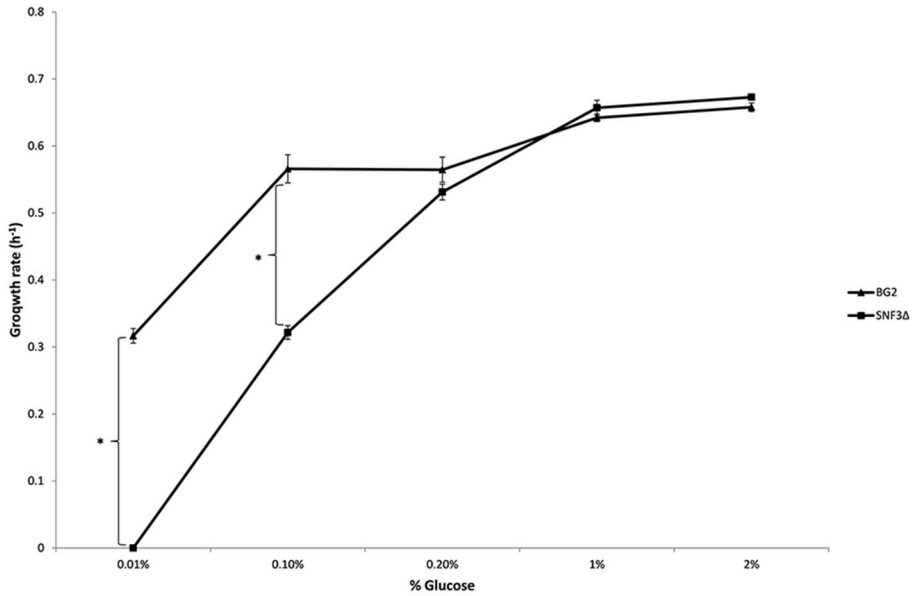

FIGURE 2 | Growth rate of *Candida glabrata* BG2 and *SNF3*Δ in five differences glucose concentrations with respiration-preferred condition. Significant differences (indicated by *) were found between wild type and mutant under low glucose environments: 0.01 and 0.1% (p-value < 0.05).

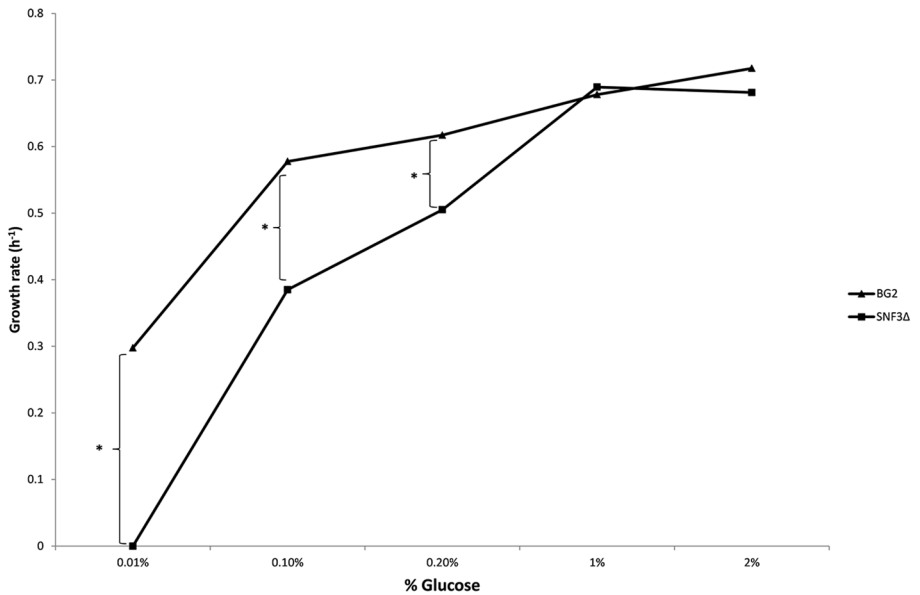

FIGURE 3 | Growth rate of *Candida glabrata* BG2 and *SNF3*Δ in five differences glucose concentrations with fermentation-preferred condition. Significant differences (indicated by *) were found between wild type and mutant under low glucose environments: 0.01, 0.1, and 0.2% (p-value < 0.05).

for both respiration and fermentation-preferred environment. Furthermore, *SNF3* is deemed to be more important in fermentation process where the growth defect of *SNF3*Δ was found to be more severe (extended up to 0.2% glucose) in fermentation-preferred condition. In respect of the data obtained, which suggested the deleterious effect of *SNF3*Δ is seen only in low glucose environment, the subsequent assays including biofilm formation and amphotericin B susceptibility assays were carried out in glucose limited environment (0.01 and 0.1%).

Deletion of *SNF3* Gives No Effect in the Biofilm Formation Capability of *C. glabrata* in Glucose-Limited Environments

Previous study demonstrated the effects of glucose levels in directing *C. albicans* to form biofilm. *Candida albicans* tends to

form biofilm in low glucose environment and lives in planktonic form in higher glucose environment (Uppuluri et al., 2010; Ng et al., 2015b). The sensitivity of *SNF3* in responding to surrounding glucose leads to the thought whether this putative high affinity glucose sensor could contribute in detecting the flow of surrounding glucose and therefore orchestrates the biofilm/planktonic living form of *Candida* species in accordance to the availability of glucose. Result showed *SNF3* did not participate in the biofilm formation of *C. glabrata* in low glucose environment as no significant differences were found between BG2 and *SNF3*Δ in the 0.01 and 0.1% glucose tested, respectively (**Figure 4**).

The Loss of *SNF3* Makes *C. glabrata* More Vulnerable to Amphotericin B Treatment in Low Glucose Concentration Environment

Previous study demonstrated the ability of *Candida* species to withstand antifungal is affected by the type of carbon sources and

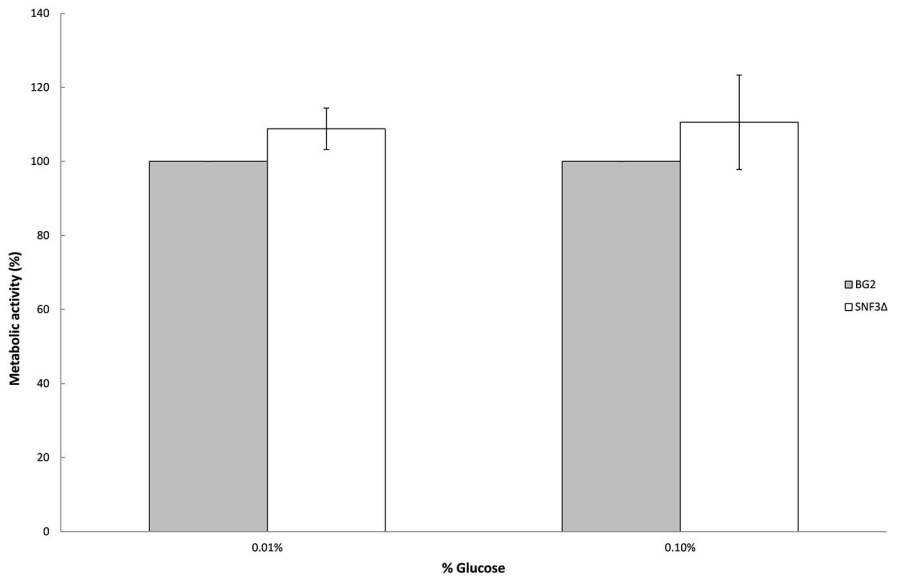

FIGURE 4 | Biofilm formation activity of *Candida glabrata* BG2 and *SNF3*Δ strains under 0.01 and 0.1% glucose concentration. Unpaired *T*-test was carried out for the statistical analysis to examine the significant difference between BG2 and *SNF3*Δ and no significant difference was found.

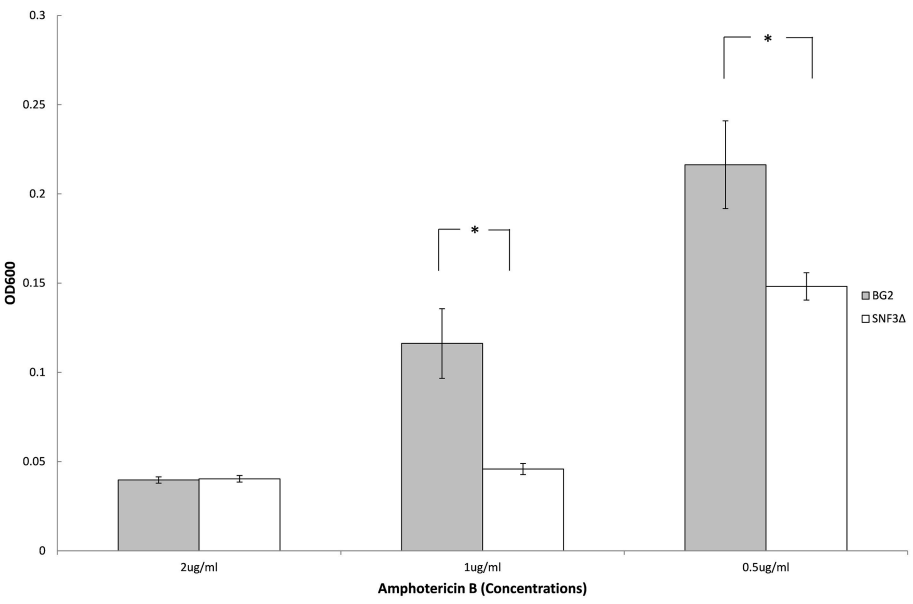

FIGURE 5 | Survivability of *Candida glabrata* BG2 and *SNF3*Δ strains under treatment of three different concentrations of amphotericin B in 0.1% glucose. Unpaired *T*-test was carried out for the statistical analysis to examine the significant differences (indicated by *) between BG2 and *SNF3*Δ (p-value < 0.01).

levels (Ene et al., 2012; Mota et al., 2015; Ng et al., 2015b). With the aim to elucidate further the possible role of *SNF3* in regulating the fitness of *C. glabrata*, the ability of both strains to withstand amphotericin B in low glucose environment was tested. However, the complete retarded growth of *SNF3*Δ in 0.01% glucose leads to the inability in the effort to set up an unstressed control for the calculation of survival percentage. Thus, only 0.1% glucose was tested in this assay. The growth of both wild type and *SNF3*Δ strain were arrested at 2 μg/mL of amphotericin B. The wild type strain was able to resist amphotericin B at 1 μg/mL while complete inhibition was observed in the *SNF3*Δ strain (p-value $<$ 0.01). The wild type showed a better growth in comparison to *SNF3*Δ strain at 0.5 μg/mL amphotericin B (**Figure 5**). These data established the fact that glucose sensing by the *SNF3* gene may contribute to the ability of *C. glabrata* in withstanding the effects of amphotericin B under low glucose environment.

*SNF3*Δ Strain Shows Reduced Growth in Macrophages

The microenvironment in macrophage is always linked to nutrient-limited environment, particularly in glucose availability. In order to validate the possible role of *SNF3* in promoting the fitness of *C. glabrata* under glucose-limited environment, the survivability of macrophage trapped- *C. glabrata* was assayed in an *ex vivo* manner. Results demonstrated a significant reduced growth (p-value $<$ 0.01) of the internalized mutant strain in comparison to wild type strain (**Figure 6**) and this suggests the essential role of *SNF3* in supporting the survivability of *C. glabrata* upon macrophage engulfment.

Deletion of *SNF3* Affects the Expression of Downstream Hexose Transporters *(HXTs)*

There are 11 hexose transporters found in *C. glabrata*. The expressions of these hexose transporters were examined and compared between wild type and *SNF3*Δ strain. Out of 11 hexose transporters, only nine hexose transporters were studied because the nucleotide sequences of putative hexose transporters CAGL0A02211 and CAGL0A02233 were found to be 96% similar while CAGL0A02662 and CAGL0A02640 displayed 100% similarity. The high similarity among these hexose transporters caused the inability in primer design for the expression study of those genes. Out of nine hexose transporters, six of them were affected with the deletion of *SNF3*, where four of them (CAGL0A1804_*HXT1*, CAGL0A01782_*HXT4,* CAGL0A02211/2233_*HXT6/7,* and CAGL0D02662/2640_*HXT2/10*) were down regulated while two (CAGL0A02321_*HXT3* and CAGL0A01826_*HXT5*) were up regulated (**Figure 7**). In addition, deletion of *SNF3* resulted in down-regulation of *STD1*, *YCK1*, and *YCK2*, which serve as the downstream messengers of *SNF3* to modulate the expression of hexose transporters (**Figure 8**). Nevertheless, the expression of *RGT2* was up regulated while expression of *RGT1*, *GRR1,* and *MIG1* did not change significantly with the deletion of *SNF3* (**Figure 8**). These observations suggest the significant role of *SNF3* in regulating the signaling pathway of glucose uptake mechanism.

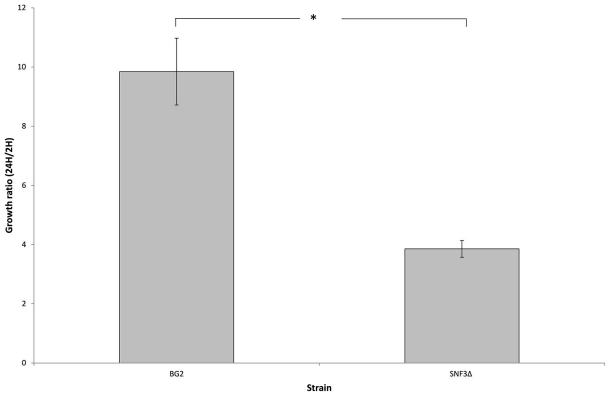

FIGURE 6 | The survival ratio of *Candida glabrata* BG2 and *SNF3*Δ strains recovered from macrophages at 24 h vs. 2 h after co-cultivation. Unpaired *T*-test was carried out for the statistical analysis to examine the significant differences (indicated by *) between BG2 and *SNF3*Δ (p-value $<$ 0.01).

DISCUSSION

Data presented in present study is suggestive of the role of *SNF3* as high affinity glucose sensor in *C. glabrata*, which is essential for it to grow in glucose-limited environment. *SNF3* appeared to be important for the growth of *C. glabrata* in both respiration and fermentation preferred condition with low glucose environments (0.01 and 0.1%) and up to 0.2% glucose in fermentation preferred condition. Brown et al. (2006) demonstrated the deletion of glucose sensor, *HGT4* in *C. albicans* attenuates its ability to grow only in the fermentation-preferred condition and low level of fermentable carbon source (0.2%). Data suggest high affinity glucose sensor appears to be more essential in *C. glabrata* than in *C. albicans*. The dissimilarities observed in both the *Candida* species could be due to the differences in their nature of glucose utilization. *C. glabrata* is identified as Crabtree-positive yeast or aerobic fermenter where, it prefers fermentation over respiration and produces ethanol even there is presence of oxygen (Van Urk et al., 1990), while *C. albicans* is known as Crabtree-negative yeast or respiratory yeast, which prefers respiration whenever there is presence of oxygen. The preferred-fermentation in Crabtree-positive yeast produces only two ATP per glucose, in comparison to 36/38 ATP per glucose produced in respiration. Owing to the preferred-inefficient mode of metabolism, Crabtree-positive yeast is found to exhibit higher glucose consumption rate than Crabtree-negative yeast (Van Urk et al., 1990; Fleck et al., 2011). Therefore, the presence of two specialized glucose sensors in *C. glabrata* (Palma et al., 2009; Ng et al., 2015a) may contribute to the higher glucose uptake sensitivity in order to fulfill its ATP demands and removal of this high affinity glucose sensor lead to detrimental effect on the growth of *C. glabrata* in low glucose environment (**Figures 1–3**). Unlike *C. glabrata*, there is only one high affinity glucose sensor (*HGT4*) found in *C. albicans* (Brown et al., 2006). The capability of *C. albicans* to assimilate both fermentable and non-fermentable carbon sources at the same time suggests *C. albicans* has evolved distinctively to adapt itself by not relying solely on glucose for its growth in hostile

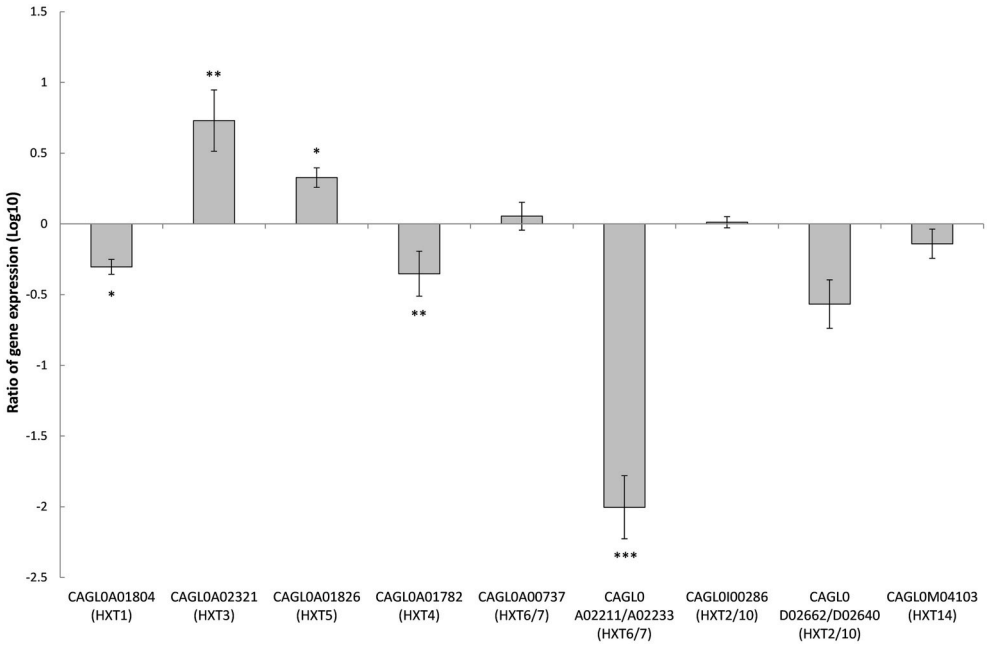

FIGURE 7 | Comparison of expression ratios (Log_{10}) for the *Candida glabrata* hexose transporters (*HXT*s) after the knockout of *SNF3*. $^{*}p < 0.1$, $^{**}p < 0.05$, $^{***}p < 0.01$.

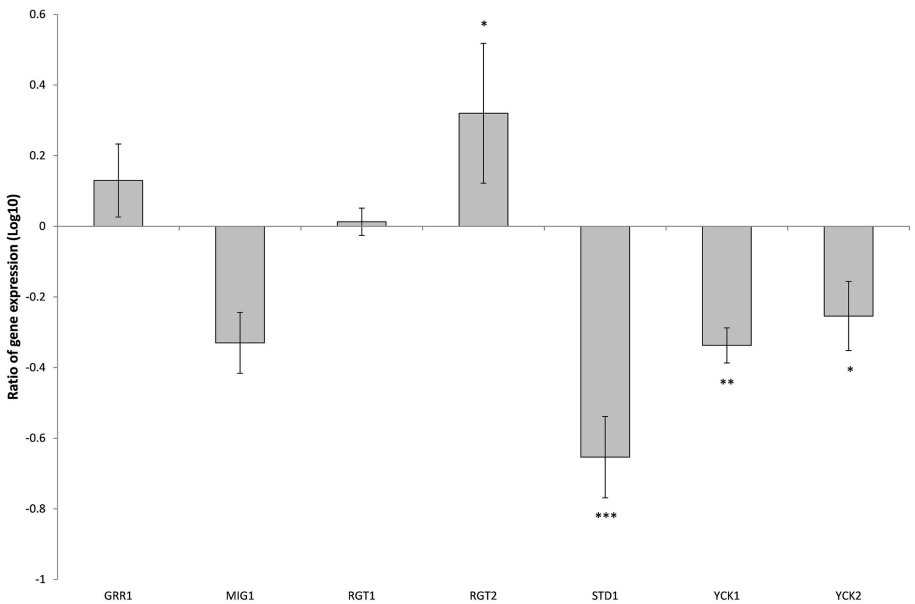

FIGURE 8 | Comparison of expression ratios (Log_{10}) for the *Candida glabrata* Sugar Receptor Repressor (SRR) related genes after the knockout of *SNF3*. $^{*}p < 0.1$, $^{**}p < 0.05$, $^{***}p < 0.01$.

host niche with limited glucose availability (Sandai et al., 2012). Thus, a single glucose sensor is probably sufficient to support the life process of *C. albicans*. However, it is still unclear whether *C. glabrata* is equipped with the same metabolic flexibility. The presence of the two glucose sensors with different affinity in *C. glabrata* similar to the ones found in *S. cerevisiae* suggests it would behave more like *S. cerevisiae*. The baker's yeast is unable to assimilate both fermentable and non-fermentable carbon sources at the same time (Sandai et al., 2012). Further investigation on the carbon metabolic flexibility in *C. glabrata* is warranted as this could provide insight into the metabolic adaptation on the disease progression of this fungal pathogen.

Apart from glucose sensing and uptake mechanism, the expression of glucose sensor gene *HGT4* in *C. albicans* is deemed to be regulated by macrophage engulfment, antifungal mechanism, and biofilm formation activity of yeast (Barker et al., 2004; Liu et al., 2005; Brown et al., 2006). The deletion of *SNF3* indeed diminished the capability of *C. glabrata* to withstand the macrophage challenge and amphotericin B treatment but did not affect its biofilm formation activity. Data presented demonstrated the importance of *SNF3* in supporting the growth of *C. glabrata* under low glucose environment in the growth profiling assay. This observation was extended further to the nutrient-limiting microenvironment of macrophage. Macrophage is critically important in building up an immunological barrier to counter infectious agents through its unique nutritional seal off and oxidative stress to destroy engulfed intruders (Kaur et al., 2006). Previous study demonstrated the capability of *C. glabrata* to perform autophagy for the nutritional scavenging and recycling in order to sustain its growth upon phagocytosis (Roetzer et al., 2010). Data (**Figure 6**) suggests in addition to autophagy mechanism, glucose sourcing and uptake are also important in aiding *C. glabrata* to sustain prolonged phagocytosis. The absence of *SNF3* may result in the inability of *C. glabrata* to absorb sufficient glucose to perform basic physiological function or even to initiate autophagy mechanism and therefore lead to the diminished growth. On the other hand, deletion of *SNF3* did not affect the capability of *C. glabrata* to form biofilm under low glucose condition as expected. Data presented suggest there is probably another sensor in *C. glabrata* but not *SNF3* that assists in detecting the nutrient flow in environment. Further investigation is warranted for a clearer picture on how this pathogenic yeast senses and alters its lifestyle to adapt itself in such environment where abrupt change of nutrients takes place.

The transcriptional analysis on selected hexose transporters (*HXT*s) revealed that almost half (four out of nine) hexose transporters were down regulated with the removal of *SNF3*, together with the down-regulation of downstream casein kinase (*YCK1* and *YCK2*) and *STD1* (**Figures 7**, **8**). The disruption of the signaling pathway for high affinity hexose transporters explained the compromised fitness of *C. glabrata* under low glucose environment (**Figures 1**–**3**) as this triggers the failure in transporting sufficient glucose to support its growth. In addition, data presented concurs with the view that the expression

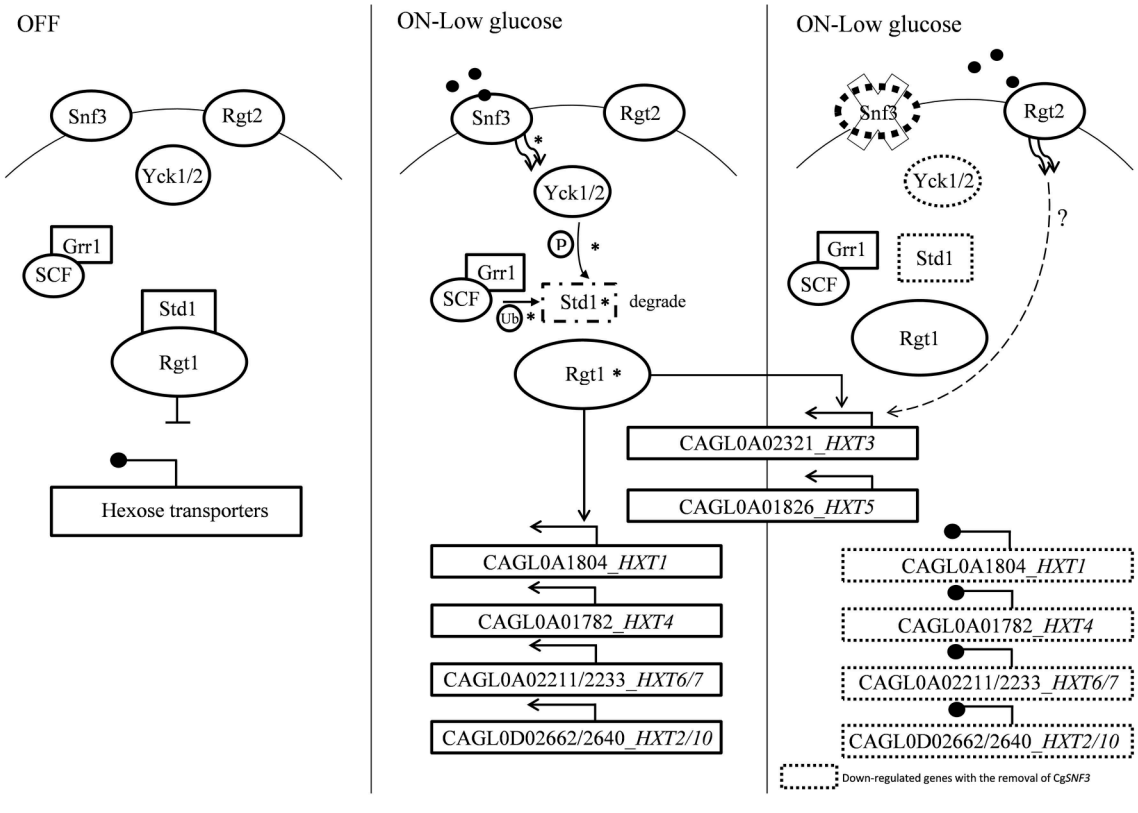

FIGURE 9 | A model of glucose sensing in *Candida glabrata* under low glucose environment. The part of the pathway labeled with asteisks inferred from published works done on *S. cerevisiae* (Rolland et al., 2002; Santangelo, 2006; Gancedo, 2008). Hexose transporters are repressed by Std1-bounded-Rgt1 when there is no stimulation from glucose sensor located in the cell membrane. Presence of low concentration of glucose induced signal from high affinity glucose sensor, Snf3 to the phosphorylation of Std1 by the Yck kinase. Phosphorylated Std1 is then subjected to the SCF^{Grr1} —mediated ubiquitination and degraded by proteasome. Degraded Std1 results in the activation of Rgt1, which then leads to derepression of downstream hexose transporters. Deletion of *SNF3* gives rise to the disruption of hexose transporters expression and glucose uptake mechanism, therefore leads to the interference of *Candida glabrata* fitness under low glucose environment. However, the possible interaction between *RGT2* and downstream *HXT3*/*HXT5* (labeled with dotted line) is remains unclear and requires further investigation.

of transcription regulator, *RGT1* is regulated by the glucose concentration but not affected by the signal generated from glucose sensors (Özcan and Johnston, 1999) as the expression of *RGT1* remain unchanged even with the missing signal from *SNF3*. However, the direct regulation of glucose concentration on the expression level of *RGT1* is still not fully understood. Nonetheless, the shutting down of these four hexose transporters did not diminish the growth of *C. glabrata* completely as there are two other hexose transporters that were still actively expressed namely the CAGL0A0232 (*HXT3*) and CAGL0A01826 (*HXT5*), together with the up regulation of *RGT2*. This could be a compensatory mechanism used by *C. glabrata* to compensate the loss of *SNF3* with the activation of *RGT2*. Notably, these *HXT3* and *HXT5* were regarded as key hexose transporters for *C. glabrata* in low glucose environment from our previous work (Ng et al., 2015a). Nevertheless, this compensatory mechanism still failed to salvage *C. glabrata* from glucose uptake crisis as the growth defect is still significant (p-value < 0.05; **Figures 1–3**) in the absence of *SNF3*. We opine the compensation of glucose uptake by *HXT3* and *HXT5* is insufficient to provide the amount of glucose needed and this highlights the importance of four other repressed *HXTs* in supporting the growth of *C. glabrata* under low glucose environment. In addition, the capability of *RGT2* to induce expression of *HXT3* and *HXT5* supports the view that *SNF3* and *RGT2* have separate but overlapping functions. Özcan et al. (1998) demonstrated the capability of *SNF3* in *S. cerevisiae* to restore the expression of *HXT1* (supposedly induce by *RGT2*) by 64%, in a *RGT2* mutant. This observation suggests a complex and interconnected regulatory pathway of glucose sensing and uptake mechanism in yeast. From the data obtained, a model of glucose sensing in *C. glabrata* through the modulation of *SNF3* is illustrated based on the understanding of the homolog and the inferred glucose sensing mechanism in *S. cerevisiae* (**Figure 9**). Further work is warranted, as the compensatory mechanism proposed here is still not fully deciphered. In addition, effort to study the transcriptional profile of the highly homologous *HXT*s genes using other approach should be carried out. With more complete information on the role of each hexose transporters present in *C. glabrata*, a clearer and more comprehensive picture on the role of *SNF3* in SRR pathway will be achieved.

In conclusion, our results thus far suggest the important role of *SNF3* in *C. glabrata* in the expression of hexose transporters under low glucose environment. We also highlight the vital role of *SNF3* in promoting *C. glabrata* growth, resistant toward amphotericin B under glucose limited environment and macrophage engulfment by governing the glucose uptake mechanism. These results suggest *SNF3* could be a potential factor for *C. glabrata* to survive and thrive in host niches with limited glucose availability. Further investigation such as RNA-sequencing and comparative proteomic study could be carried out for the analysis of global transcriptomes and validation of the obtained result. Owing to the essential role of glucose on metabolic network of organism, further exploration on the glucose sensing mechanism highlighted in current study could contribute in the discovery of novel drug target and help in controlling the emergence of *C. glabrata*.

AUTHOR CONTRIBUTIONS

TS, LT, and DS designed the experiments. TS, SY, and PR carried out the experiments. TS analyzed and interpreted the data. TS and LT wrote the manuscript with critical revision for important intellectual content from MD, DS, and PP.

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REVIEW
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Optimizing Outcomes in Immunocompromised Hosts: Understanding the Role of Immunotherapy in Invasive Fungal Diseases

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A major global concern is the emergence and spread of systemic life-threatening fungal infections in critically ill patients. The increase in invasive fungal infections, caused most commonly by *Candida* and *Aspergillus* species, occurs in patients with impaired defenses due to a number of reasons such as underlying disease, the use of chemotherapeutic and immunosuppressive agents, broad-spectrum antibiotics, prosthetic devices and grafts, burns, neutropenia and HIV infection. The high morbidity and mortality associated with these infections is compounded by the limited therapeutic options and the emergence of drug resistant fungi. Hence, creative approaches to bridge the significant gap in antifungal drug development needs to be explored. Here, we review the potential anti-fungal targets for patient-centered therapies and immune-enhancing strategies for the prevention and treatment of invasive fungal diseases.

Keywords: invasive fungal infections, immunocompromised, immune regulation, immune enhancement, cytokines

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INTRODUCTION

From among more than a million species of fungi present in nature, only a few 100 of them are capable of causing infections in humans (O'Brien et al., 2005). Of these, only a handful can cause diseases in healthy people, which is mostly superficial in nature (Kohler et al., 2015; LIFE at www.life-worldwide.org). Invasive fungal diseases (IFD) usually occur in susceptible individuals who are immunocompromised due to serious illnesses such as leukemia, neutropenia, AIDS, etc. In addition, medical advances have created vulnerable populations such as patients undergoing chemotherapy, solid and hematopoietic stem cell transplantation (HSCT), complex surgeries, immunosuppressive therapies for auto-immune and auto-inflammatory diseases, antibiotic therapies and treatment in intensive care units.

The major fungi responsible for these invasive infections, which kill about one and a half million people every year, are *Candida*, *Aspergillus*, and *Zygomycetes* species. Invasive candidiasis is the fourth and sixth most common nosocomial infection in US and Europe respectively with a high mortality rate ranging from 36 to 63% (Wisplinghoff et al., 2004; Brown et al., 2012; Rodrigues et al., 2014). The risk factors for candidemia include prior antibiotic usage, abdominal surgery, *Candida* colonization, central lines and parenteral nutrition (Wey et al., 1989; Blumberg et al., 2001) *Aspergillus* is a ubiquitous filamentous saprophytic mold whose conidia are dispersed in the air. Like the Zygomycetes, these molds cause several invasive diseases in hosts with markedly

suppressed immunity and have a mortality rate in excess of 50–60% despite treatment (Herbrecht et al., 2002; Neofytos et al., 2009).

Such is the concern of the impact of IFD in immunocompromised patients. This is despite the ever wider availability of anti-microbials beyond the conventional amphotericin-B based preparations and in the recent decade especially, the newer generation and classes of anti-fungals like voriconazole, posaconazole, and isavuconazole (of the azole family) and the echinocandins (caspofungin, anidulafungin, and micafungin). Some of the reasons for the high mortality are the difficulties in the early and correct diagnosis of invasive fungal infections (de Pauw and Picazo, 2008; Erjavec et al., 2009) as well as drug resistance profiles among specific fungal pathogens (Perlin, 2007; Verweij et al., 2007; Xie et al., 2014). The main reason for the poor outcomes from invasive disease nonetheless, is the incapacity of the patient's compromised immune system to respond appropriately to the invading pathogen despite the presence of antimicrobials.

The response to such a challenge faced by the clinician at the bedside has led to exploration of novel therapeutic modalities beyond conventional antimicrobials; specifically, the manipulation and augmentation of the host immune response in the face of IFD. Through understanding how the immune system can detect the fungi, immunotherapeutic strategies may be formulated as adjuncts in the management of IFD.

IMMUNE RECOGNITION AND RESPONSE BY THE HOST

The susceptibility and outcome of fungal infections depend on two main factors: the pathogen and the host. Pathogen factors may include the dose of the infecting fungi and its virulence. The efficacy of the immune response and the degree of the immune suppression in the patient are the major host determinants. The host defense capacity to fungal infection range from the protective mechanisms provided by skin, mucosa and innate immunity to the humoral response and adaptive immunity (Mueller-Loebnitz et al., 2013). The innate immune system despite its lack of specificity has been considered to bear significant importance in the defense mechanism against fungi. Monocytes, macrophages, neutrophils, and natural killer (NK) cells effect anti-fungal capabilities through phagocytosis, and directed pathogen killing. The fungal cell wall is the first structure encountered by host cells. Fungal cell wall is made up of various polysaccharides that have immune activating and modulatory properties. These pathogen associated molecular patterns (PAMPs); such as alpha and beta glucans, chitins, mannans, β- 1, 2-oligomannosides and galactomannan of varying constitutions in the cell wall of various fungi allow recognition by the innate immune cells; mainly monocytes, macrophages, dendritic cells (DCs) and endothelial cells (Netea et al., 2008). Pathogen recognition receptors (PRRs), a protein family of cellular receptors that mediate recognition of microbial pathogens and subsequent inflammatory response are present on the surface of DCs and macrophages (Hamad, 2012).

IMMUNE RECOGNITION

One of the main PRRs are the Toll-like receptors (TLRs), whose role in the recognition of *Aspergillus* and *Candida* has been well documented especially, TLR2, TLR4, and TLR9 (Pasare and Medzhitov, 2005; Takeda and Akira, 2005; Goodridge and Underhill, 2008; Uematsu and Akira, 2008; Loures et al., 2010). The PRRs mounted on the host cells recognize specific fungi cell wall moieties of polysaccharide origin, namely the PAMPs. Fungal PAMPs for cell surface TLRs have been identified mainly through studies involving fungi with cell wall mutations. For instance, fungal phospholipomannans (PLMs), linear beta-1, 2-oligomannosides and glucuronoxylomannan (GXM) are known to bind with TLR2, while, O-linked mannans have been shown to activate TLR4 (Netea et al., 2006). Apart from cell surface PAMPs, nucleic acids released from the fungi in the phagosome also stimulate TLRs and modulate the host responses. TLR 9 activation occurs through interaction of genomic DNA whereas double stranded and single stranded RNA stimulate TLR3 and TLR7 respectively (Bourgeois and Kuchler, 2012).

Recognition of fungal antigen by TLR4 leads to pro-inflammatory cytokine production by NF-κB activation mediated by the adaptor protein Myd88. Bellocchio et al. (2004) supported that TLR4-mediated pro-inflammatory effects are protective against invasive aspergillosis by showing increased susceptibility of $TLR4^{-/-}$ mice to *Aspergillus fumigatus* infection. Mutation of Asp299Gly in TLR4 is associated with increased incidence of pulmonary aspergillosis (Carvalho et al., 2008). It was subsequently demonstrated that HSCT patients in possession of the D299G/T399I haplotype were at higher risks of invasive aspergillosis (Bochud et al., 2008). TLR2 was shown to influence early recruitment and killing capacity of neutrophils against *A. fumigatus* (Bellocchio et al., 2004). $TLR2^{-/-}$ mice infected intraperitoneally with *Candida albicans* were found to have lesser recruitment of neutrophils and monocytes (Tessarolli et al., 2010). However, $TLR2^{-/-}$ mice had decreased fungal burden compared to the control mice accompanied by increased production of interleukin 12 (IL12) and decreased production of IL10. The role of TLR2 is still under debate as studies based on targeted patient genotype of TLR2 did not reveal enhanced susceptibility. $TLR9^{-/-}$ mice are reported to have higher fungal burden than control mice and found to be producing more IL10 and lower IL12 which is in contrast to findings in $TLR2^{-/-}$ mice. Mutations in TLR9 are associated with increased incidence of allergic bronchopulmonary aspergillosis (Carvalho et al., 2008; Mezger et al., 2010). An association of invasive aspergillosis was also seen in patients undergoing HSCT with SNPs in TLR1 and TLR6 (Kesh et al., 2005). It should be noted that TLR response may vary depending on fungal species and morphotype, and route of infection as well as the specific fungal infection (Romani, 2011).

Another family of PRRs that are important in the recognition of fungal PAMPs are C-type lectin receptors, otherwise known as CLRs. β-glucans present on the cell walls of *Candida* and *Aspergillus* species activate Dectin-1 receptor, while Dectin-2, and Dectin-3 mainly recognize hyphal α-mannan (Saijo et al., 2010). N-mannan is recognized by mannose receptor while galectin-3

binds to β-mannans. Fungal N- linked mannans also bind to DC-SIGN and mannose binding lectin (MBL) receptors present on phagocytes (Becker et al., 2015).

Dectin-1 is the most widely known CLR associated with fungal recognition. Dectin-1 recognition of β-glucan activates canonical and non-canonical NF-κB activation by two pathways, Syk-CARD9 and RAF pathways, resulting in increase in the pro-inflammatory cytokine production. Stimulation of Dectin-1 also increases IL1β and IL18 production through NLRP3 inflammasome pathway. Dectin-1 also collaborates with TLR2 to trigger pro-inflammatory cytokine production upon recognition of *Candida albicans* and zymosan. Dectin-1 deficient and CARD 9 deficient mice have predisposition to *Candida* infections (Ferwerda et al., 2009; Drewniak et al., 2013). Dectin-2 pairs with FcRγ to induce pro-inflammatory cytokine release. Dectin-1 plays an important role in human fungal infections too. It is evident from the polymorphism Y238X noticed in a Dutch family whose members were subject to recurrent vulvovaginal candidiasis and/or onychomycosis, while increased oral and gastrointestinal colonization of *Candida* was observed in HSCT recipients. In addition, it was noticed that there were defects in the expression of Dectin-1 and β-glucan recognition by phagocytes coupled with decrease in the production of cytokines, especially IL17 (Ferwerda et al., 2009; Plantinga et al., 2009). Similarly CARD9$^{-/-}$ patients show increased susceptibility to chronic mucocutaneous candidiasis and reduced Th17 cells (Glocker et al., 2009). MINCLE, which is mainly expressed by macrophages, also induce NF-κB activation through Syk-CARD9 signaling. Mannose receptors are involved in the phagocytosis of un-opsonized *Candida* yeasts. Mannose receptor interacts with galectin-3, a PRR which recognizes carbohydrate moieties on fungal cell wall, to induce TNFα production (Esteban et al., 2011; Kawai and Akira, 2011).

Both *Candida* and *Aspergillus* also trigger an immune response through activation of the inflammasome—most well described through NLRP3 and caspase-1 activation, with the involvement of the tyrosine kinase Syk and Dectin-1 (Gross et al., 2009; Said-Sadier et al., 2010). The non-canonical caspase-8 pathway is also implicated in the context of *Candida* (Gringhuis et al., 2012). Both result in the cleaving and production of IL1β, a pivotal mediator of inflammatory response together with interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα). The invocation of a "pro-inflammatory" response necessitates a "counter-regulatory" component which is maintained by IL4 and IL10 and more recently, possibly through the inhibitory group of NLR (nucleotide-binding domain, leucine-rich repeat containing) proteins (Ting et al., 2010). It is believed that it is in the context of such a conventional paradigm of a balance between a "pro- and anti-inflammatory milieu" that host susceptibility and outcome of an IFD episode may be determined (Chai et al., 2011).

IMMUNE REGULATION

Role of Neutrophils

The state of neutropenia is a well-established risk factor for invasive aspergillosis (Marr et al., 2002). Neutrophils, being the primary effector cells of innate immune system, efficiently and rapidly kill fungi by various mechanisms. Neutrophils are capable of recognizing fungi by TLR2, TLR4, Dectin-1, and complement receptors such as CR1 and CR3 (Braem et al., 2015). MAP kinase signaling is reported to mediate neutrophil activation, especially ERK signaling pathway, since inhibition of ERK signaling pathway abolishes *C. albicans* induced neutrophil migration (Wozniok et al., 2008). Once activated, neutrophils are able to release neutrophil extracellular traps as well as an array of cytokines and chemokines. Neutrophils recruitment, activation and survival in inflammatory sites are affected by Th17 controlled pathway in fungal infections. Neutrophils are also the source of pattern recognition molecule, pentraxin 3 (PTX3) which forms complexes on the conidial surface of the fungus and acts as an opsonin, enhancing recognition and phagocytosis of conidia through mechanisms that depend on Fcγ receptor, CD11b and complement (Mantovani et al., 2011; Cunha et al., 2014).

Role of Dendritic Cells/Monocytes/ Macrophages

Dendritic cells serve the bridge between innate and adaptive immunity since they can present antigen to T cells, activate both innate and adaptive immune system by release of cytokines and chemokines. DCs can recognize fungal pathogens by the receptors such as Dectin-1, TLR2, and TLR4. Production of CCL20 as well as PTX3 increased with the activation of DCs (Mezger et al., 2008). DCs also mature after phagocytosis of fungal cells and promote the differentiation of naive T cells to CD4^{+} T cells which are essential for antifungal defense. *Aspergillus* conidia and hyphae induce NF-κB translocation and release of proinflammatory cytokine TNFα, and MIP2 in TLR2 and TLR4 dependent manner via adaptor protein Myd88.

Monocytes are macrophage and DC precursors; they serve as phagocytes as well as antigen presenting cells. Monocytes produce CCL20 which activates neutrophils, monocytes and naive T cells. Alveolar macrophages destroy *Aspergillus* conidia via non-oxidative mechanisms. The activity of macrophages can be enhanced by GM-CSF or IFNγ (Mueller-Loebnitz et al., 2013).

IMMUNE RESISTANCE VS IMMUNE TOLERANCE

T cells act as the immune modulators and master effectors in the immune response against fungal pathogens. Conventionally, Th1 response is associated with TLR4 signaling resulting in secretion of IFNγ and TNFα (for protection against fungal pathogen), while Th2 response is associated with TLR2 signaling resulting in the production of anti-inflammatory cytokines (IL4 and IL10) to regulate the inflammatory response (which unfortunately leads to more susceptibility against fungal infection). Th17 cells have been increasingly recognized to serve one of the central roles in the anti-*Candida* response especially the mucosal immunity. Th17 has long been attributed to autoimmune diseases while defective Th17 response results in mucocutaneous candidiasis in patients with primary immunodeficiencies (Zelante et al., 2009). In fungal infections, Th17 activation occurs through Syk-CARD9, Myd88 and mannose receptor signaling pathways in DCs and macrophages (Romani, 2011). Activation of IL17 results

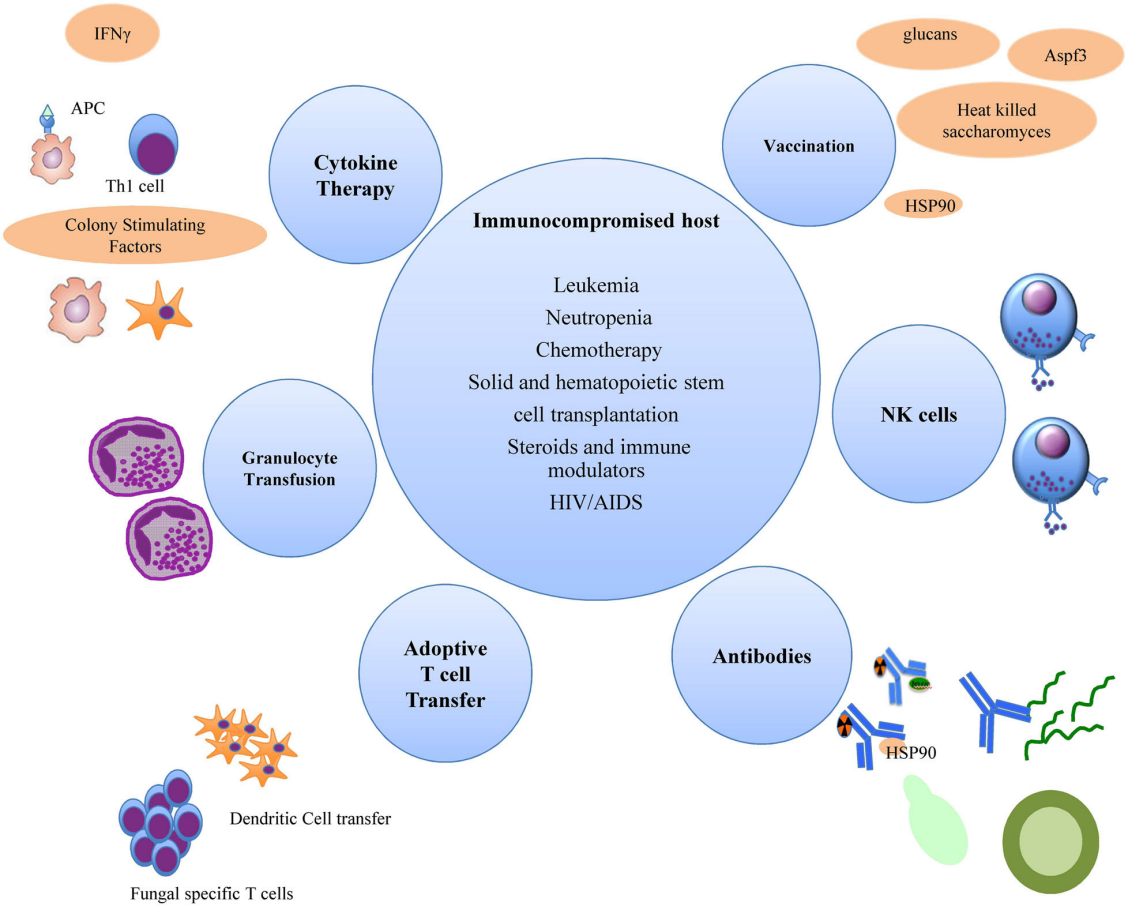

FIGURE 1 | Immune enhancement strategies for invasive fungal infections in immunocompromised hosts.

in the recruitment of neutrophils, defensins and ultimately results in inflammation. However, IL17 activation is also associated with high inflammatory pathology and inhibitory effects on the IFNγ related activation of indolamine 2, 3-dioxygenase (IDO) that is important for immune tolerance function (Romani and Puccetti, 2008). *Candida albicans* is known to dampen Th17 response resulting in chronic inflammation due to the impairment of IL17 dependent neutrophil recruitment leading to fungal persistence and immune dysregulation (Cheng et al., 2010).

While inflammation and immune response is necessary to eliminate the fungus, it is also important to limit the collateral damage to tissue and restore homeostasis to the environment. IL10, a major suppressive cytokine produced by $CD4^+$ T regulatory cells plays an important role in keeping inflammation under control. However, the delicate balance of IL10/IFNγ needs to be in check since high level of IL10 suppresses the activity of IFNγ which provides the main Th1 defense against fungal infections. IDO which is a product of tryptophan metabolism is also increasingly recognized as the master regulator of immune resistance and tolerance since it can induce T regulatory cells and inhibit Th17. IDO and kynurenines balance immune tolerance and resistance by providing adequate elimination of fungal pathogen while preventing the unacceptable level of inflammation and allergy (Zelante et al., 2009).

IMMUNE ENHANCEMENT STRATEGIES

The increased understanding of anti-fungal host responses has facilitated novel approaches into molecular and cell-based immunotherapeutics for invasive fungal infections (**Figure 1**). Notably, the major protective host response against fungi is the effective induction of Th1 and IFNγ responses, which in turn, activates effector phagocytic cells that kill the fungi. A cautionary note, however, is that this inflammatory response needs to be appropriately regulated or curbed when the pathogen or stimulatory ligand is contained, to minimize progression into a chronic inflammatory state which may induce collateral tissue damage.

Cytokine Therapy

The use of recombinant cytokines such as human granulocyte macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF) and interferon-gamma (IFNγ) have been explored as immune enhancing agents. GM-CSF, G-CSF,

and M-CSF belong to the family of hematopoietic cytokines. They stimulate the proliferation of granulocyte and/or macrophage progenitor cells, induce differentiation and maturation, and stimulate functional activity of mature hematopoietic cells (Pikman and Ben-Ami, 2012). GM-CSF alone or in combination with IFN-γ has been shown to enhance the fungicidal activity of innate phagocytic cells *in vitro* and *in vivo*. GM-CSF has been shown to preferentially enhance both the numbers and activity of type 1 DCs and cause upregulation of macrophage dectin-1 expression (Willment et al., 2003; Van de Veerdonk et al., 2010). Human M-CSF enhanced the activity of phagocytic cells and prolonged survival alone or in combination with amphotericin B in immunosuppressed mice with systemic *Candida* infection (Kuhara et al., 2000). Similarly, M-CSF when added to standard antifungal treatment of 46 stem cell transplantation recipients with progressive fungal infections showed better overall survival rates (Nemunaitis et al., 1993).

G-CSF is widely used in clinical practice during chemotherapy induced neutropenia. While G-CSF clearly reduces neutropenic days and neutropenia-related hospitalization, its efficacy in clinical outcomes including infection and mortality rates remain less clear (Smith et al., 2006). In a review of 925 mucormycosis cases, 15 of 18 patients showed favorable clinical response when given G-CSF adjunctive therapy (Roden et al., 2005). Clinical data on the use of GM-CSF as adjuvant antifungal therapy are scarce. Few case reports or small patient series with drug-refractory invasive aspergillosis infection have been published but provide limited information. Recently, a retrospective assessment of 66 patients was performed in whom GM-CSF was given during antifungal therapy to high-risk cancer patients and stem cell transplant recipients with IFD. A complete or partial response occurred in more than half of the patients treated with GM-CSF despite recent treatment with antineoplastic therapy and presence of other predictors of poor outcomes (Safdar et al., 2013). Further prospective studies to assess CSFs efficacy in the treatment of established fungal disease are needed.

IFNγ, produced by T and NK cells, increases the cytotoxic capacity of antigen presenting cells and intracellular killing. In a recent prospective case series, eight patients with invasive *Candida* and/or *Aspergillus* infections were treated with recombinant IFN-γ for 2 weeks in addition to standard antifungal therapy. Recombinant IFN-γ treatment in patients with invasive *Candida* and/or *Aspergillus* infections partially restored immune function, as characterized by an increased HLA-DR expression in those patients and an enhanced production of pro-inflammatory cytokines involved in antifungal defense (Delsing et al., 2014). IFNγ is also used in the treatment of recalcitrant aspergillosis (Kelleher et al., 2006; Bandera et al., 2008; Estrada et al., 2012). Further large-scale clinical studies to assess the potential clinical benefit of IFNγ is needed, but the cost of the drug remains a major concern.

Preclinical trials have assessed other pro-inflammatory cytokines that upregulate the antifungal Th1 response such as IL12, IL15, and TNFα as candidate adjuvants. IL12 is required for *Candida*-induced differentiation of Th1 cells *in vivo* (Romani et al., 1997) and for the antifungal activity of monocytes against *A. fumigatus* hyphae *in vitro* (Roilides et al., 1999). The usefulness of IL12 as immune enhancer is controversial. Invasive mold infections were reported in two autologous stem cell transplantation recipients treated with IL12 (Toren et al., 1997), raising concern that IL12 may paradoxically provoke an immune flare to fungal pathogens.

IL15 is also a potential new drug candidate. This cytokine, shares biological activities with IL2, in enhancing antifungal granulocyte activity in cell cultures (Vazquez et al., 1998; Winn et al., 2003). Neutralization of TNFα, a signature cytokine of Th1 cells, increases the susceptibility of mice to invasive aspergillosis, whereas intratracheal instillation of TNFα agonist peptides confers protection against *A. fumigatus* conidia (Mehrad et al., 1999). Further preclinical investigation is required not only for these cytokines, but also for IL18 and IL36 belonging to the interleukin 1 family (Gresnigt et al., 2013; Ketelut-Carneiro et al., 2015).

Granulocyte Transfusion

Transfusion of granulocytes from healthy donors has been used anecdotally for immune enhancement in patients with neutropenia who suffer from invasive fungal infections. Earlier attempts were beset by the lower yield and quality of granulocytes recovered from steroid treated donors. However, with advances in apheresis methods, better sedimenting agents and the recent use of recombinant cytokines like G-CSF and IFN-γ1b in addition to steroids, the yield and quality of leukocytes from healthy donors have improved.

The efficacy of granulocyte transfusion has been shown by the increased survival rates following its use in the treatment of cancer patients with candidemia (Price et al., 2000). In an uncontrolled prospective study of 23 patients treated for IFD with granulocyte transfusion, no recurrent infection was observed (Mousset et al., 2005). However, in a Phase III randomized trial of 74 patients with febrile neutropenia, 55 of whom had IFD and 39 had received stem cell transplantation, there was no clear effect of granulocyte transfusion on survival up to day 100 (Seidel et al., 2008). Though major randomized trials are lacking for patients with invasive aspergillosis and mucormycosis, good clinical efficacy and safety using appropriate granulocytes is evident through various small case series and case reports (Dignani et al., 1997; Illerhaus et al., 2002; Slavin et al., 2002; Safdar et al., 2006). Therefore, the use of granulocyte transfusions in patients with severe neutropenia and uncontrolled infection, in spite of appropriate antifungal therapy might be considered as a potential life-saving treatment option.

Antibodies

The era of antibody-based therapy for invasive fungal infections dawned with the discovery of protective monoclonal antibodies (mAbs) against the capsular polysaccharide of *Cryptococcus neoformans* (Dromer et al., 1987). Subsequently, protective antibodies against *Candida albicans* (Han and Cutler, 1995; Moragues et al., 2003), *Aspergillus fumigatus* (Chaturvedi et al., 2005) and other fungi were elucidated.

Two antifungal mAbs have been evaluated in clinical trials. 18B7, a mAb against the capsular polysaccharide of C. neoformans was found to be safe in a Phase I study (Larsen et al., 2005) but there is a lack of efficacy data. Efungumab (Mycograb) is

a genetically engineered human recombinant antibody against fungal heat shock protein 90. HSP90 is an immunodominant antigen of the *Candida* cell wall and is required for its survival. In preclinical studies, Mycograb showed activity against a wide range of *Candida* species and synergized with antifungal drugs (Matthews et al., 2003; Hodgetts et al., 2008). But the role of Mycograb at the bedside remains still controversial. Results of a double-blind clinical trial in 117 patients with invasive candidiasis receiving liposomal Amphotericin B with or without Mycograb, showed that by day 10, the patient group receiving Mycograb combination (84 vs 48%; $p < 0.001$) had complete response with more rapid clearance of fungal cultures and reduced *Candida*-attributable mortality rate (Pachl et al., 2006). However, due to methodological and safety issues (Herbrecht et al., 2006), the drug has not gained licensure yet.

On a similar note, monoclonal antibodies mAb C7 and mAb A9, against *Candida* cell wall mannoprotein and *A. fumigatus* cell wall glycoprotein respectively, exhibit direct fungicidal activity (Moragues et al., 2003; Chaturvedi et al., 2005) with reduced fungal burden and increased survival rate in murine models of invasive infection.

Killer anti-idiotypic antibodies, which mimic broad spectrum antimicrobial peptides have been developed. These antibodies, upon intranasal administration to immunosuppressed mice with invasive aspergillosis have resulted in cure and long-term survival (Cenci et al., 2002).

Radioimmunotherapy is another novel antibody-based concept, whereby radiolabeled antibodies that recognize fungal antigens are used to deliver microbicidal radiation with less systemic toxicity (Bryan et al., 2010). It is hoped that radiolabeled mAbs that bind antigens shared by many pathogenic fungi, such as HSP60 and β1, 3 glucan, may act as adjuncts in tandem with conventional antifungals (Bryan et al., 2012).

Vaccination

Antifungal vaccines is an area that has drawn increasing interest and research in recent years. The effective usage of fungal vaccines is limited in the immunocompromised hosts as they not only tend to mount weak protective responses to vaccines but are also at risk from live attenuated formulations. Hence fungal vaccines are often based on standardized cellular subunits which require an adjuvant to induce protective immunity. Heat shock proteins may serve as powerful adjuvants while the immune response may be enhanced by mannosylation of antigens (Spellberg, 2011). Protective immunity arises from both T-cell responses, specifically Th1 and/or Th17 (Wuthrich et al., 2011) and antibody responses.

Preclinical evaluation of vaccines to a number of important fungal pathogens have been performed and at least two have been subject to Phase I clinical trials (Pikman and Ben-Ami, 2012). Universal fungal vaccines may be on the horizon with a conjugate vaccine that evokes antibodies to β-glucans offering cross-protection against three major fungal pathogens: *C. albicans*, *A. fumigatus*, and *C. neoformans* (Torosantucci et al., 2009). Another promising panfungal vaccine preparation originates from heat-killed *Saccharomyces* and is found to confer protection against *Aspergillus*, *Coccidioides*, and *Candida* infections (Stevens et al., 2011).

Though animal studies with crude *A. fumigatus* antigens are promising, the ideal dose that can be safely administered to humans is not well understood (Stevens, 2004). Vaccination of mice with a distinct *Aspergillus* antigen Aspf 3 prior to immunosuppression was shown to confer protection against subsequent inhalational challenge with *A. fumigatus* (Ito et al., 2006). It was shown that immunization confers cellular rather than humoral immunity since naive mice were protected from invasive aspergillosis by passive transfer of $CD4^+$ cells rather than anti-Aspf 3 antibodies from immunized mice (Diaz-Arevalo et al., 2011). Additional vaccine candidates include secreted protein Pep1p and anchored proteins Gel1p and Crf1p (Bozza et al., 2009) of which, Crf1p proved to be immunogenic with cross-reactivity and protection against *C. albicans* (Stuehler et al., 2011).

Natural Killer Cell Treatment

Recently the role of NK cells in antifungal immunity is being investigated. It has been found that IL2-primed NK cells are cytotoxic toward *A. fumigatus* germlings and hyphae, an effect that is not mediated through degranulation of its cytotoxic proteins like perforin, granzymes etc., but mediated by IFNγ and TNFα secretion (Bouzani et al., 2011; Schmidt et al., 2011). NK cells have been shown to be the most important source of IFN-γ in the lungs of neutropenic hosts during the early stages of invasive aspergillosis (Park et al., 2009). It was also shown that the chemokine ligand MCP1/CCL2 mediates recruitment of NK cells resulting in more rapid clearance of *Aspergillus* from the lungs (Morrison et al., 2003) implicating the potential for NK-based therapeutic applications.

Adoptive T cell Transfer

Defective T-cell immunity is a hurdle in the path to a robust immune response to vaccines and antimicrobial treatment. Conceptually, this problem could be overcome by T-cell-independent vaccination, wherein the $CD4^+$ T-cell-derived factor CD40L, required for DC costimulation of B cells, is replaced (Zheng et al., 2005).

One of the strategies to reduce the risk of invasive aspergillosis is the induction of Th1-type immune response that may be achieved by either transferring *Aspergillus*-specific Th clones or DCs that have been primed to trigger *Aspergillus*-specific immunity (Pikman and Ben-Ami, 2012). Adoptive T-cell transfer has been shown to decrease galactomannan levels significantly with higher survival rates as compared with patients who did not receive immunotherapy (Perruccio et al., 2004). Specific *Candida* cell wall proteins expressed during invasive infection have been synthesized as immunogenic peptide epitope–β-mannan conjugates. DCs pulsed with three of these epitopes conferred protection against disseminated candidiasis in mice. Of note is one epitope, derived from fructose-bisphosphate aldolase, which was shown to induce robust antibody dependent protective responses to *C. albicans* (Xin et al., 2008).

Various vaccine formulations using DCs to induce adoptive immunity to *Aspergillus* have been studied. DCs pulsed with live conidia, transfected with conidial RNA or primed with unmethylated CpG oligodeoxynucleotides and pulsed with Aspf16 antigens trigger specific Th1-type responses and

protective immunity against invasive aspergillosis in a mouse model. DC infusion was shown to be more effective and superior to that of *Aspergillus*-specific T cells (Bozza et al., 2002, 2003). Subsequently, it was shown that DCs transfected with IL-12 DNA and pulsed with heat-inactivated *A. fumigatus* induced protective immunity against invasive pulmonary aspergillosis, as reflected by decreased fungal burden and increased survival (Shao et al., 2005).

CONCLUSIONS AND FUTURE PERSPECTIVES

Despite the advances in our knowledge and understanding in pathogenesis, IFD continues to result in significant morbidity and mortality in immunocompromised patients. The current conventional therapeutic modalities have not been fully effective. In addition, prolonged use of antifungal agents pose the risk of emergence of fungi resistant to conventional drugs.

The urgent need of the hour is to improve treatment options for patients with IFD by the usage of newer and more effective drugs, alone or combined together that can cure the infection. The other promising solution would be the use of immunotherapeutic modalities to improve and enhance the host defense system against fungal pathogens. The increase in knowledge of the pathogenesis of fungal infections has ushered in a new era of immunotherapeutic options. It is of utmost importance that further relevant clinical trials be conducted to explore the various immunotherapeutic strategies that hold promise for the better treatment and control of IFD in the near future.

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Heat Shock Protein 90 (Hsp90) as a Molecular Target for the Development of Novel Drugs Against the Dermatophyte *Trichophyton rubrum*

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Treatment of fungal infections is difficult due to several reasons, such as side effects of drugs, emergence of resistant strains, and limited number of molecular targets for the drug compounds. In fungi, heat shock proteins (Hsps) have been implicated in several processes with the conserved molecular chaperone Hsp90 emerging as a potential target for antifungal therapy. It plays key cellular roles by eliciting molecular response to environmental changes, morphogenesis, antifungal resistance, and fungal pathogenicity. Here, we evaluated the transcription profiles of *hsp* genes of the most prevalent dermatophyte *Trichophyton rubrum* in response to different environmental challenges including nutrient availability, interaction with cells and molecules of the host tissue, and drug exposure. The results suggest that each Hsp responds to a specific stress condition and that the cohort of Hsps facilitates fungal survival under various environmental challenges. Chemical inhibition of Hsp90 resulted in increased susceptibility of the fungus to itraconazole and micafungin, and decreased its growth in human nails *in vitro*. Moreover, some *hsp* and related genes were modulated by Hsp90 at the transcriptional level. We are suggesting a role of Hsp90 in the pathogenicity and drug susceptibility of *T. rubrum* as well as the regulation of other Hsps. The synergism observed between the inhibition of Hsp90 and the effect of itraconazole or micafungin in reducing the fungal growth is of great interest as a novel and potential strategy to treat dermatophytoses.

Keywords: Hsp, antifungal therapy, molecular target, drug synergism, itraconazole, micafungin, 17-AAG

INTRODUCTION

Dermatophytes are pathogenic fungi and primary causative agents of superficial mycoses in humans (Brown et al., 2012). These fungi are keratinolytic and infect keratinized structures such as skin, nails, and hair in the host, giving rise to diseases (also known as dermatophytoses or tineas) such as athlete's foot, onychomycosis, ringworm, and jock itch. Among the species belonging to this group of filamentous fungi, *Trichophyton rubrum* is the leading cause of human skin and nail mycoses and has high prevalence worldwide (Havlickova et al., 2008; Seebacher et al., 2008; Nenoff et al., 2014). Although rare, disseminated or deep dermatophytoses have been reported

in immunocompromised or immunosuppressed patients (Gong et al., 2007; Marconi et al., 2010; Lanternier et al., 2013). Depending on the amplitude and the site of infection, dermatophytoses can be difficult to cure and often relapse post-treatment, even in immunocompetent individuals (Gupta and Cooper, 2008; Ghannoum and Isham, 2014).

Although a reasonable number of antifungal drugs are commercially available, majority of the clinical drugs act on the ergosterol biosynthesis pathway, thus restricting the number of cellular targets. Besides, resistance to commonly used antifungal drugs has been reported in dermatophytes and other human pathogens, rendering the choice of drug challenging and exacerbating the prospect of successful treatment (Martinez-Rossi et al., 2008; Pfaller, 2012). Therefore, novel antifungal targets have become the prime focus of several researchers in the field of medical mycology. Drug combinations and synergism have been proposed as therapeutically desirable approaches to decrease the development of resistance (Kontoyiannis and Lewis, 2002).

Evaluation of the interconnection among drug resistance, stress response, and the signaling pathways activated in these processes has been revealing key elements or the core circuitry as targets for antifungal therapy (Cowen and Steinbach, 2008; Shapiro et al., 2011). One such promising cellular candidate is the heat shock protein 90 (Hsp90) (Wirk, 2011), a molecular chaperone belonging to the highly conserved family of heat shock proteins (Hsps). These proteins rapidly accumulate in the cytosol in response to heat and environmental challenges such as antifungal drugs, oxidative stress, and heavy metal exposure among others. The heat shock response (HSR) is considered a rescue mechanism that enables the cells to cope under stressful conditions and protects from severe damage. The primary role of Hsps is to sense and assist proper protein folding and refolding, and direct them for degradation in case of misfolding, thereby assuring proteome integrity and homeostasis (Lindquist and Craig, 1988). Hsps act as molecular chaperones or transcriptional regulators in a myriad of physiological functions. These proteins are classified into several families based on their function and molecular weight, which ranges from 9 to 110 kDa. Hsps are also found in all organisms (Lindquist and Craig, 1988; De Maio et al., 2012), and are involved in the assembly of protein complexes, transport and sorting of proteins into the proper cellular compartments, cell-cycle control, and protein fate, among other functions. In fungi, Hsps have been implicated in several processes, including pathogenicity, phase transition in dimorphic fungi, and antifungal drug resistance. Hsps are synthesized as an adaptive response to stress that contributes to the survival of pathogenic microorganisms in the host (Burnie et al., 2006; Brown et al., 2010).

Heat shock protein 90 is highly abundant in cells even in non-stressful state and increases further in response to different forms of stress. However, some eukaryotes present two *hsp90* genes, one inducible and the other constitutively expressed (Taipale et al., 2010). Hsp90 can associate with several proteins involved in signaling, metabolism, cell growth, transcription, protein trafficking, chromatin remodeling, and stress response, among others (Leach et al., 2012b). It is an ATP-dependent chaperone and functions as a dimer. Each monomer presents an amino-terminal domain (NTD) that binds ATP and hydrolyzes upon association with the target proteins, a middle domain (MD) crucial for the interaction with the target proteins, and a carboxyl-terminal domain (CTD) responsible for dimerization. The energy produced by the hydrolysis of ATP is used by Hsp90 to fold the target proteins to their active conformations (Taipale et al., 2010). By chaperoning the target proteins, Hsp90 can modulate several downstream processes and regulatory cascades, thus controlling the responses to dynamic environments (Shapiro et al., 2011; Leach et al., 2012b).

Inhibitors of Hsp90 have been thoroughly searched for and some natural compounds produced by microorganisms have been isolated. These include geldanamycin, which is a benzoquinone ansamycin derived from actinomycetes, and a resorcyclic acid lactone called radicicol produced by certain fungal species (Piper and Millson, 2012). Some Hsp90 inhibitors are in clinical trial for cancer therapy and derivatives of natural compounds have been synthesized to increase efficacy and decrease side effects and toxicity (Gorska et al., 2012). In general, these inhibitors act as ATP competitors and interfere with the ATP-binding domain, which turns Hsp90 non-functional and leads to the ubiquitination and proteasome degradation of target proteins because of their aberrant conformation (Wirk, 2011). Besides their therapeutic potential, geldanamycin and its derivatives have been used to characterize the role of Hsp90 in fungal adaptation to host environment and antifungal resistance, as well as to understand their synergism with other antifungal drugs. A promising and interesting consequence of Hsp90 inhibition was that the emergence of resistance to azoles and echinocandins were reduced *in vitro* in human pathogens *Candida albicans* and *Aspergillus fumigatus*, respectively, thus validating the efficiency of these antifungal drugs in experimental infection models (Cowen, 2008; Cowen et al., 2009). Compromising the Hsp90 function was also effective against *C. albicans* and *A. fumigatus* biofilms, which are highly drug-resistant recalcitrant structures and an important cause of mortality. Targeting Hsp90 with chemical inhibitors increased the susceptibility of *C. albicans* biofilms to azoles *in vitro* and in an animal infection model, and the efficacy of azoles and echinocandins against *A. fumigatus* biofilms (Robbins et al., 2011). In *C. albicans*, inhibition of Hsp90 also affected cell wall biogenesis by disrupting the signaling pathways involved in cell wall remodeling (Leach et al., 2012a). Additionally, this also impaired the Hsf1–Hsp90 auto-regulatory circuit in *C. albicans*. The heat shock transcription factor Hsf1 governs the HSR and is a target of Hsp90. Thus, Hsp90 inhibition affected the Hsf1 regulon, consequently the regulation of HSPs and the resistance to proteotoxic stress (Leach et al., 2012a).

In this work, we have chemically inhibited Hsp90 in the dermatophyte *T. rubrum* and analyzed the effects to assess the roles played by this molecular chaperone in response to antifungal drugs, fungal pathogenicity, and regulation of other genes. To analyze drug susceptibility, three molecules with different modes of action were tested; itraconazole (ITRA), 5-Fluorocytosine (5-FC), and micafungin (MCFG) act on ergosterol biosynthesis, nucleic acids, and glucan synthesis,

respectively. The antifungal effects of these drugs in synergy with the Hsp90 inhibitor against the growth of *T. rubrum* were evaluated. In order to analyze the role of Hsp90 in pathogenicity, the ability of *T. rubrum* to colonize human skin and nail in the presence of Hsp90 inhibitor was tested in an *ex vivo* model of infection. The influence of Hsp90 in the regulation of *hsp* genes and other related genes such as those encoding for the heat shock factor Hsf1 and the pH responsive regulator PacC was evaluated by transcription profile analyses in response to nutritional sources. Finally, in order to assess the adaptive response to various stress conditions, the transcriptional profile of Hsps, including Hsp90, was evaluated after exposure of *T. rubrum* to antifungal drugs and substrates present in the host.

MATERIALS AND METHODS

Trichophyton rubrum Strain

Trichophyton rubrum strain CBS118892 (CBS-KNAW Fungal Biodiversity Centre) was cultivated in malt extract agar (MEA: 2% peptone, 2% glucose, 2% agar, pH 5.7) at 28°C. To prepare the conidia suspension, 15-day-old plates were flooded with sterile 0.9% NaCl and the suspension filtered through fiber glass to remove mycelia debris. The conidia concentration in the filtrate was estimated using a Neubauer chamber, as previously described (Persinoti et al., 2014).

Antifungal Drug Susceptibility Test

The synergistic effect between chemical inhibition of Hsp90 and antifungal agents was tested by the following method: *T. rubrum* conidia (1×10^6 per plate) were spread on the surface of MEA containing 10, 100, and 300 μM of the inhibitor 17-AAG (17-allylamino-17-demethoxygeldanamycin; InvivoGen, San Diego, CA). E-test (AB Biodisk, Solna, Sweden) gradient strips of ITRA, 5-Fluorocytosine (5-FC), or MCFG were then placed on these plates. Gradient concentration of the tested antifungal drugs ranged from 0.002 to 32 μg/mL and the results were observed after incubation at 28°C for 5 days. Plates without antifungal agents were used to assess fungal development in the presence of 17-AAG. Three independent experiments were conducted.

Ex vivo Pathogenicity Test

The *ex vivo* nail and skin interaction assays were performed as described here. Autoclaved small pieces of human nail obtained from healthy donors were infected with 1×10^4 *T. rubrum* conidia and incubated at 28°C for 5 days, in the absence or presence of 50, 100, or 200 μM of Hsp90 inhibitor 17-AAG. After incubation, nail fragments were observed under a light microscope to evaluate the hyphal development and fungal morphology.

Small pieces of human skin were obtained from patients who underwent abdominal surgery at the University Hospital of Ribeirão Preto Medical School, University of São Paulo, Brazil (HC-FMRP-USP). After removal of the adipose tissue, the small pieces of human skin were infected with 1×10^4 *T. rubrum* conidia in the absence or presence of 200 μM 17-AAG and incubated at 28°C for 5 days. Infected skin fragments were maintained in skin graft fluid (SGF; Duek et al., 2004) supplemented with or without 200 μM 17-AAG. Scanning electron microscopy (SEM) was employed to visualize hyphal development. For this purpose, the skin fragments were fixed with 3% glutaraldehyde in 0.1% phosphate buffer (pH 7.2) at 4°C for 2 h, rinsed with 0.1% phosphate buffer (pH 7.2), and post-fixed with 1% osmium tetroxide for 2 h. Samples were dehydrated by a graded ethanol series and sputter-coated with gold to obtain a layer of approximately 200 μm thickness. The samples were viewed under a Jeol JSM -6610 LV scanning electron microscope at an acceleration voltage of 25 kV.

Growth Conditions for Gene Expression Assays

T. rubrum conidia (1×10^6) were inoculated in 100 mL of malt extract (ME) medium (pH 5.0) or keratin medium (KM: 2.5 g/L keratin powder, MP Biomedicals, suspended in water, pH 5.0). After shaking at 28°C for 96 h, the resultant mycelia were filtered, frozen in liquid nitrogen, and stored at -80°C for expression studies. For the Hsp90 chemical inhibition assay, fresh mycelia grown for 96 h at 28°C in ME or KM were incubated for 30 or 90 min at 28°C with 100- or 300 μM 17-AAG. For antifungal drug response assays mycelia grown in ME at 28°C for 96 h were aseptically transferred to RPMI 1640 (Life Biotechnologies—buffered with 0.167M MOPS, pH 7.0) in the absence (control) and presence of sub-inhibitory concentrations of acriflavine (ACR; 1.75 μg/mL) or terbinafine (TRB; 0.2 μg/mL), and incubated for 3 h at 28°C.

For the interaction assays, the small pieces of human skin were cleaned, infected with 1×10^4 *T. rubrum* conidia, and incubated at 28°C for 96 h. The fungus was then harvested and used for total RNA extraction. For the nail interaction assay, each human nail fragment was exposed to 1×10^4 *T. rubrum* conidia and incubated at 28°C for 96 h. The infected nail fragments were vortexed to release fungal mycelia for total RNA extraction and the nails discarded. The interaction assays were approved by the local Ethics Committee (Protocol No. 046/2009). Three independent experiments were conducted for each growth condition and interaction assay.

Gene Expression Analysis

Total RNA was isolated from frozen mycelia using Illustra RNAspin Mini RNA Isolation Kit (GE Healthcare). First-strand cDNA was synthesized using the SuperScriptIII First-Strand Synthesis Super Mix for qRT-PCR kit (Invitrogen). Both RNA extraction and cDNA synthesis were performed according to the manufacturer's recommendations. An intron flanking region of the β-tubulin gene was used as positive control to verify DNA contamination and proper cDNA synthesis, as previously described (Jacob et al., 2012).

Specific primer pairs for each *T. rubrum* gene were designed using the Primer Express v.3 software (Life Technologies) and are listed in **Table 1**. qRT-PCR reactions were carried out in a final volume of 12.5 μL, containing 6.25 μL Power SYBRGreen PCR

TABLE 1 | Primers used for qPCR assays.

Gene	Accession number[a]	Primer sequences (5'– 3')	Amplicon length (bp)	Efficiency(%)
hsp20	TERG_01659	F: GCCAAGGAGGAGTTGAATCCT R: AGCCCTCTCCAATCTCGTCTT	57	95.29
hsp60	TERG_04141	F: AAGCGTCGTTGTCGGTAAGC R: TGTCGAAGCCACGGTTAAAGT	62	92.6
hsp70	TERG_01883	F: CCGCCATGAACCCTGAGA R: CGAATCGTCGTCCGATAAGAC	60	96.0
hsp70-like	TERG_06505	F: CACGTACTCCTGCGTGGGTAT R: TGCGGTTTCCCTGATCGT	71	96.0
hsp clpa	TERG_07049	F: CCGGCAGTCTCCCAAGTCT R: GTAGGCAGCAGCCATGACTTC	60	91.8
hsp88-like	TERG_07658	F: AAGGGTGTCACCGCTGATG R: TCAGTCTAGCCTTGAGCTTGCA	61	95.8
hsp90	TERG_06963	F: ACCGTGCTGCCCTTGCT R: GTGATCTCGTCGCCAGACTTG	61	96.0
cdc37	TERG_06398	F: GAGATCGCAACTCTAGGGTACGA R: GCCCGTCAATCCGTTTCA	64	93.5
hsp ssc1	TERG_03206	F: ACCGAGTCCGTCAAGAGCAT R: TCGTCGGGATTAACGGACTT	59	96.1
hsp78	TERG_07949	F: CCGGTCTCAGCGGTGAAA R: GGTGGGCCCAAGAAACATG	56	92.6
hsf1	TERG_04406	F: AGTGCTGGAGGCCGAGAAG R: TCCCGACCCGAGAGCAA	60	97.4
pacC	TERG_00838	F: TCCCAGCAGCCCCAAC R: ATGTGGGAGGTGATGTGGT	63	98.3

[a]*Dermatophytes genome database accession number (http://www.broadinstitute.org/annotation/genome/dermatophytecomparative).*

Master Mix (Life Technologies), 1.0 μL of each primer (*hsp20*, 250 nM; *hsp60*, 500 nM; *hsp70*, 450 nM; *hsp70-like*, 350 nM; *hsp clpa*, 300 nM; *hsp88-like*, 350 nM; *hsp90*, 300 nM; *cdc37*, 400 nM; *hsp ssc1*, 400 nM; *hsp78*, 350 nM; *hsf1*, 350 nM; *pacC*, 350 nM), 2.0-μL template cDNA (50 ng), and 3.25-μL ultra-pure water. Thermal conditions for qRT-PCR were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All reactions were performed in triplicate in 96-well reaction plates using the StepOnePlus Real-Time PCR System (Life Technologies). A melting curve for each gene was obtained and 2% agarose gel electrophoresis was performed to confirm the amplification of the unique product of expected size for each *hsp* gene. To determine PCR efficiency, standard curves were generated using cDNA sample at five-point, twofold dilutions and measured in triplicates. The reference genes *rpb2* and *actin* were used for data normalization as previously described (Jacob et al., 2012). Relative expression was calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Statistical significance was evaluated by one-way ANOVA followed by the Tukey's *ad hoc* test, using the GraphPad Prism v 5.1 Software.

RESULTS

Inhibition of Hsp90 by 17-AAG

In order to evaluate the synergism of Hsp90 with other antifungal drugs, as well as its role in *T. rubrum* pathogenicity, 17-AAG was used to chemically inhibit Hsp90 expression in *T. rubrum*. Although inhibition of Hsp90 using 300 μM of 17-AAG had no effect on *T. rubrum* growth in MEA, an increase in fungal susceptibility to ITRA and MCFG was observed (Supplementary Figure S1). This was demonstrated by 10- and fourfold decreases in minimal inhibitory concentration (MIC) values, respectively. Moreover, there was no effect when the fungus was challenged with 5-FC (**Table 2**). The role of Hsp90 in *T. rubrum* pathogenicity was also analyzed using an *ex vivo* nail interaction assay. Inhibition of Hsp90 decreased *T. rubrum* growth on human nail *in vitro*. This decrease was dependent on the concentration of 17-AAG used, and at 200 μM, *T. rubrum* growth was almost entirely inhibited (**Figure 1**), indicating the attenuation of fungal virulence. However, SEM of *ex vivo* human skin inoculated with *T. rubrum* conidia showed no significant difference in fungal growth when Hsp90 was inhibited at the same 17-AAG concentration. (Supplementary Figure S2). This suggests that other virulence factors or incomplete inhibition of Hsp90 activity might foster fungal growth in the *ex vivo* skin model.

TABLE 2 | Synergism of heat shock protein 90 (Hsp90) inhibition and antifungal drugs.

17-AAG (μM)	MIC (μg/mL)		
	5-Fluorocytosine (5-FC)	Itraconazole (ITRA)	Micafungin (MCFG)
0	>32	0.125	0.008
10	>32	0.125	0.008
100	>32	0.125	0.004
300	>32	0.012	0.002

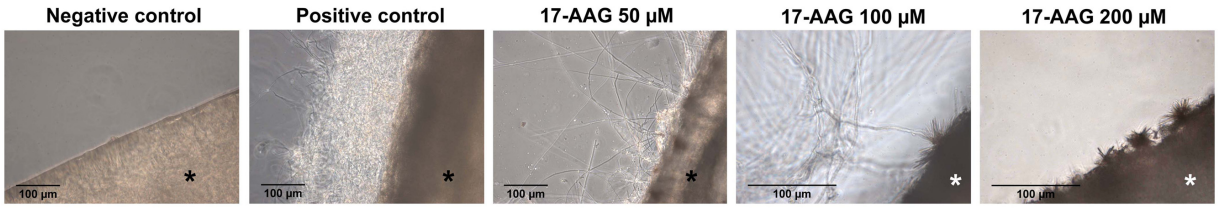

FIGURE 1 | Effect of heat shock protein (Hsp90) inhibition on *Trichophyton rubrum* growth in human nail. Light microscopy was used to analyze hyphal development in human nail fragments infected with *T. rubrum* conidia in the absence (positive control) or presence of the Hsp90 inhibitor 17-AAG (50, 100, or 200 μM) after 5 days at 28°C. Negative control consists of uninoculated and untreated nails. Asterisks indicate nail fragments.

Expression Profile of *hsps* and Related Genes

The expression of several *hsp*s and related genes is modulated during the growth of *T. rubrum* in KM and ME in the presence of Hsp90 inhibitor 17-AAG. For some genes, this effect is time- and concentration-dependent (**Figure 2**). While the inhibition of Hsp90 led to decreased accumulation of *hsp70-like* and *hsf1* transcripts, regardless of the growth medium, the levels of *hsp60* and *hsp88-like* transcripts were decreased in response to ME and KM, respectively. However, *hsp70* and *hsp clpa* genes were upregulated in the absence of Hsp90 activity, which led to increased accumulation of *hsp90* transcripts, perhaps in an attempt to compensate for the chemical inhibition of the protein. Interestingly, *pacC* was downregulated when Hsp90 was inhibited in the presence of keratin. Moreover, the gene coding for the Hsp90 co-chaperone cdc37 was upregulated in ME but not in KM, whereas *hsp ssc1* was upregulated only in KM (**Figure 2**) upon Hsp90 inhibition. These results suggest a regulatory role for Hsp90 in the expression of *pacC* and other *hsp* genes in *T. rubrum*, depending on the growth medium.

Given the involvement of Hsps in the pathogenicity of several fungal pathogens and in drug susceptibility, the expression profile of some of these *hsp* genes was evaluated in *T. rubrum* in response to other antifungal drugs, such as TRB and ACR, and during its growth on nail and skin fragments (**Figure 3**). The analysis revealed different expression profiles in response to these environmental challenges. While *hsp clpa* gene was not modulated in response to any stimuli analyzed (**Figures 3A,B**), *hsp90*, *hsp88-like*, and *hsp20* transcripts accumulated in response to drug exposure (**Figure 3B**). However, *hsp60* and *hsp78* presented a slightly broad range of modulation with transcripts accumulating during growth on nails and in response to one or both drugs (**Figures 3A,B**).

DISCUSSION

Dermatophytes affect millions of individuals annually and have become an important public health concern because of their refractivity to therapy, which prolongs the duration of treatment especially in aging populations (Coelho et al., 2008; Martinez et al., 2012). Because both the host and the pathogen are eukaryotic organisms, treatment of fungal infections is difficult due to the limited number of antifungal targets available (Martinez-Rossi et al., 2008). Another concern is the emergence of resistance to antifungal drugs currently in clinical use. Thus, it is necessary to identify new strategies for therapy against fungal infections. In this study, we observed that it is possible to increase the efficacy of antifungal drugs, ITRA and MCFG, by targeting the molecular chaperone Hsp90 with 17-AAG, an inhibitor of the Hsp90 ATPase activity. Interestingly, these antifungal agents have different mechanisms of action. While ITRA inhibits the enzyme lanosterol 14α-demethylase, thus preventing the biosynthesis of ergosterol, a key sterol in the fungal membrane (Lupetti et al., 2002; Odds et al., 2003), MCFG inhibits the enzyme 1, 3-β-D-glucan synthase, thereby blocking the biosynthesis of a key linker molecule in the fungal cell wall (Onishi et al., 2000). When challenged with antifungal drugs, several dermatophytes generally react by activating stress responses (Paião et al., 2007; Yu et al., 2007; Zhang et al., 2009; Peres et al., 2010), which often depends on the Hsp90 chaperone. Hsp90 can associate itself with a myriad of target proteins such as its co-chaperones and functional regulators, and modulate the activation and stability of the complex. Thus, functional inhibitors of the Hsp90 that act on its ATPase-coupled conformation (Siligardi et al., 2002; Lotz et al., 2003) disassemble the molecular complex of Hsp90 with co-chaperones and target proteins, thereby abrogating drug resistance and increasing the efficacy of traditional antifungal drugs (Veri and Cowen, 2014). Although Hsp90 is conserved among eukaryotes, it presents some conformational differences in fungi, especially in regions such as the ATP binding and the MDs that could be selectively targeted by the chemical inhibitors (Wider et al., 2009; Shahinas et al., 2015). Alternatively, it is possible to interfere with the Hsp90 targets or functional regulators, thus expanding the possibility to find clearer discrepancies between the pathogen and the host (Veri and Cowen, 2014). Therefore, targeting Hsp90 or other related proteins may be a viable alternative to treat fungal infections caused by *T. rubrum* and probably by other dermatophytes as well. We have also shown that the Hsp90 chaperone has a role in conferring the fungus with the ability to colonize human nails *in vitro*. The involvement of this chaperone in the pathogenicity of other pathogens such as *C. albicans* and *C. glabrata* has been demonstrated (Noble et al., 2010; Leach et al., 2012b; Singh-Babak et al., 2012). Thus, blocking the action of Hsp90 in dermatophytes becomes

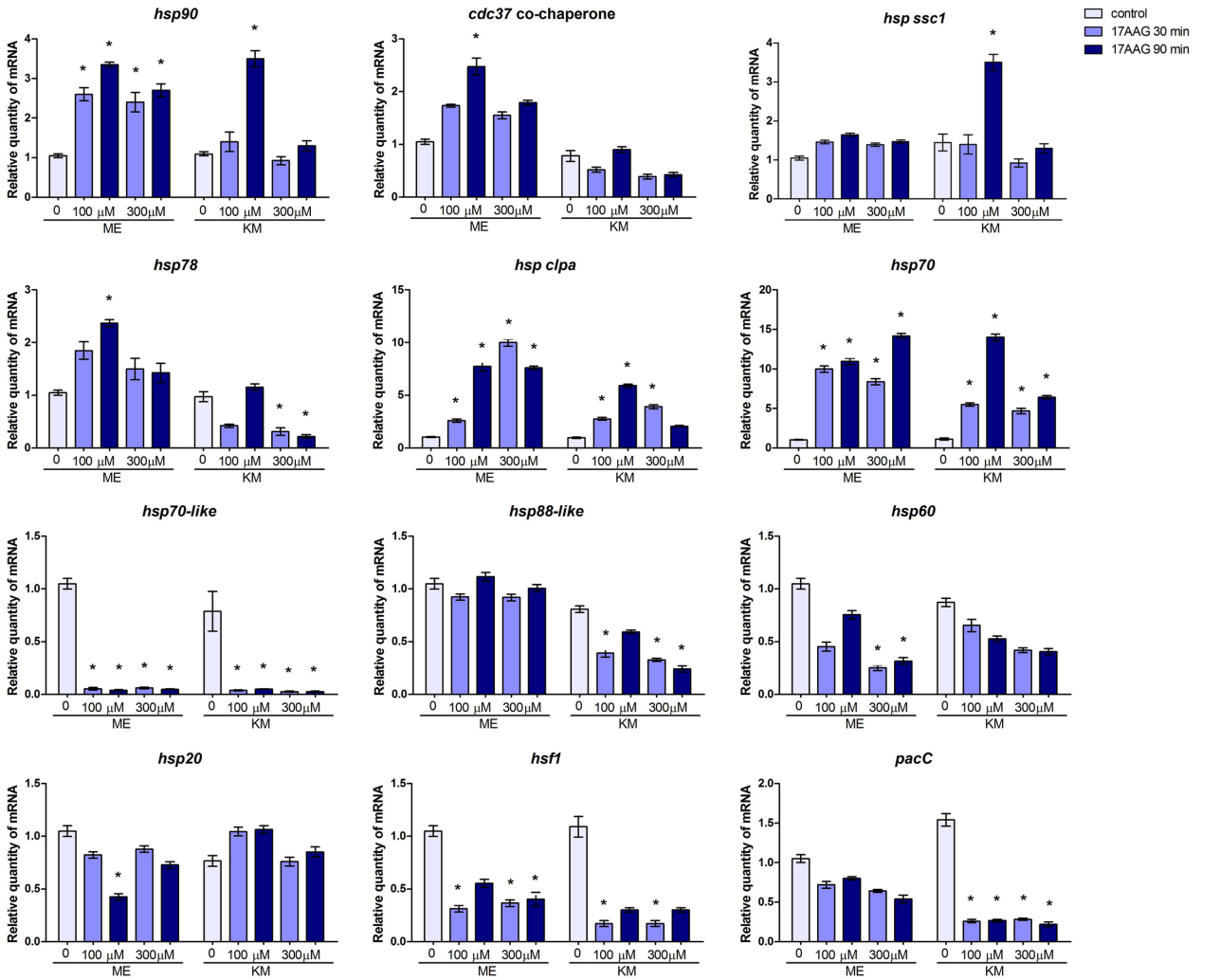

FIGURE 2 | Effect of Hsp90 on the expression of *hsp* and related genes in *T. rubrum*. qRT-PCR analyses of the *hsp* and related genes of *T. rubrum* grown in malt extract (ME) or keratin medium (KM) in the absence or presence of Hsp90 inhibitor. Gene expression levels are represented by the quantities of mRNA in each condition relative to the control (ME). Data are represented as mean ± *SD* from three independent experiments with reactions performed in triplicate. Tukey's *ad hoc* test was used for statistical analysis ; $^*P < 0.05$.

a potential strategy that combines this therapy with conventional antifungal drugs, which would enhance the overall outcome of the treatment.

The broad spectrum of Hsp90 functions were confirmed by changes in the expression profile of various *hsp* and related genes upon chemical inhibition of this chaperone in *T. rubrum*. Moreover, these changes in gene expression in the fungus were nutrient-dependent (ME and KM medium). The heat shock transcription factor Hsf1 positively regulates the transcription of the *hsp90* gene in *C. albicans* and *Saccharomyces cerevisiae*; both pharmacological inhibition and genetic depletion of Hsp90 correlate with Hsf1 activation in response to thermal stress (Wu, 1995; Leach et al., 2012b). However, *hsf1* transcript levels decreased when *T. rubrum* was challenged with the Hsp90 inhibitor in MEA or KM, under non-stressful temperature conditions (**Figure 2**). Additionally, there was an evident increase in *hsp90* transcripts in *T. rubrum* treated with the Hsp90 inhibitor, which was likely to compensate for the absence of Hsp90 function (**Figure 2**). This suggests a regulatory role for Hsp90 over *hsf1* transcript levels or a compensatory mechanism upon Hsp90 inhibition, in which most of the *hsf1* transcripts in the cell are efficiently transduced into protein, in turn aiding *hsp90* transcription. In *T. rubrum*, putative DNA-binding sites for Hsf1 in the *hsp90* promoter region enable direct regulation. Another interesting observation from this study is the decreased amount of the *pacC* transcripts during growth in keratin cultures containing 17-AAG (**Figure 2**). The transcription factor PacC mediates diverse metabolic events, including virulence and keratinolytic activity, in *T. rubrum* (Ferreira-Nozawa et al., 2006; Silveira et al., 2010; Martinez-Rossi et al., 2012), suggesting a correlation between the *pacC* and *hsp90* genes and fungal virulence.

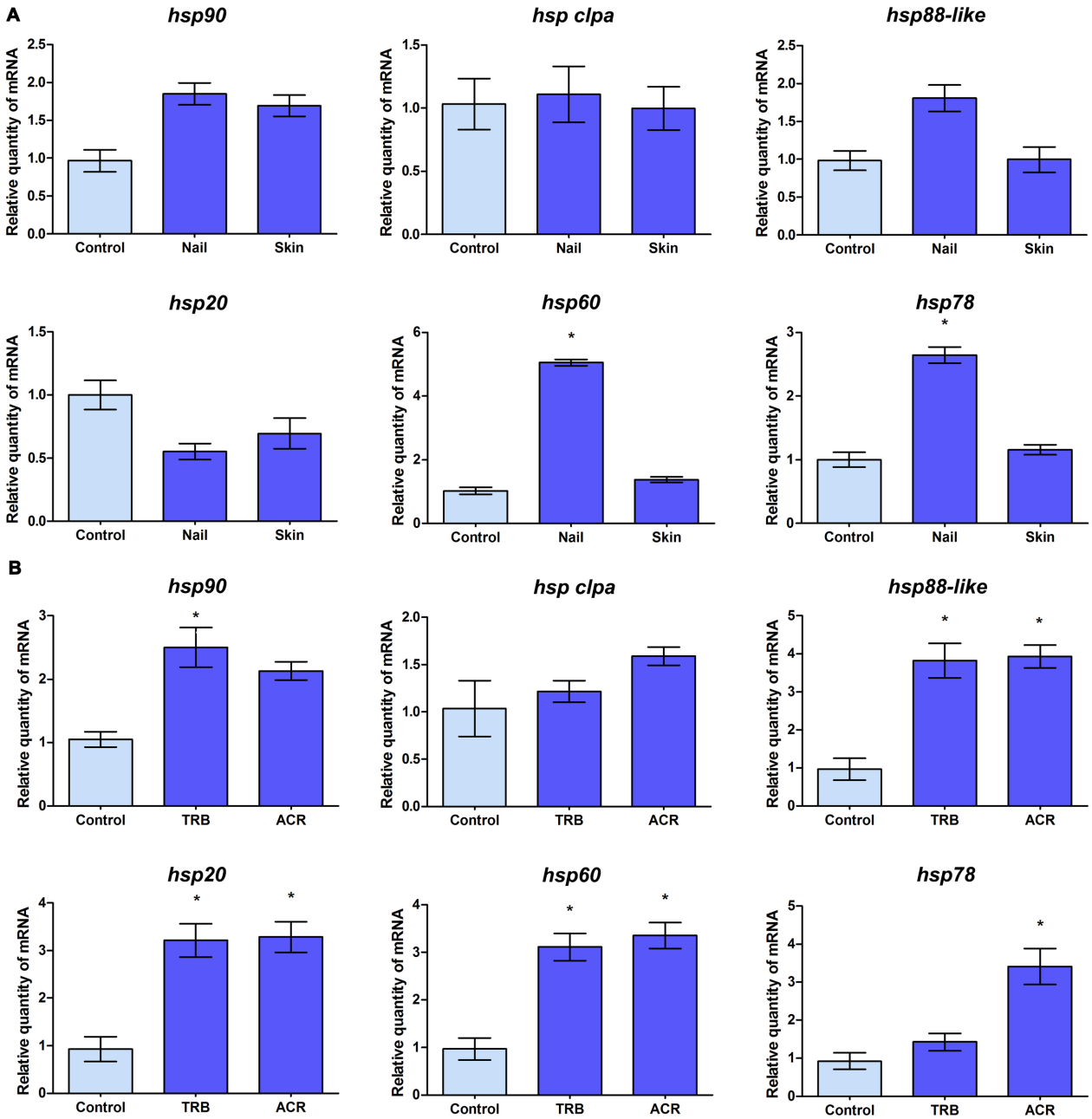

FIGURE 3 | Transcript levels of *T. rubrum hsp* genes in response to environmental challenges. (A) qRT-PCR analyses of the transcript levels of *hsp* genes of *T. rubrum* grown in malt extract (control), human nail, or human skin fragments for 96 h. **(B)** Mycelia were transferred to RPMI medium in the absence (control) or presence of sub-inhibitory doses of terbinafine (TRB) or acriflavine (ACR) for 3 h for assessment of drug response. mRNA quantity in each condition relative to the control are represented as mean ± *SD* from three independent experiments with reactions performed in triplicate. Tukey's *ad hoc* test was used for statistical analysis, $^*P < 0.05$.

Additionally, we observed an increased accumulation of some *hsp* transcripts on Hsp90 inhibition, while others, including those belonging to the same family, suffered drastic decline. For example, regardless of the culture condition, transcription of the *hsp70-like* gene showed a significant reduction, whereas *hsp70* transcripts increased significantly and *hssc1* (Hsp70 family protein) transcripts practically accumulated to the same amount, suggesting different roles for each Hsp (**Figure 2**).

In order to evaluate the expression profiles of some *hsp* genes in *ex vivo* models, as well as during treatment with antifungal drugs, *T. rubrum* was cultured in MEA or in nail or skin fragments (**Figure 3A**) and in RPMI medium containing sub-inhibitory concentrations of TRB or ACR (**Figure 3B**). *hsp90*

gene expression increased only in response to TRB, an antifungal used to treat dermatophytosis. Unexpectedly, *hsp90* transcript levels were similar to those observed in other experimental conditions tested, including the human nail, in which the fungus was dependent on Hsp90 function when this substrate was its sole nutrient source. It is possible that the fungus depends on similar amounts of the Hsp90 protein to cope with varying culture conditions. The transcription profile of the *hsp clpA* gene was unchanged in the analyzed conditions, demonstrating constitutive expression in response to various antifungal drugs and growth conditions. However, there was an increase in *hsp20*, *hsp60, hsp78,* and *hsp88-like* gene transcript levels when *T. rubrum* was challenged with ACR and/or TRB, suggesting their involvement in cellular stress responses. An increase in *hsp60* and *hsp78* transcripts was also observed when the fungus was grown in nail fragments, suggesting that Hsp60 and Hsp78 proteins act as Hsp90 co-chaperones in nail infections.

CONCLUSION

Complex formation of Hsp90 and its chaperones depends on an ATPase-coupled conformational cycle, which links ATP binding and hydrolysis. This is a highly conserved mechanism in eukaryotic organisms, including *C. albicans* and other human pathogenic fungi, since ATP binding and hydrolysis are essential for Hsp90 function. Functional inhibition of ATP binding to Hsp90 disassembles the molecular complex between Hsp90 with target proteins and co-chaperones, consequently abrogating drug resistance and increasing the efficacy of traditional antifungal drugs such as ITRA and MCFG. Blocking the Hsp90 activity drastically decreased the ability of *T. rubrum* to grow on human nail fragments and interfered with the modulation of some *hsp* genes and the *pacC* gene, a regulator involved in *T. rubrum* virulence. Thus, blocking Hsp90 function in dermatophytes is suggested as a potential strategy for combination of this therapy with traditional antifungal drugs, which would enhance the antifungal efficacy. This is an attractive hypothesis to be explored further probably using *in vitro* and/or *ex vivo* models of infection as well as new inhibitors for Hsp90 and other co-chaperones.

AUTHOR CONTRIBUTIONS

TJ performed most of the experimental procedures, such as fungal cultivation, RNA extraction, synergism assays, and qPCR. NP participated in the experimental design and procedures, and drafted the manuscript. PS performed computational analyses to identify the genes used in this work, their promoter regions, identifying DNA-binding domains and the putative proteins. He also performed the statistical analyses of qPCR. EL performed some experimental procedures, such as skin infections, image acquisition, and drafted the manuscript. MM performed the nail infections, synergism assays and image acquisition. AR and NM-R designed the project, supervised the research study, and prepared the manuscript. All authors participated in data analysis and interpretation, read, revised critically the manuscript and approved the final version. Also, all authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01241

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New strategic insights into managing fungal biofilms

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Fungal infections have dramatically increased in the last decades in parallel with an increase of populations with impaired immunity, resulting from medical conditions such as cancer, transplantation, or other chronic diseases. Such opportunistic infections result from a complex relationship between fungi and host, and can range from self-limiting to chronic or life-threatening infections. Modern medicine, characterized by a wide use of biomedical devices, offers new niches for fungi to colonize and form biofilm communities. The capability of fungi to form biofilms is well documented and associated with increased drug tolerance and resistance. In addition, biofilm formation facilitates persistence in the host promoting a persistent inflammatory condition. With a limited availability of antifungals within our arsenal, new therapeutic approaches able to address both host and pathogenic factors that promote fungal disease progression, i.e., chronic inflammation and biofilm formation, could represent an advantage in the clinical setting. In this paper we discuss the antifungal properties of myriocin, fulvic acid, and acetylcholine in light of their already known anti-inflammatory activity and as candidate dual action therapeutics to treat opportunistic fungal infections.

Keywords: biofilm-related infections, antifungal resistance, myriocin, fulvic acid, acetylcholine

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Introduction

The population of subjects at risk of developing fungal infections is steadily increasing due to rising life expectancy and the continuous medical progress in the treatment of serious diseases such as cancer, transplantation or impairment of immune system (Brown et al., 2012).

Even though advanced medical treatments allow these patients to live longer, the exposure to surgery and medical devices composed of polymeric materials results in evolved ecological niches for biofilm-producing microorganisms and increases the risk for infectious diseases, including those caused by opportunistic fungi (Ramage et al., 2006). *Candida albicans* amongst yeasts and *Aspergillus fumigatus* amongst molds are still the most common pathogens in the clinical setting (Morace and Borghi, 2010; Kriengkauykiat et al., 2011; Guinea, 2014), and continue to carry a high mortality despite the antifungal treatment.

Antifungal resistance is emerging in *Candida* and *Aspergillus* species (Arendrup, 2014), and together with intrinsic or acquired mechanisms, the drug tolerance related to biofilm formation is emerging as having a crucial role in the failure of treatments (Ramage et al., 2014). Fungal cells within the biofilms display resistance to azoles and polyenes, at least at therapeutic doses (Taff et al., 2013). Echinocandins seem to achieve better results against *Candida* biofilms, but not against *A. fumigatus* (Pierce et al., 2013). Thus, the development of new compounds able to overcome the drug-resistance of biofilms is undoubtedly a current and, even more, a future medical need for the treatment of such infections.

Recently, some compounds with known anti-inflammatory properties have been investigated for their antifungal activity. This is of particular relevance in the context of fungal infections. The interplay between fungus and host, i.e., immune system and inflammatory milieu, is crucial in determining the tolerance or the disease status (Romani, 2011). Although inflammation is required to control of fungal infections, its resolution is necessary to avoid collateral damage to tissues and to restore a homeostatic environment (Romani, 2011). Drugs displaying dual activity, antifungal and anti-inflammatory, could thus represent novel approaches to treat biofilm-related infections. In this work we discuss the anti-biofilm properties of myriocin, fulvic acid, and acetylcholine, three compounds recently investigated for their antifungal activity in the context of fungal biofilms.

Myriocin

Sphingolipids (SPLs) are a class of molecules with structural and signaling activities conserved from fungi to humans. Many studies have demonstrated that SPL mediators are involved in infection-related mechanisms (Mor et al., 2015). Both microbial and mammalian dysregulation of SPLs play a role in the delicate relationship between pathogen and host during the infection process, having an impact on signaling pathways that eventually lead to commensalism or host damage (Heung et al., 2006).

Fungal SPLs have been implicated in several cellular processes such as endocytosis, apoptosis, heat stress response, and fungal pathogenesis (Lattif et al., 2011). In fact, SPLs are part, together with ergosterol, of plasma membrane domains named lipid rafts that are crucial for cell signaling and membrane trafficking, and mediate protein–protein interactions (Farnoud et al., 2015).

Changes in the SPLs content could thus strongly impact the local membrane structure and alter specific protein localization such as the GPI-anchored proteins (Singh and Del Poeta, 2011). These have been extensively studied in *C. albicans* and are crucial for adhesion to substrates in the early phases of biofilm formation (Cabral et al., 2014). Differences in SPLs content have been observed in planktonic and sessile cells of *C. albicans*, suggesting a role for the lipid moiety in biofilm formation and maturation (Lattif et al., 2011). Lipid rafts have been found to localize at the hyphal tip, and drugs affecting SPLs biosynthesis, such as myriocin, lead to defects in hypha formation (Martin and Konopka, 2004).

Myriocin targets the first step of SPLs *de novo* biosynthesis, by inhibiting the enzyme serine palmitoyl transferase (SPT) that catalyzes the condensation of a fatty acyl CoA with serine, a common step to both fungal and mammalian SPLs biosynthesis.

Many cell-stress responses cause ceramide, the central molecule of SPL metabolism, to accumulate and trigger the activation of inflammatory processes (Hannun and Obeid, 2008). High levels of ceramide are characteristic of several inflammatory diseases. Animal models showed that myriocin treatment is able to reduce inflammation by down-regulating ceramide and its related pro-inflammatory cascade (Jiang et al., 2011; Lee et al., 2012; Caretti et al., 2014).

Besides this action and similarly to other SPLs metabolism inhibitors (Groll et al., 1998; Mormeneo et al., 2008), myriocin has a direct antifungal activity (Martin and Konopka, 2004; Lattif et al., 2011; de Melo et al., 2013; Sharma et al., 2014). Recently, Lattif et al. (2011) assessed a potential antibiofilm activity for the drug. The authors grew *C. albicans* biofilms in the presence and absence of various myriocin concentrations and observed a progressive reduction in biofilm biomass and metabolic activity. In addition, lipid raft formation was strongly reduced as well as the *C. albicans* filamentation (Lattif et al., 2011).

Myriocin has been found to be also active against *A. fumigatus* (Cirasola et al., 2014). Administration of myriocin to conidia resulted in a dose-dependent inhibition of germination, whereas the treatment of 24 h pre-formed biofilms strongly reduced the biofilm biomass, as determined by crystal violet assay, and the metabolic activity. In particular, myriocin led to the presence of aberrant hyphal structures in *A. fumigatus*, with increased branching and reduction in apical hyphal growth. Hyphal polarization and branching in *A. fumigatus*, as well as filamentation in *C. albicans*, have been shown to be crucial for virulence and biofilm formation, resulting in more stable biofilms (Brand, 2012; Riquelme, 2013). The inhibition of SPL metabolism disrupts the actin organization at the tip, impacting on normal hyphal growth and differentiation (Cheng et al., 2001). Moreover, a deprived quantity of SPLs results in a decrease of SPLs in lipid rafts with a subsequent reduction of plasma membrane-anchored proteins that participate in the maintenance of polarized growth (Momany, 2002). Although the compound is also active against planktonic fungal cells, all the major SPLs classes seem to be over-represented in the biofilm-organized cells (Lattif et al., 2011), suggesting a key role for SPLs in modulating biofilm formation.

To improve the delivery of myriocin, a highly lipophilic compound, Strettoi et al. (2010) explored the use of solid lipid nanocarriers in a mice model of retinitis pigmentosa. Similarly, other authors observed a decrease in the effective drug concentration compared with pure compound when using nanocarrier delivery in a cystic fibrosis mouse model (Caretti et al., 2014). By treating mice with intratrachea myriocin-loaded nanocarriers, Caretti et al. (2014) were able to achieve a reduction of lung infection and inflammation after *Pseudomonas aeruginosa* infection.

Due to the poor penetration of biofilm matrix by drugs, the same nanocarriers were investigated on fungal biofilms. Nanocarriers improved myriocin delivery into *A. fumigatus* biofilms, allowing its distribution within few hours even in bottom layers (Cirasola et al., 2014).

Due to its dual action, anti-inflammatory and antifungal, myriocin might represent a useful treatment for patients suffering from chronic diseases that increase the risk of fungal infections. However, deeper investigations into its administration need to be performed. Recently de Melo et al. (2013) observed that prophylaxis treatment with myriocin, in an invertebrate model of systemic candidiasis, reduces the insect survival (de Melo et al., 2013). The optimal scenario for the myriocin use could be late phases of fungal infection as well as pathological situations characterized by ceramide mediated hyper-inflammation. On the other hand, the development of myriocin derivatives as well as other compounds targeting downstream steps in the fungal

SPL synthesis could increase the specificity of these compounds against fungal enzymes avoiding host side effects.

Fulvic Acid

Humic substances are commonly found in decaying organic matter including plants, animal residues, sewage and soil (Snyman et al., 2002). Although fulvic acids account for ∼90% of all humic substances and their biological significance recognized for many years (van Rensburg et al., 2000), there is still minimal scientific understanding on which to support the claims of its biological properties. Oxifulvic acid, a derivate of fulvic acid, has been shown to elicit antibacterial and antifungal properties (van Rensburg et al., 2000). However, these formulations contain numerous toxic elements that make their use clinically impossible. Recently, there has been the development of a pure form of fulvic acid, carbohydrate derived fulvic acid (CHD-FA), that has been shown to be safe to use clinically and absent from environmental contaminants known to be harmful to the host (Gandy et al., 2011).

An initial randomized double blind controlled trial indicated that fulvic acid was well-tolerated in patients with eczema, where side effects were minimal and severity and erythema were significantly reduced compared with the placebo control (Gandy et al., 2011). A subsequent phase 1 clinical study carried out to determine the safety profile of CHD-FA, showed that this agent was able to elicit anti-inflammatory properties in addition to being non-toxic when used as an oral formulation (Gandy et al., 2012). This anti-inflammatory activity was also shown in a rat wound model, where the use of a topical cream enhanced wound healing and was non-toxic during both acute and chronic treatments (Sabi et al., 2012). However, so far the mechanism by which CHD-FA elicits the observed immunomodulatory effects is unknown.

Although the anti-inflammatory properties of CHD-FA have been studied, there are very few reports of the antimicrobial properties of this agent. Recent studies have shown CHD-FA to be fungicidal against *C. albicans* planktonic and sessile cells at similar concentrations, indicating good biofilm activity unlike azole antifungals (Sherry et al., 2012). Time-kill analysis of CHD-FA was performed in comparison to the other classes of antifungals, and whilst caspofungin achieved the greatest kill, CHD-FA elicited its maximum activity quicker than any of the other agents, which is of particular benefit in treating systemic infections such as candidemia, where delayed antifungal therapy coincides with mortality rates (Morrell et al., 2005). The rapid killing action was further analyzed by visualizing the uptake of propidium iodide by the cells, only feasible when the cell membrane has been compromised. Membrane damage was recorded as early as 10 min following CHD-FA exposure, which also correlates with the release of intracellular ATP from the cell (Sherry et al., 2012). To further test the hypothesis of a membrane active compound, the activity against the *C. albicans* cell membrane was investigated using a chitin synthase inhibitor. Chitin is a simple polysaccharide found in the cell walls of fungi that provides cell structure and rigidity (Lenardon et al., 2010). It was argued that if the cell membrane was the target of CHD-FA, then by weakening the cell by inhibiting its chitin production would increase the exposure of the cell membrane to the agent and would increase CHD-FA sensitivity (Sherry et al., 2012). Here it was demonstrated that *C. albicans* cells were hyper-susceptible to CHD-FA in the presence of a chitin synthase inhibitor, a finding that was also observed in voriconazole treated biofilms (Kaneko et al., 2010). Collectively, these data suggest that CHD-FA acts through disruption to the cell membrane. It is therefore feasible to suggest that this agent may have broad-spectrum antimicrobial activity against a variety of fungi and bacteria. Indeed, this was the case when CHD-FA was shown to possess antibacterial activity toward a range of oral bacterial biofilms, including an *in vitro* four-species periodontal biofilm model (Sherry et al., 2013).

Additionally, fulvic acid was shown to be minimally affected by characterized biofilm resistance mechanisms, including the extracellular matrix (ECM) and efflux pumps. For example, it is known that glucans within the cellular matrix hinder the penetration of azoles through biofilms, with the depletion of *FKS1*, encoding a β-1,3 glucan synthase, increasing the susceptibility of fluconazole within these communities (Nett et al., 2010a,b). Overexpression of *FKS1*, as well as a deletion mutant, was used to determine the impact of CHD-FA activity. Here it was shown that this agent's sensitivity was not compromised by the elevated expression of *FKS1*, which is in contrast to azoles, polyenes and echinocandins, where the matrix sequesters these agents and their activity is significantly reduced against *C. albicans* biofilms (Nett et al., 2010a).

Efflux pumps have been widely shown to play a role in azole resistance within *Candida* biofilms, particularly during early biofilm development both *in vitro* and *in vivo* (Ramage et al., 2002; Mukherjee et al., 2003; Nett et al., 2009). Although CHD-FA was shown to induce efflux pump activity in *C. albicans* biofilms, there was no change in the minimum inhibitory concentration (MIC) when an efflux pump inhibitor was used, demonstrating that CHD-FA activity is not compromised by these pumps unlike other antifungals (Sherry et al., 2012).

Overall, whilst our knowledge base for CHD-FA is relatively limited, it does appear to have appropriate biological properties of a broad-spectrum antimicrobial agent and not compromised by know biofilm resistance mechanisms, which has yet undefined immunomodulatory capacity. Further *in vitro* and *in vivo* studies are required to determine its safety profile.

Acetylcholine

Bi-directional neurochemical interactions occur between the host and colonizing microorganisms (Lyte, 2013, 2014a,b; Sandrini et al., 2015). Many microorganisms share neuro-endocrine mediator synthesis pathways and recognition receptors with their human hosts (Lyte, 2013). Therefore, it is hypothesized that there is constant communication between a vertebrate host and its microbiota, and a bi-directional influence on behavior (Freestone, 2013). However, many of the inter-kingdom signaling molecules and receptors, particularly from the fungal perspective, remain to be characterized in detail. Furthermore, the biological consequences of neuro-endocrine signaling in fungi, with respect to growth and pathogenicity, are only just beginning to be determined.

Acetylcholine (ACh) is widely distributed in both prokaryotic and eukaryotic cells. In mammalian systems, ACh has two major roles: (1) neuronal ACh acts as a neurotransmitter to mediate rapid communication between neurons and effector cells and (2) non-neuronal ACh acts as a local signaling molecule involved in the regulation of cellular phenotype, modification of ciliary activity, and modification of cell-cell contact (Wessler and Kirkpatrick, 2008). In recent years ACh has received greater attention due to the discovery of the "cholinergic anti-inflammatory pathway" that has been demonstrated to regulate immune responses (Borovikova et al., 2000). In this pathway, ACh released from efferent vagus nerve terminals interacts with the alpha 7 nicotinic receptor (α7nAChR) on proximal immune cells resulting in down regulated localized immune responses. In addition, the efferent vagus nerve interacts with the splenic nerve to activate a unique ACh-producing memory phenotype T-cell population, which can propagate ACh mediated immune-regulation throughout the body (Rosas-Ballina et al., 2011). Furthermore, as ACh is also produced by cells out with neural networks, non-neuronal ACh can also play a vital role in localized immune-regulation through its cytotransmitter capabilities (de Jonge et al., 2005; Macpherson et al., 2014). In addition, evidence also suggests that ACh signaling through other cholinergic receptor subtypes, such as the muscarinic receptors, can also modulate inflammatory responses in mammalian systems (Verbout and Jacoby, 2012).

Interestingly, in a recent study, ACh was found to play multiple roles in the pathogenesis of fungal infections in a primitive *Galleria mellonella* infection model. Specifically, ACh was found to: (i) inhibit *C. albicans* yeast–to-hyphae transition and biofilm formation; (ii) promote a rapid and effective cellular immune response to *C. albicans* infection; and (iii) regulate antifungal defenses to limit sepsis induced damage of host tissues (Rajendran et al., 2015). The fact that ACh can directly act on *C. albicans* to inhibit yeast–to-hyphae transition suggests that this organism possesses a functional ACh receptor. However, the ACh receptor(s) and the downstream signaling pathway(s) that are involved in inhibiting *C. albicans* yeast–to-hyphae transition have yet to be characterized in detail.

Sequencing of the *C. albicans* genome has suggested this organism possesses putative cholinergic receptor genes (Inglis et al., 2012). Furthermore, pharmacological evidence suggests that *C. albicans* may possess a receptor that is homologous to human muscarinic (M) receptors. Midkiff et al. (2011) demonstrated that the dopamine receptor antagonist clozapine could inhibit *C. albicans* budding-to-hyphal transition by inhibiting a component of the Efg1 pathway, upstream of the Gpa2 G-alpha subunit, which the authors hypothesized to be the Gpr1 G-protein-coupled receptor (GPCR). However, clozapine has a broad range complex pharmacological profile. Indeed, it is now known that clozapine is a weak dopamine D2 receptor inverse agonist/antagonist and has mixed agonist-antagonist properties on human muscarinic receptors, with strong evidence that it can act as a potent agonist of the M1 and M4 receptors in mammalian systems (Zorn et al., 1994; Olianas et al., 1997, 1999; Miller, 2009; Wiebelhaus et al., 2012). Therefore, it is interesting to speculate that the observed effects on *C. albicans* budded-to-hyphal transition in the study of Midkiff et al. (2011) may be in fact due to clozapine acting upon a putative *C. albicans* cholinergic receptor homologous to human muscarinic receptors. However, further research aimed at characterizing the cholinergic receptor mediated signaling pathways of *C. albicans* is required to confirm this hypothesis.

There is also substantial evidence to suggest that fungi can synthesize and release ACh (Horiuchi et al., 2003; Kawashima and Fujii, 2008). Indeed, sequencing of the *C. albicans* genome revealed this organism to possess putative genes for the enzymes responsible for ACh synthesis; choline acetyltransferase (ChAT) and carnitine acetyltransferase (CrAT; Inglis et al., 2012). However, the ACh synthesis machinery of *C. albicans* remains to be characterized. Furthermore, the biological functions of fungal derived ACh remain to be elucidated.

The fact that both *C. albicans* and its human host both synthesize ACh and possess cholinergic receptors lead to speculate that there is cholinergic mediated bi-directional communication between the two species *in vivo*. The role of this cholinergic bi-directional communication in the maintenance of health and/or the pathogenesis of *C. albicans* infections are at present unknown. The evidence to date suggests the host may utilize ACh to protect against candidiasis (Rajendran et al., 2015). Although, the fact that ACh can modulate host immunity (Tracey, 2010) and also mucosal integrity through the regulation of epithelial cell phenotype and cell–cell contact (Wessler and Kirkpatrick, 2008), may also suggest that *C. albicans* derived ACh may be a potential virulence factor. Either way, further research into the role of bi-directional cholinergic signaling mechanisms between *C. albicans* and the colonized host is required.

The preliminary data to date imply that cholinergic mechanisms may be rational novel therapeutic targets to prevent or treat candidiasis (Rajendran et al., 2015). Indeed, there are a number of pharmacological agonists and antagonists already marketed for the treatment of neurodegenerative disorders, cancers and chronic inflammatory diseases that target cholinergic receptors (Pohanka, 2012; Zoheir et al., 2012; Sales, 2013; Matera and Tata, 2014; Russo et al., 2014). Many of these molecules have already undergone extensive safety and efficacy testing in human trials. Therefore, one or more of these molecules may be worthy of investigation for the prevention or treatment of candidiasis and may offer novel therapeutic approaches beyond conventional antifungals.

Concluding Remarks

The opportunistic nature of fungal infections highlights the crucial role of the host immune system in regulating host–fungus interactions.

Humans suffer from a range of fungal biofilm diseases that cause high levels of morbidity and mortality. Conventional antifungal drugs have been demonstrated to ineffective against fungal biofilms, and alternative strategies are needed to overcome their intrinsic resistance.

Therefore molecules targeting both fungal biofilm formation and the host inflammatory response could represent a new therapeutic approach to treat fungal biofilm-related infections with broader implications for healthcare applications.

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Histatin 5 inhibits adhesion of *C. albicans* to Reconstructed Human Oral Epithelium

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Candida albicans is the most pathogenic fungal species, commonly colonizing on human mucosal surfaces. As a polymorphic species, *C. albicans* is capable of switching between yeast and hyphal forms, causing an array of mucosal and disseminated infections with high mortality. While the yeast form is most commonly associated with systemic disease, the hyphae are more adept at adhering to and penetrating host tissue and are therefore frequently observed in mucosal fungal infections, most commonly oral candidiasis. The formation of a saliva-derived protein pellicle on the mucosa surface can provide protection against *C. albicans* on oral epithelial cells, and narrow information is available on the mucosal pellicle composition. Histatins are one of the most abundant salivary proteins and presents antifungal and antibacterial activities against many species of the oral microbiota, however, its presence has never been studied in oral mucosa pellicle. The objective of this study was to evaluate the potential of histatin 5 to protect the Human Oral Epithelium against *C. albicans* adhesion. Human Oral Epithelial Tissues (HOET) were incubated with PBS containing histatin 5 for 2 h, followed by incubation with *C. albicans* for 1 h at 37°C. The tissues were then washed several times in PBS, transferred to fresh RPMI and incubated for 16 h at 37°C at 5% CO_2. HOET were then prepared for histopathological analysis using light microscopy. In addition, the TUNEL assay was employed to evaluate the apoptosis of epithelial cells using fluorescent microscopy. HOET pre-incubated with histatin 5 showed a lower rate of *C. albicans* growth and cell apoptosis when compared to the control groups (HOET alone and HOET incubated with *C. albicans*). The data suggest that the coating with histatin 5 is able to reduce *C. albicans* colonization on epithelial cell surfaces and also protect the basal cell layers from undergoing apoptosis.

Keywords: histatins, salivary proteins, mucosal pellicle, oral mucosa, *Candida albicans*

Introduction

Removable dentures provide edentulous patients with the rehabilitation of masticatory and esthetic functions (Dhir et al., 2007); however, one consequence of the continual use of dentures is the adhesion of microorganisms and biofilm formation (Lazarin et al., 2014). *Candida* sp. are opportunistic pathogens that are frequently isolated from the oral cavity, and its biofilms are often

associated with candidiasis (Gomes et al., 2011; Hahnel et al., 2012; Lazarin et al., 2014). These biofilms are extremely resistant to antimicrobial agents compared to planktonic microorganisms due to the presence of extracellular polymeric substance (EPS) generated by microorganisms themselves, which acts as an impervious and protective covering of biofilms. They are not only resistant to the action of most of the available antifungal substances, but they also resist the phagocytic action of our immune cells (Chandra et al., 2001b). Salivary pellicle is a thin, biological film of selective salivary proteins, lipids, and carbohydrates which coats oral surfaces and acts as an interface between the oral surface and the first layer of microorganisms. When the salivary proteins adsorb on the tooth surface, it is called acquired enamel pellicle (AEP). A mature AEP has more than 130 different proteins, ranging from protein to peptide size (Siqueira et al., 2007). The formation of a saliva-derived protein pellicle on the mucosa surface can provide protection against the colonization and invasion of *Candida albicans* on oral epithelial cells, which leads to candidiasis. In the literature, narrow information is available on the mucosal pellicle composition, and few studies have reported the presence of salivary proteins such as mucins, cystatins, IgA, amylase, and statherin (Bradway et al., 1992; Gibbins et al., 2013) on the oral epithelial cell.

Histatins are one of the most abundant salivary proteins and have been shown to be multifunctional in the oral cavity due to their strong antifungal and antibacterial activities against many species of the oral microbiota (Pollock et al., 1984). Histatin 5, for example, adsorbed as a protein integument on PMMA and hydroxyapatite, effectively inhibits *C. albicans* colonization (Vukosavljevic et al., 2012). Potentially, histatin 5 could be one of the salivary components in mucosal pellicle that protects the oral cavity against infections caused by pathogenic microorganisms. The purpose of this study was to assess the potential effect of histatin 5 human oral mucosa coating to protect epithelial cells against *C. albicans* colonization.

Materials and Methods

Microorganisms and Growing Conditions

Stock culture of *C. albicans* (ATCC 90028) maintained at −80°C was used in each experiment. After recovery, *C. albicans* was maintained on Sabouraud Dextrose Agar (SDA; BDTM Difco, Franklin Lakes, NJ, USA), stored at 4°C. A loopful of the stock culture of *C. albicans* was streaked onto SDA and incubated aerobically at 37°C for 48 h to prepare the yeast inoculum. One loopful of this young culture was then transferred to 20 mL of YNB broth (BioShop®, Canada Inc., Burlington, ON, Canada) supplemented with 50 mM glucose and incubated at 37°C for 21 h. Cells of the resultant culture were harvested, washed twice with PBS at pH 7.4, and centrifuged at 4,000 × *g* for 10 min. The final concentration was adjusted to 10^7 cells mL^{-1} (Chandra et al., 2001a; Pereira-Cenci et al., 2008).

Candida albicans Killing Assay

A total of 50 μL from the suspension was added to 50 μL of a 10-fold serial dilution of histatin 5 in a 96-well polystyrene microtiter plate (Corning Inc., Corning, NY, USA) with an initial concentration of 800 μg/mL. For the control group, 50 μL of *C. albicans* suspension was added to 50 μL of PBS. After 1.5 h of incubation at 37°C, 50 μL of suspension from the selected wells were diluted in 9 mL of PBS. After that, 25 μL aliquot of the diluted suspension was plated on SDA and incubated at 30°C for 48 h (Helmerhorst et al., 2006). Colony counting was used to assess cell viability (CFU mL^{-1}). This experiment was carried out in triplicate.

The Effect of Histatin 5 When Adsorpted on a Microtiter Plate Prior C. *albicans* Biofilm Formation

Prior to the *C. albicans* assay, histatin 5 (protein purity > 95%, GenScript, Piscataway, NJ, USA) was re-suspended in distilled water with a concentration of 15 μg/mL. A total volume of 200 μL of histatin 5 solution was added to each well of a 96-well polystyrene microtiter plate, and the wells were incubated for 2 h at 37°C under gentle agitation. The wells were then washed with distilled water to remove the non-adsorbed histatin 5, and subsequently used for the formation of *C. albicans* biofilm at different time periods: 90 min, 24 h, 48 h, and 72 h. Non-adherent *C. albicans* cells were removed by washing them with PBS. At each time period, the adherent cells were harvested from the microtiter plate and plated onto SDA as described above. This experiment was carried out in triplicate.

Effect of Histatin 5 When Incubated Over a 48 h *C. albicans* Biofilm Formation

A 48 h *C. albicans* biofilm was developed as described above. The only difference was the absence of histatin 5 as a solid surface. After the *C. albicans* biofilms formation, histatin 5 was added with different concentrations, ranging from 6.3 to 12,800 μg/mL. After 24 h of contact with histatin 5, the *C. albicans* were washed three times with PBS and the cells were harvested, which was followed by 10-fold serial dilutions from 10^{-1} to 10^{-4} and plated onto SDA. The experiment was carried out in triplicate.

For the three tests described above, the number of CFU mL^{-1} was calculated and the analyses of variance (ANOVA) follow by the Tukey Honestly Significant Difference (HSD) *post hoc* test was used to determine differences between means ($a = 0.05$).

Cell Culture

The cytotoxicity effect of histatin 5 was evaluated on gingival fibroblasts grown in Dulbeccco's Modified Eagle Medium (gibco® by life technologies), supplemented with antibiotic–antimycotic solution (Sigma–Aldrich) and 10% v/v fetal bovine serum (gibco® by life technologies). The culture was maintained at 37°C in an atmosphere of 5% CO_2 in 95% air. (Thermo Scientific, USA). Cells were cultured until reaching confluence (90%) and removed with trypsin (0.05%)/EDTA (0.02%), (gibco® by life technologies) in 1X PBS. The trypsin was inactivated by the addition of culture medium, and the cells were then subjected to centrifugation at 2000 rpm for 5 min, resuspended and re-plated. The medium was changed two to three times per week. Total viable cell counts were made in a Neubauer chamber (New Optics), and a suspension containing 2.0 × 10^4 cells/ml was

placed in 24 well plates (TPP) and incubated in a humidified atmosphere containing 5% CO_2 at 37°C for 48 h. After the incubation period, the culture medium was disposed, and attached cells remained at the bottom of the plates. A serial dilution of histatin 5 was performed using fresh culture medium, and 1 mL was added to each well. The plates were maintained at 37°C in an atmosphere of 5% CO_2 in 95% air for 24 h. Five wells were used for each experimental group. Five wells received only 1 ml of culture medium Dulbeccco's Modified Eagle Medium (gibco® by life technologies), supplemented with antibiotic–antimycotic solution (Sigma–Aldrich) and 10% v/v fetal bovine serum (gibco® by life technologies), which served as the negative control.

Cytotoxicity Assay (MTT)

Mitochondrial dehydrogenase activity was measured using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay from Sigma–Aldrich. After 24 h of cell growth in either control or test culture media, 100 μl of MTT stock solution (MTT, Sigma Chemical Co., St. Louis, MO, USA) was added to each well. The plates were incubated for 4 h at 37°C in 5% CO_2. After the incubation period, the cultures were removed from the incubator, and the resulting formazan crystals were dissolved by adding 100 μL of MTT solubilization solution (MTT, Sigma Chemical Co., St. Louis, MO, USA). Plates were then shaken until the crystals were completely dissolved and the absorbance was spectrophotometrically measured at a wavelength of 570 nm (Labsystems Multiscan Ascent, Thermo Labsystems, Vantaa, Finland). All experiments were performed three times.

The results were submitted to ANOVA and Bonferroni tests. In addition, the results were also evaluated in accordance with ISO standard 10993-5:

0: not cytotoxic (inhibition below 25%)
1: slightly cytotoxic (inhibition between 25 and 50%)
2: moderately cytotoxic (inhibition between 50 and 75%)
3: intensely cytotoxic (inhibition higher than 75%).

Human Oral Epithelium Infection

Reconstructed human oral epithelium (HOE) tissues (SkinEthic Laboratories, Lyon, France) were incubated overnight in 12-well polystyrene microtiter plates containing 1.0 mL of Maintenance Medium at 37°C in a humidified atmosphere with 5% CO_2. After the incubation period, the tissues were washed three times with PBS. Before the infection procedure, the HOE tissues were divided into four experimental groups ($n = 3$), with G1 and G2 incubated with PBS, and G3 and G4 incubated with histatin 5 (50 μg mL^{-1}). The incubation time was performed for 2 h at 37°C under 5% CO_2 (Vukosavljevic et al., 2012). Following, the tissues were washed three times with PBS and then transferred to a new microtiter plate containing 5 mL of RPMI-1640 medium (gibco® by life technologies) in each well. For G2 and G4, aliquots of 50 μL of 10^7 mL^{-1} C. *albicans* cells were transferred into each well and incubated for 60 min at 37°C in an orbital shaker at 75 rev min^{-1} for the adhesion phase. Tissues were then washed three times with PBS, transferred to a new microtiter plate filled with fresh RPMI-1640 medium and incubated for 24 h at 37°C with 5% CO_2. The same protocol was followed for G1 and G3 but without adding the microorganism.

Histology and Light Microscopy

The HOE tissues were excised around the circumference with a blade, fixed immediately in 4% buffered formalin, and then embedded in paraffin wax. For each HOE tissue, 30 of 3 μm paraffin wax sections were prepared. After deparaffinized in xylene, the sections were stained using hematoxylin and eosin (H&E) technique, mounted in DPX mountant (VWR, Lutterworth, UK), and examined by light microscopy with a Leica DFC295 camera connected to a Leica DM1000 microscope (Leica Microsystems, Wetzlar, Germany). To determine which cells were in apoptosis, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was assayed fluorescently using *in situ* cell death detection kit (Roche, West Sussex, UK) and HOE sections were imaged under the fluorescent microscope Zeiss Axio Imager M.1 Axio coupled with a Qimaging Retiga EXi CCD camera (Zeiss, Jena, Germany). Histological changes during infection were examined by microscopy at ×40 magnification.

Results

For the purposes of analysis, CFU mL^{-1} values were transformed into logarithm values (log10). The results of number of colony-forming units per milliliter (CFU mL^{-1}) were evaluated statistically by ANOVA. Tukey HSD *post hoc* test was used to determine differences between means ($a = 0.05$). **Figure 1** presents the mean (M) and standard deviation (SD) of the logarithm cell count in CFU/mL for the killing assay test. There were no statistically significant differences between the control and the group treated with 12.5 μg/mL of histatin 5. Moreover, the control and the group treated with 12.5 μg/mL of histatin 5 produced higher mean values of logarithm cell count. This was statistically different ($p = 0.00$) when compared to the other groups, which are statistically similar to each other (**Figure 1**).

The effect of histatin 5 when adsorbed to microtiter plate prior *C. albicans* biofilm formation was summarized in **Figure 2**. The logarithm cell counts in CFU/mL were compared with the two groups (control and group coated with histatin 5) at each time point. The results revealed that the group coated with histatin 5 in a concentration of 15 μg/mL previous to microorganisms colonization exhibited lower mean values of *C. albicans* for all time points (90 min, 24 h, 48 h, and 72 h) when compared with control group ($p = 0.00$). On the other hand, according to the results presented in **Figure 3**, when histatin 5 was incubated for 24 h over a prior formed 48 h *C. albicans* biofilm, there was no difference in cell counts in all tested concentrations of histatin 5 (6.3–12,800 μg/mL) when compared with each other and with the control ($p = 0.454$).

The ANOVA followed by Bonferroni test shows statistically significant differences between groups, where we can observe that the concentrations of 12800, 6400, and 3200 ug/mL are statistically similar and have the highest cytotoxicity by reducing the viability of the fibroblasts to up to 94%.

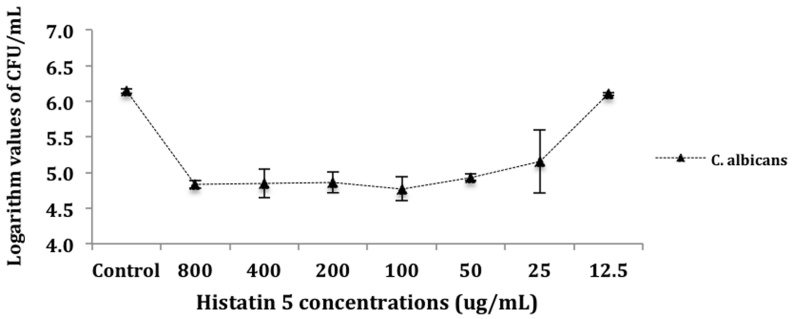

FIGURE 1 | Killing of *Candida albicans* by histatin 5. Cells were incubated for 1.5 h at 37°C with a dilution series of histatin 5 or without histatin 5 (control group). The dilutions were then plated in Sabouraud Dextrose Agar (SDA) media and the logarithm values of CFU/mL^{-1} were calculated.

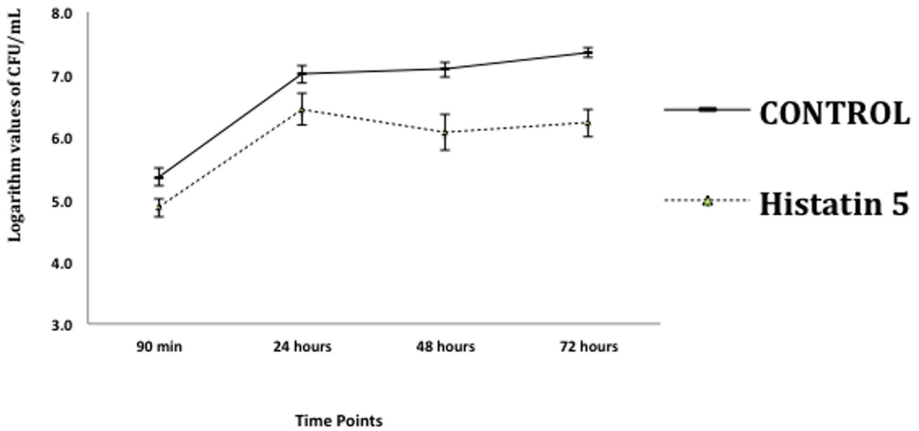

FIGURE 2 | Logarithm values of CFU/mL^{-1} when histatin 5, in a concentration of 15 μg/mL, is adsorpted on a microtiter plate prior to *C. albicans* biofilm formation in different stages of growth: 90 min, 24, 48 and 72 h, and their respective controls.

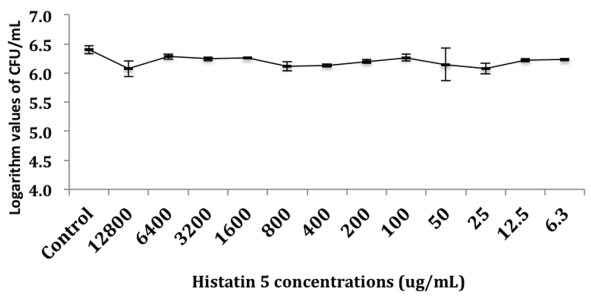

FIGURE 3 | Logarithm values of CFU/mL^{-1} when histatin 5 was in different concentrations, ranged from 12,800 to 6.3 μg/mL, were incubated for 24 h over a 48 h *C. albicans* biofilm.

The results show that concentrations ranging from 800 to 6.25 μg/mL have statistically similar values to the control group, may be considered non-cytotoxicity to human cells (**Table 1**).

After identifying the minimal concentration range needed to coat a solid surface with histatin 5 in order to obtain a significant inhibition effect on *C. albicans* biofilm, our approach was to evaluate the effect of histatin 5 adsorbed to HOE cells using a similar concentration. **Figure 4** shows that untreated HOE (G1) and the group treated with 50 μg/mL of histatin 5 (G3), both without *C. albicans*, exhibited a normal HOE morphology (**Figures 4A,C**). Our results also demonstrated that the epithelium cells treated according to G1 and G3 are not in apoptotic stage (**Figures 4E,G**).

In order to assess the potential implications of histatin 5 as a protein that is able to protect the oral epithelium against microorganism adhesion, the tissues from G2 and G4 were infected with *C. albicans*. As expected, examination of H&E stained tissue sections demonstrated efficient adherence of *C. albicans* on the outer layer of the reconstructed HOE (**Figures 4B,D**). For G2, which was not treated with histatin 5, a higher number of *C. albicans* in hyphae form (**Figure 4B**) was observed. Interestingly, for the group treated with histatin 5 (G4), we can observe that *C. albicans* cells are in the non-invasive form. These images were consistent with those observed with TUNEL assay, where the group treated with histatin 5 showed that the presence of the apoptotic cells were restricted to the outer layer (**Figure 4H**). In contrast, the group without histatin

TABLE 1 | Mitochondrial dehydrogenase activity of gingival fibroblast exposed to histatin 5 solutions.

Group	Mean	SD	% of inhibition
Control	0.230^{AB}	0.043	0
12800	0.015^{C}	0.002	94
6400	0.015^{C}	0.001	94
3200	0.025^{C}	0.003	89
1600	0.173^{B}	0.008	25
800	0.199^{AB}	0.013	14
400	0.220^{AB}	0.021	4
200	0.211^{AB}	0.025	8
100	0.231^{A}	0.017	0
50	0.204^{AB}	0.026	9
25	0.142^{AB}	0.020	7
12.5	0.221^{AB}	0.075	4
6.25	0.208^{AB}	0.076	9

Cytotoxicity mean values designated with vertically identical capital letters were not statistically different ($P > 0.05$).

5 clearly displays the presence of apoptotic cells in different layers.

Discussion

The processes that lead to the development of oral infections have been extensively studied. In the cases when there is balance between the virulence of a microorganism and the host ability to prevent microbial colonization, both host and microorganism can leave in a commensal state. On the other hand, when there is an imbalance, the oral cavity becomes an opportune environment for the development of infection diseases such as candidiasis, *C. albicans* being one of the most successful oral pathogens (Gow et al., 2012).

The fungicidal activity of histatin 5 has been shown to compromise residues 11–24 (Raj et al., 1990) where histatin 5 enters the *C. albicans* cell by involving specific receptors and/or driven by the transmembrane potential causing mitochondrial swelling, inhibiting the Krebs cycle, reducing the expression of an ATPase complex, and leading to a decrease in ATP production (Komatsu et al., 2011). The final result is the release of ATP and other essential energy storage molecules from the cell and cellular demise.

The data from killing assay (**Figure 1**) indicates that *C. albicans* are highly susceptible to histatin 5 solutions ranged from 800 to 25 μg/mL, leading to an approximated 2-log reduction in CFU mL^{-1}. Our results are in accordance with Konopka et al. (2010), which treated different strains of *C. albicans* with histatin 5, and concluded that in a concentration of 4.8 μM, histatin 5 was able to reduce the yeast growth by 50%. In addition, it was observed that there is no statistical significant difference between the solutions ranged from 800 to 25 μg/mL. This observation might be due to the fact that histatin precipitates at > 64 μg/mL (Pettit et al., 2005). According to Jori et al. (2006), the efficiency of a treatment can be expressed when it induces a 4-log decrease in the survival of microorganisms; however, herein is an *in vitro* study, and in the oral cavity histatin 5 is secreted continuously.

The effect of histatin 5 when adsorpted to microtiter plate prior *C. albicans* biofilm formation (90 min, 24, 48, and 72 h) are summarized in **Figure 2**. The reduction of *C. albicans* was significantly higher for the group treated with histatin 5, irrespective of the evaluated time point ($p = 0.00$). Interestingly, 48 h histatin 5 coating resulted in a reduction of biofilm development compared to the 24 and 72 h, suggesting that histatin 5 is effective in reducing *C. albicans* growth during a later stage. According to Pusateri et al. (2009), PMMA disks treated with histatin 5 did not present an effect in reducing biofilm until 72 h. Similar results showed that the amount of *C. albicans* initially attached to PMMA surface was not significantly different between the control group and a group treated with histatin 5 until after 24 h (Yoshinari et al., 2006). However, herein, in the initial stage of *C. albicans* adhesion (90 min), a decrease in the number of cells was observed, showing a clear effect of histatin 5. In another study, histatin 5 adsorbed on PMMA or hydroxyapatite effectively inhibited *C. albicans* adhesion in initial stages and continued this inhibitory effect after 24 h (Vukosavljevic et al., 2012).

When histatin 5 was incubated for 24 h over a prior formed 48 h *C. albicans* biofilm, even with high concentrations of histatin 5, no effect against the *C. albicans* was observed (**Figure 3**). Development of *C. albicans* biofilms are associated with an increasing presence of extracellular polysaccharides (EPS), which is known to physically interact with antibiotics and contributes to resistance against these drugs Hawser and Douglas (1995). Moreover, *Candida* sp. EPS has a hydrophobic characteristic, which can diminish the penetration of histatin 5 into *C. albicans* biofilm (Chandra et al., 2001b).

Merely the presence of *C. albicans* cannot be related to the candidiasis establishment. The change of yeast to hyphae is a critical step for the host invasion by *C. albicans* and colonization of host tissue (Berman and Sudbery, 2002). Interestingly, our study demonstrated that pre exposition of histatin 5 to oral epithelial cells diminished the adhesion of *C. albicans* to the epithelium. In addition, a change to hyphae form was significantly inhibited when histatin 5 was adsorbed to HOE. This outcome suggests that histatin 5 interfere in the not totally characterized mechanism of *C. albicans* adhesion on the oral mucosa.

Candida albicans has the ability to invade and damage oral epithelial cells, which is critical for infection establishment. Indeed, oral epithelial cells after 18 h of candidal infection demonstrate significant death prevalence. The invasion for *C. albicans* stimulates oral epithelial signaling pathways and causes early apoptotic cell death, which is followed by secondary necrosis (Villar and Zhao, 2010). Our results verified that pre incubation of histatin 5 to oral epithelium drastically decreased the oral epithelium apoptosis caused by *C. albicans*, which was restricted to the outer layer of HOE. This event can most likely be explained by inhibition of hyphae formation when histatin 5 is adsorbed to HOE. In addition, only histatin 5 in the tested concentration did not damage the epithelial cell, which suggests a low cytotoxicity effect of this protein against

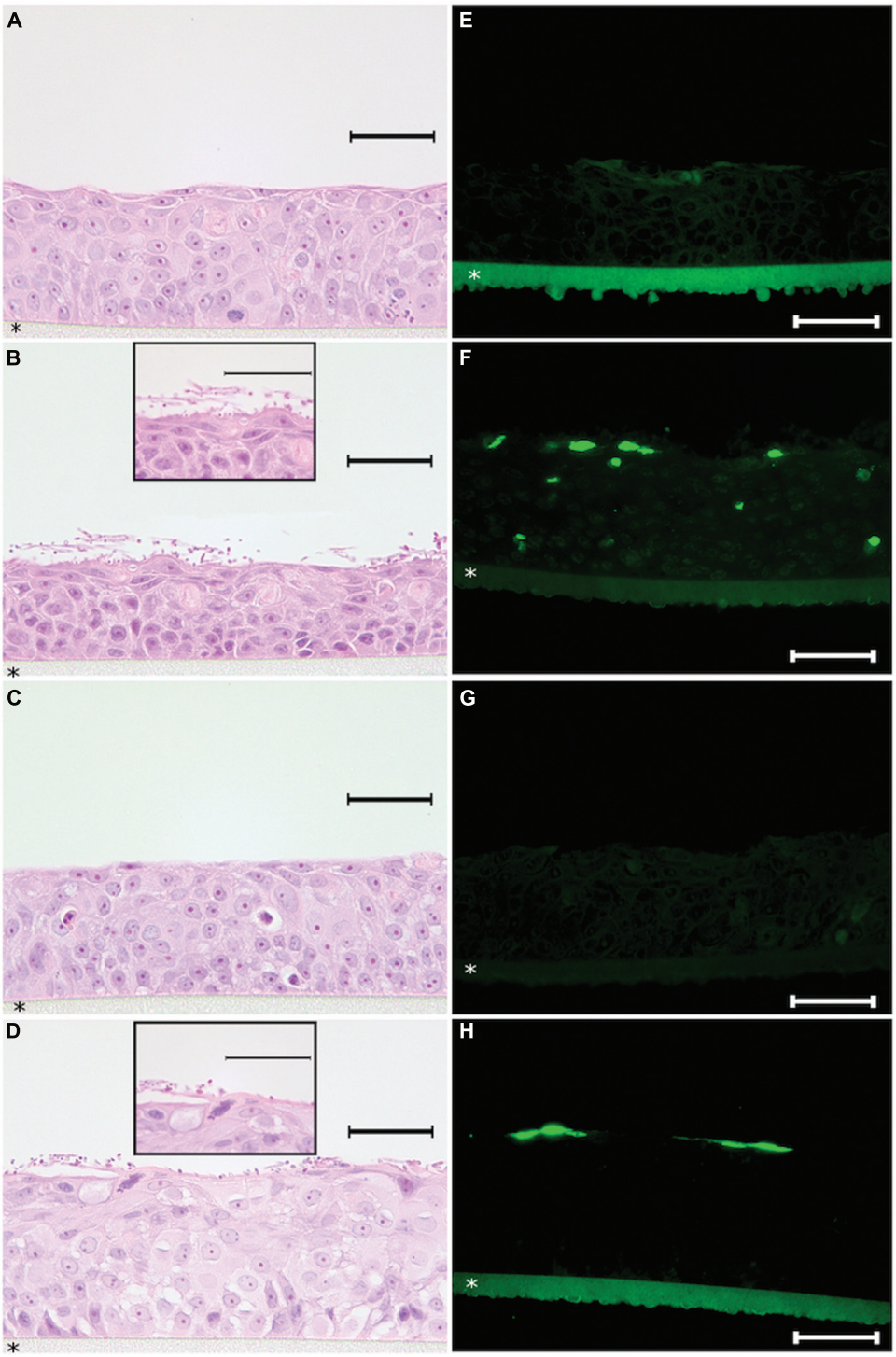

FIGURE 4 | Histopathology analysis of reconstructed human oral epithelium, exposed or not to histatin 5, both without *C. albicans*, exhibited a normal morphology (A,C) and no apoptotic cells (E,G). For the tissues exposed to *C. albicans*, hematoxylin and eosin (H&E) stained sections demonstrated efficient adherence of *C. albicans* on the outer layer of the reconstructed human oral epithelium (HOE; **B,D**). In the group which was not treated with histatin 5, a higher number of *C. albicans* in hyphae form **(B)** was observed. Interestingly, for the group treated with histatin 5 (50 μg ml^{-1}), we can observe that *C. albicans* cells are in the non-invasive form **(D)**. For the TUNEL assay, HOE treated with histatin 5 showed presence of apoptotic cells restricted to the outer layer **(H)**. In contrast, the group without histatin 5 clearly displays the presence of apoptotic cells in different layers **(F)**. Scale bars = 50 μm. All pictures were taken with $\times$40 magnification with exception of the details presents in figure **(B,D)** that are $\times$100 magnification. HOE is formed on polycarbonate filter (*).

host cell. Moreover, our cytotoxicity data showed that histatin 5 at the concentration of 50 μg/mL was able to cause an inhibition of 9% on cell viability of gingival fibroblast, and according to ISO standard 10993-5, a inhibition below 25% is considered not cytotoxic.

In the present study, different assays were used to quantify the activity of histatin 5 against *C. albicans* planktonic cells, biofilm and an *in vitro* formation of a histatin 5 oral mucosal pellicle. Histatin 5 in a physiological concentration was able to protect the HOE against *C. albicans* colonization and, at the same time, not interfere in the host cell homeostasis. This exciting outcome prepares a base for clinical research where the protection of the human oral mucosa against yeast infection could be evaluated by using a native protein.

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Chemosensitization of multidrug resistant *Candida albicans* by the oxathiolone fused chalcone derivatives

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Three structurally related oxathiolone fused chalcone derivatives appeared effective chemosensitizers, able to restore in part sensitivity to fluconazole of multidrug-resistant *C. albicans* strains. Compound **21** effectively chemosensitized cells resistant due to the overexpression of the *MDR1* gene, compound **6** reduced resistance of cells overexpressing the ABC-type drug transporters *CDR1/CDR2* and derivative **18** partially reversed fluconazole resistance mediated by both types of yeast drug efflux pumps. The observed effect of sensitization of resistant strains of *Candida albicans* to fluconazole activity in the presence of active compounds most likely resulted from inhibition of the pump-mediated efflux, as was revealed by the results of studies involving the fluorescent probes, Nile Red, Rhodamine 6G and diS-C_3(3).

Keywords: multidrug resistance, chalcones, antifungals, chemosensitization, *Candida albicans*

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Introduction

Opportunistic fungal infections in immunocompromised hosts have become an important clinical problem, with *Candida* species remaining one of the leading causes of hospital-acquired bloodstream infections. The attributable frequency of deaths from candidemia remains close to 40% and *Candida albicans* comprises nearly half of the isolated fungal pathogens (Pfaller and Diekema, 2007). The main factors determining high mortality from candidal infections are: a limited repertoire of clinically used antimycotics and an emerging appearance of drug resistance, including its multidrug form (Sanglard and Odds, 2002; Pfaller, 2012; Srinivasan et al., 2012). Among molecular mechanisms underlying multidrug resistance (MDR), the most important is an overproduction of membrane proteins belonging to the ATP-binding cassette (ABC) transporters or the major facilitator superfamily (MFS). A number of efflux pumps have been identified in fungi, including Cdr1p, Cdr2p, Mdr1p, and Flu1p in *C. albicans* (Prasad et al., 2002; Prasad and Goffeau, 2012). In view of these facts, the search for new antimycotics active against MDR fungi and/or chemosensitizers, i.e., compounds able to render MDR strains sensitive to clinically used antifungals, is an urgent need. Chemosensitization has been postulated as one of the ways of overcoming fungal resistance to the most popular triazole antifungals, including fluconazole (FLC). Reported examples of compounds effectively chemosensitizing FLC-resistant human pathogenic

fungi include Cdr1p/Cdr2p–specific curcumin (Sharma et al., 2009), ibuprofen (Ricardo et al., 2009), or cyclosporine (Marchetti et al., 2000), inhibitors of MFS-type drug transporters, like cerulenin analogs (Diwischek et al., 2009) or synthetic heterocycles containing a cyclobutene-dione core (Keniya et al., 2015) and clorgyline, targeting both types of fungal drug efflux pumps (Holmes et al., 2012).

Chalcones, compounds constituting a subclass of flavonoids, exhibit a number of biological effects, including antimicrobial activity (Dimmock et al., 1999; Nowakowska, 2007). Antifungal properties of some chalcones were demonstrated and it was suggested that the observed activity might be related to the inhibition of biosynthesis of cell wall components, β(1→3)glucan and chitin (López et al., 2001). It was also shown that some of the chalcone derivatives inhibited drug extrusion by the yeast drug transporters of the ABC type (Conseil et al., 2000; Wink et al., 2012).

We reported previously that a synthetic oxathiolone fused chalcone derivative AMG-148 exhibited *in* antifungal activity (Łącka et al., 2011). In the present communication, results of our studies on structural analogs of AMG-148, concerning especially their chemosensitizing effect on MDR yeast cells, are described.

Materials and Methods

Compounds and Reagents

The oxathiolone fused chalcone derivatives were synthesized as described (Konieczny et al., 2007a,b,c). Fluconazole was kindly provided by Pliva Krakow (Cracow, Poland). All other chemicals were from Sigma-Aldrich, St. Louis, MO.

Strains and Culture Conditions

The reference strain used in this study was *Candida albicans* ATCC 10231. Non-reference strains are listed in **Table 1**. *C. albicans* F2, F5, B3, B4, Gu4, and Gu5 clinical isolates (Franz et al., 1998, 1999) were kindly provided by J. Morschhäuser, Würzburg, Germany, while DSY2039 and DSY750 by D. Sanglard, Lausanne, Switzerland. *S. cerevisiae* AD1-8u$^-$ and US50-18C mutants AD1-3, AD12, AD13, and AD23 were kindly provided by A. Goffeau, Louvain-la-Neuve, Belgium. The AD-derived strains ADCDR1, ADCDR2, and ADMDR1 were constructed by the previously described methods (Gupta et al., 1998; Prasad et al., 1998; Smriti et al., 2002). Strains were grown at 30°C in Sabouraud medium (2% glucose, 1% yeast extract, and 2% bactopeptone) and stored on Sabouraud plates containing 2% agar.

Susceptibility Testing Procedures

MIC values of tested compounds were determined in RPMI-1640 medium by the slightly modified serial dilution microtiter plate method recommended by CLSI (Clinical Laboratory Standards Institute, 2008). Turbidity in individual wells was measured with a microplate reader (Victor^{3}V, Perkin Elmer). The MIC was defined as the lowest drug concentration at which at least 80% decrease in turbidity, in comparison to the drug-free control, was observed.

TABLE 1 | Non-reference yeast strains used in this study.

Strains	Description	Source/ references
SACCHAROMYCES CEREVISIAE		
US50-18C	*MATα, PDR1-3, ura3, his1* (parent strain)	Balzi et al., 1987
AD1-8u$^-$	*MATα, PDR1-3, ura3, his1, Δyor1::hisG, Δsnq2::hisG, Δpdr5::hisG, Δpdr10::hisG, Δpdr11::hisG, Δycf1::hisG, Δpdr3::hisG, Δpdr15::hisG*	Decottignies et al., 1998
ADCDR1	AD1-8u$^-$ transformed with *CaCDR1*	Smriti et al., 2002
ADCDR2	AD1-8u$^-$ transformed with *CaCDR2*	Smriti et al., 2002
ADMDR1	AD1-8u$^-$ transformed with *CaMDR1*	Gupta et al., 1998
AD1-3	*MATα, PDR1-3, ura3, his1, Δyor1::hisG, Δsnq2::hisG, Δpdr5::hisG*	Decottignies et al., 1998
AD12	*MATα, PDR1-3, ura3, his1, Δyor1::hisG, Δsnq2::hisG*	Decottignies et al., 1998
AD13	*MATα, PDR1-3, ura3, his1, Δyor1::hisG, Δpdr5::hisG*	Decottignies et al., 1998
AD23	*MATα, PDR1-3, ura3, his1, Δsnq2::hisG, Δpdr5::hisG*	Decottignies et al., 1998
***CANDIDA ALBICANS* CLINICAL ISOLATES**		
Gu4	Fluconazole sensitive	Franz et al., 1998
Gu5	Fluconazole-resistant due to the overexpression of *CDR1* and *CDR2*	Franz et al., 1998
F2	Fluconazole sensitive	Franz et al., 1999
F5	Fluconazole-resistant due to the overexpression of *CaMDR1* and *ERG11*	Franz et al., 1999
B3	Fluconazole sensitive	Franz et al., 1998
B4	Fluconazole-resistant due to the overexpression of *CaMDR1*	Franz et al., 1998
DSY2039	Fluconazole sensitive	D.S.[a]
DSY750	Fluconazole-resistant due to the overexpression of *CaMDR1*	D.S.

[a] *strains provided by Dominique Sanglard, Lausanne, Switzerland.*

The same conditions were applied for quantification of an antifungal effect of chalcones in combination with Fluconazole (FLC), using the checkerboard microdilution assay. The final concentrations of chalcones ranged from 2 to 64 μg/mL for all chalcones but **11**, for which the concentration range was 0.0625 to 2 μg/mL. FLC was tested in the 0.03125–8 μg/mL range. The data obtained by the checkerboard microdilution assays were analyzed using the model-fractional inhibitory concentration (FIC) index method based on the Loewe theory. The FIC index is defined as the sum of the MIC of each drug when used in combination divided by the MIC of the drug used alone. Synergy and antagonism were defined by FIC indexes of $\leq$0.5 and $>$4, respectively. A FIC index value $>$0.5 but $\leq$4 was considered indifferent (Odds, 2003).

ATPase Activity Assay

The ATPase activity of the plasma membrane fractions was measured in terms of oligomycin-sensitive release of inorganic phosphate, as described previously (Smriti et al., 2002), either alone or in the presence of compounds tested.

Quantification of Energy-dependent Rhodamine 6G Efflux

Preparation of yeast cells was performed as described previously (Sharma et al., 2009). Rhodamine 6G (R6G) solution was added to 1 ml aliquots of 2% cell suspension in PBS (to get the 10 μM final concentration of R6G) along with the compound tested and the mixtures were incubated for 1 h at 30°C. The cells were washed twice with PBS and re-energized by re-suspending them in 1 ml of PBS containing 2% glucose and incubated at 30°C for 30 min. After incubation, the samples were centrifuged at 9000 × g for 2 min and absorption of the supernatant was measured at 527 nm.

Nile Red Accumulation Assay

The accumulation of Nile Red (NR) was determined by modification of the method described elsewhere (Ivnitski-Steele et al., 2009) and measured with a FACSort flow cytometer (Becton-Dickinson Immunocytometry Systems, San Jose, CA). Exponential phase yeast cells were collected, washed 3 × with water and suspended in PBS, pH 7.4, containing 2% glucose to the final cell density 2% (w/v). The NR solution was added to 1 ml portions of the cell suspension in PBS/glucose to get the 7 μM final concentration of NR, along with the compound tested. After 30 min incubation at 30°C, the samples were excited with a 488-nm laser and PE–Texas Red filter was used to detect NR-derived fluorescence. The mean fluorescence intensity was calculated using the histogram stat program. Analysis was performed with the CellQuest software (Becton-Dickinson Immunocytometry Systems).

DiS-C_3(3) Accumulation Assay

Fluorescence measurement of diS-C_3(3) accumulation in cells was performed using the procedure described previously (Hendrych et al., 2009). Briefly, the fluorescent probe diS-C_3(3) (final concentration 2×10^{-8} M) was added to the cell suspension 10 min after compounds tested and fluorescence emission spectra of the cell suspensions were measured ($\lambda_{ex} = 531$ nm) at the time of staining. In each experiment, the CD cocktail (5 μM CCCP plus 10 μM DM-11) was added, usually after 40 min of staining.

Transmission Electron Microscopy

C. albicans cells from the overnight cultures were harvested, washed and suspended in Sabouraud medium to the final cell density of $\approx 10^6$ cfu/mL. The compounds tested were added and cultures were incubated for 9 h at 30°C. For ultrastructural studies, the cells were fixed with 2% glutaraldehyde in 0.1% phosphate buffer for 3 h at 25°C, washed with 0.1 M phosphate buffer (pH 7.2) and post-fixed with 1% OsO_4 in 0.1 M phosphate buffer for 1 h at 4°C. Samples were dehydrated with graded acetone, cleared with toluene, infiltered consequently with toluene and araldite mixture at room temperature and pure araldite at 50°C and finally embedded in an Eppendorff tube with pure araldite mixture at 60°C. Semithin and ultrathin section cutting was done with ultramicrotome (Ultramicotome Lecia EM UC6). Sections were taken on the 3.05 mm diameter, 200 mesh copper grid, stained with uranyl acetate.

Results

Growth Inhibitory Effect of Chalcone Derivatives

In the previous study, AMG-148, an oxathiolone fused chalcone derivative, was found to exhibit *in vitro* antifungal activity against several strains of human pathogenic yeasts, with MIC values within the range of 1–16 μg/mL and a fungicidal effect was observed at concentrations 4–32-fold higher than the MICs (Łącka et al., 2011). In this work, a growth inhibitory effect of AMG-148 (here compound **11**) was compared to that of its 26 structural analogs, using the serial dilution microtiter plate method employing *C. albicans* ATCC 10231 as a reference microorganism. Results presented in **Table 2** indicate that all compounds but **11** exhibited poor anticandidal activity, with MICs in the 64 − >256 μg/mL range. MIC of the known antifungal drug FLC in this assay was 2 μg/mL.

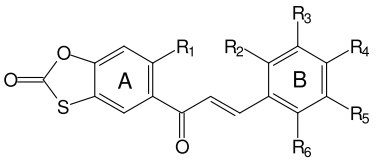
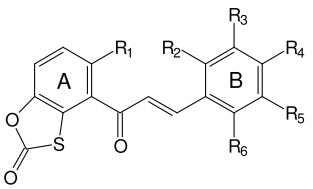
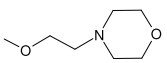
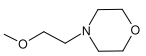
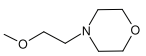
Combined Antifungal Effect of Chalcone Derivatives and Fluconazole

Antifungal effect of 11 chalcone derivatives with MIC values ≤ 64 μg/mL (**Table 2**) in combination with FLC was quantified using the checkerboard serial dilution assay. The only case of a slight synergistic effect was noted for combination of FLC with compound **11**, where a FIC index = 0.22 was determined. For combinations of all the other 10 chalcones tested with FLC, the FIC indexes were in the 0.92–1.36 range, thus indicating neither synergy nor antagonism.

Modulation of Multidrug Resistance

Some natural flavonoids and their synthetic derivatives were reported to be effective modulators of microbial multidrug resistance (Ivanova et al., 2008; Liu et al., 2008; Sharma et al., 2010). To check whether chalcones tested in this work were able to restore the antifungal potency of FLC against FLC-resistant human pathogenic yeasts, an *in vitro* assay was performed employing *C. albicans* clinical isolates resistant to fluconazole, due to the FLC-induced overexpression of genes encoding multidrug efflux pumps. The Gu5 and B4 isolates are FLC-resistant, due to the documented overexpression of *CDR1* and/or *CDR2* in the former and *MDR1* in the latter. Their FLC-sensitive counterparts, Gu4 and B3, respectively, exhibit a basal expression of these resistance genes. The antifungal activity of FLC against *Candida* isolates was determined in the presence of a fixed concentration of each chalcone. All compounds were tested at concentrations that did not interfere with fungal growth (< 1/2MIC; 0.5 μg/mL for **11** and 25 μg/mL for the other compounds). Sixteen out of twenty seven chalcones did not show any effect but the remaining 11 were able to decrease the MIC_{FLC} value of at least one of the FLC-resistant isolates (**Table 3**). Eight derivatives demonstrated ability to enhance sensitivity of *C. albicans* B4 to FLC. This effect was significant in the case of compounds **11**, **18,** and **21**. Seven compounds were able to enhance sensitivity of *C. albicans* Gu5 to FLC, however this change was significant only for compounds **6** and **18**. The chemosensitizing efficiency of compounds **6**, **18,** and **21** is thus comparable to that of the known chemosensitizers of fungal drug efflux pumps, verapamil and trifuoperazine. On the other hand,

TABLE 2 | Fungistatic activity of oxathiolone-fused chalcones.

Compound	Structure						MIC[a] (μg mL^{-1}) *C. albicans* ATCC 10231
	R_1	R_2	R_3	R_4	R_5	R_6	
Type 1							
1	$-OCH_3$	–H	–H	–H	–H	–H	64
2	$-OCH_3$	–H	–H	–H	–Cl	–H	64
3	$-OCH_3$	–H	–H	$-OCH_2CH_2N(C_2H_5)_2$	–H	–H	64
4	$-OCH_2CH_2N(C_2H_5)_2$	–H	–H	–H	–H	–H	128
5	$-OCH_2CH_2N(C_2H_5)_2$	–H	–H	–Br	–H	–H	128
6	$-OCH_2CH_2N(C_2H_5)_2$	–H	–H	$-OCH_3$	–H	–H	64
Type 2							
7	$-OCH_3$	–H	–H	$-OCH_3$	$-OCH_3$	–H	>256
8	$-OCH_3$	–H	–H	$-N(CH_3)_2$	–H	–H	>256
9	$-OCH_3$	–H	–H	$-NO_2$	–H	–H	>256
10	$-OCH_3$	–H	–H	–H	–Cl	–H	>256
11	$-OCH_3$	–H	–H	$-OCH_2CH_2N(CH_3)_2$	–H	–H	2
12	$-OCH_2CH_2N(C_2H_5)_2$	–H	–H	–Cl	–H	–H	128
13	$-OCH_2CH_2N(C_2H_5)_2$	–H	–H	–H	–Cl	–H	128
14	$-OCH_2CH_2N(C_2H_5)_2$	–H	–H	–H	–H	–Cl	128
15	$-OCH_2CH_2N(C_2H_5)_2$	–H	–H	$-OCH_3$	–H	–H	64
16	$-OCH_2CH_2N(C_2H_5)_2$	–H	–H	$-OCH_2CH_2N(CH_3)_2$	–H	–H	64
17	$-OCH_2CH_2N(C_2H_5)_2$	–H	–H	–H		–H	64
18	$-OCH_3$	–H	–H	$-OCH_2CH_2CH_2N(CH_3)_2$	$-OCH_3$	–H	64
19	$-OCH_2CH_2CH_3$	–H	–H	–H		–H	128
20	$-OCH_3$	–H	–H	–H		–H	128
21	$-OCH_2CH_2CH_3$	–H	–H	$-OCH_2CH_2N(CH_3)_2$	–H	–H	64
22	$-OCH_2CH_2N(C_2H_5)_2$	–H	$-OCH_3$	$-OCH_3$	$-OCH_3$	–H	128
23	$-OCH_2CH_2N(C_2H_5)_2$	–H	–H	$-OCH_3$	$-OCH_3$	–H	128
24	$-OCH_2CH_2CH_3$	–H	–H	$-OCH_3$	–H	–H	128
25	$-OCH_2CH_2CH_3$	–H	–H	$-OCH_3$	$-OCH_3$	–H	128
26	$-OCH_2CH_2CH_3$	$-OCH_3$	–H	$-OCH_3$	–H	$-OCH_3$	128
27	$-OCH_2CH_2N(CH_3)_2$	–H	–H	$-OCH_3$	–H	–H	64

[a]*MICs were determined in RPMI-1640 buffered medium, as described in Materials and Methods.*

the observed substantial reduction of MIC_{FLC} of the B4 strain in presence of **11** may result from chemosensitization, but at least in part could be also attributed to the observed synergism between FLC and **11** as antifungals.

Several lower concentrations of compounds listed in **Table 3** were examined in order to find the lowest concentrations at which the FLC-sensitizing effect was observed. In the case of *CDR1/CDR2*-overexpressing *C. albicans* Gu5, a two-fold

TABLE 3 | Influence of chalcones on MIC_{FLC} values determined for *Candida albicans* clinical isolates.

Compound	MIC of FLC (μg/mL)[a]			
	B3	B4	Gu4	Gu5
–	1	16	4	256
5	1	4	4	256
6	1	16	4	**32**
11	0.5	**1**	2	128
15	1	8	4	64
18	1	**2**	4	**32**
19	1	8	4	256
20	1	16	4	128
21	1	**2**	4	64
22	1	16	4	128
23	1	8	4	128
25	1	8	4	128
VP[b]	1	**0.5**	4	64
TFP[b]	1	**1**	4	128

[a] *MIC values for FLC were determined by the serial dilution method as described Materials and Methods, in the presence of a fixed concentration of a compound tested (0.5 μg/mL for **11** and 25 μg/mL for the other compounds).*
[b] *Verapamil (50 μg/mL) and trifluoroperazine (20 μg/mL) were used as positive controls. Cases of significant (>4-fold) MIC_{FLC} reduction by a given compound are highlighted in bold.*

TABLE 4 | Activity of compounds 6, 18, 21 and fluconazole against MDR *C. albicans* clinical isolates and their drug-sensitive counterparts.

Strain	MIC/IC_{50}^{a} (μg/mL)			
	6	18	21	FLC
C. albicans B3	64/38.5	64/40.0	64/42.8	1
C. albicans B4 (MDR1)	64/37.3	64/41.2	64/43.9	16
C. albicans Gu4	32/24.2	64/36.6	64/38.4	4
C. albicans Gu5 (CDR1/CDR2)	64/35.5	64/36.2	64/39.6	256

[a] *MICs and IC_{50}s were determined by using RPMI-1640 buffered medium, as described in Materials and Methods.*

reduction of MIC_{FLC} was found for **6** at 5 μg/mL, while **18** did the same at 6.25 μg/mL. In the case of *C. albicans* B4, compounds **21** and **18** caused the twofold reduction of MIC_{FLC} at 0.25 μg/mL and 0.1 μg/mL, respectively.

Compounds **6**, **18,** and **21** were also tested for their intrinsic antifungal activity against FLC-resistant and FLC-sensitive *C. albicans* clinical isolates. Comparison of MIC or IC_{50} values determined for B3/B4 and Gu4/Gu5 pairs indicates, how the enhanced activity of a particular transporter affects drug susceptibility. Data presented in **Table 4** confirm resistance of B4 and Gu5 strains to FLC. On the other hand, the antifungal activity of chalcones was in most cases not affected by presence/absence of drug transporters, except for the slight effect observed for compound **6** in the case of the Gu4/Gu5 pair, while no difference in MIC values was found for **18** and **21**. These results suggest that **18** and **21** are not effluxed by both ABC-type and MFS-type drug transporters of *C. albicans*, while **6** may be a poor substrate of Cdr1p or Cdr2p but not of Mdr1p.

Effect on ATPase Activity of Cdr1p/Cdr2p

The effect of selected compounds (**6**, **21,** and **18**) on the ATPase activity of Cdr1p/Cdr2p was studied by determination of the oligomycin-sensitive ATP hydrolysis by plasma membrane preparations isolated from *C. albicans* Gu5 clinical isolate overproducing the ABC pumps. No significant reduction of the ATPase activity in presence of the tested compounds was found up to 50 μg/mL. A very slight reduction, about 20% was noted for **18** (50 μg/mL), however at 25 μg/mL the reduction was lower than 5%. It seems therefore that the oxathiolone-fused chalcones studied are not inhibitors of the ATPase activity of the ABC-type *C. albicans* drug transporters.

Changes in Membrane Potential and Cell Integrity Monitored with the diS-C_3(3) Probe

Using a set of five isogenic mutant strains, the effect of selected chalcone derivatives on membrane potential and activity of Pdr5p and Snq2p ABC-type drug exporters in *S. cerevisiae* was tested by the fluorescence method, with diS-C_3(3) as a probe. Intracellular accumulation of the probe is accompanied by a gradual shift of its λ_{max} toward longer wavelengths (red shift), while any possible efflux results in a blue shift. DiS-C_3(3) is a substrate for both Pdr5p and Snq2p (Čadek et al., 2004; Hendrych et al., 2009), so that comparison of the probe accumulation curves obtained for Pdr5p- and/or Snq2p-expressing and Pdr5p- and Snq2p-deficient cells measured in the presence of any compound may provide information about its effect on a given drug efflux pump. On the other hand, analysis of the level of staining of pump-deficient cells treated with any compound may reveal its influence on membrane potential, as the blue shift indicates plasma membrane depolarization, while a red shift is usually a consequence of hyperpolarization or permeabilization of the cell membrane. Finally, the cell destruction upon the action of any compound may be confirmed by the consequences of inclusion of the CD cocktail (5 μM CCCP with 10 μM DM-11) into the diS-C_3(3) assay. Addition of the lipophilic, weak acid (CCCP) plus the H^+-ATPase blocker (DM-11) results in the rapid blue shift for the suspension of intact cells, while the shift does not occur if the cells are broken.

Selected chalcones **6**, **11**, **18,** and **21** were tested in a broad range of concentrations, from 0.1 μM to 20 μM. The representative staining curves obtained for chalcone derivatives are presented in **Figure 1**. Cells treated with **11** (**Figure 1A**) demonstrated the highest initial rate of staining, indicating rapidly increasing cell surface permeability for the probe. It should be noted that the magnitude of the red shift induced by **11** action on AD1-3 cells (drug efflux pump-free) was concentration-dependent and was observed even at concentration as low as 0.1 μM (graphs not shown). Addition of the CD cocktail caused lower drop of λ_{max}, indicating partial cell damage.

Three compounds, **6**, **18,** and **21**, caused hyperpolarization of the cell membrane but did not damage the cells. Increased staining of pump-expressing cells after their exposure to

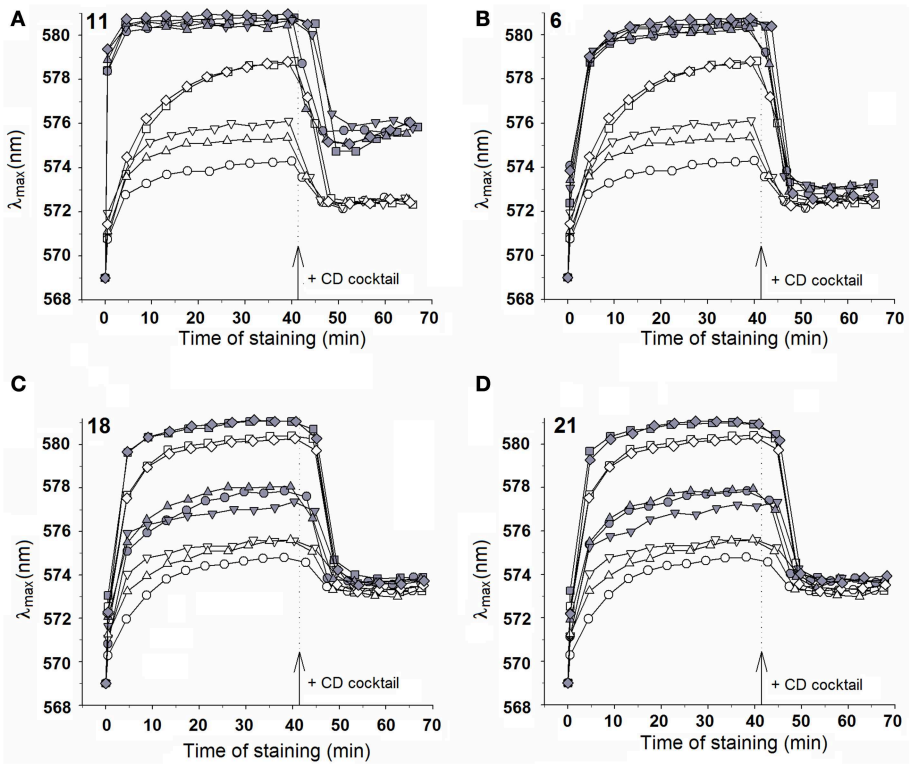


FIGURE 1 | Effect of selected compounds on the membrane potential and activity of MDR pumps of *S. cerevisiae*. Staining curves of AD1-3 (squares), AD23 (diamonds), AD12 (circles), AD13 (inverted triangles), and US50-18C (triangles) cells. Empty symbols–no compound added; full symbols–compounds added 10 min before diS-C_3(3) at following concentrations: 1 μM **(A)**, 10 μM **(B–D)**. Dotted lines with arrows indicate the addition of the CD cocktail.

compounds in comparison to chalcone-free controls was caused by both hyperpolarization and inhibition of the probe export. A strong inhibition of diS-C_3(3) efflux by **6** and **11** was observed in the case of cells expressing Pdr5p or Snq2 (**Figures 1A,B**), presence of chalcones **18** and **21** led only to a partial inhibition of the probe export (**Figures 1C,D**).

Effect of Chalcone Derivatives on Nile Red Accumulation

Nile Red (NR) is a fluorogenic substrate of *C. albicans* ABC transporters Cdr1p and Cdr2p and the MFS transporter Mdr1p (Ivnitski-Steele et al., 2009). The probe was used in a flow cytometry-based assay to measure influence of chalcone derivatives on NR accumulation in yeast cells. Biological models used in these studies were: the *Saccharomyces cerevisiae* AD1-8u$^-$ strain and its fluconazole-resistant transformants: ADCDR1, ADCDR2, and ADMDR1, along with the matched pairs of clinical *Candida* isolates, F2/F5, Gu4/Gu5, and DSY2039/DSY750. Cells were loaded with NR and levels of fluorescence derived from NR accumulated by chalcone treated pump-expressing cells was compared to that of the pump-deficient cells. As shown in **Figure 2**, significantly lower level of NR-derived fluorescence was measured in all resistant cells, comparing to their pump-deficient counterparts, what indicates an active efflux of NR from the former. The ADMDR1 cells accumulated approximately tenfold more, the ADCDR2 cells threefold more and ADCDR1 twofold more of NR in the presence of compound **18** at 70 μM (∼ 28 μg/mL) than the AD1-8u$^-$ cells. Significant accumulation NR in ADMDR1 was also induced by **21**. Accumulation of NR in ADCDR1 and ADCDR2 cells remained unaffected by **11**. Further studies showed that **21** and **18** at concentrations as low as 0.5 μg/mL still strongly inhibited NR efflux from ADMDR1, causing a twofold higher accumulation of the probe in comparison AD1-8u$^-$ (data not shown). Compound **6** at 70 μM caused significant accumulation of NR exclusively in ADCDR2 and ADCDR1 (2.5 × and 2 ×, respectively in comparison to AD1-8u$^-$), with no effect on ADMDR1 (details not shown).

The inhibitory effect of **21** and **18** on MDR1p-mediated efflux was confirmed in the model of clinical *Candida* isolates. Compound **21** inhibited NR efflux only from the cells of the F5 and DSY750 strains overexpressing the *MDR1* gene, where respectively fivefold and twofold increase in NR-derived fluorescence was observed. Surprisingly enough, compound **18** was also found to interfere only with Mdr1p-mediated efflux. In F5 cells, accumulation of NR increased two times and in DSY750, three times. Both compounds were not able to inhibit NR efflux from Gu5 isolate overproducing Cdr1p and Cdr2p proteins. Some accumulation of NR in Gu5 but not in F5 and DS750 was observed in the presence of **6** (details not shown).

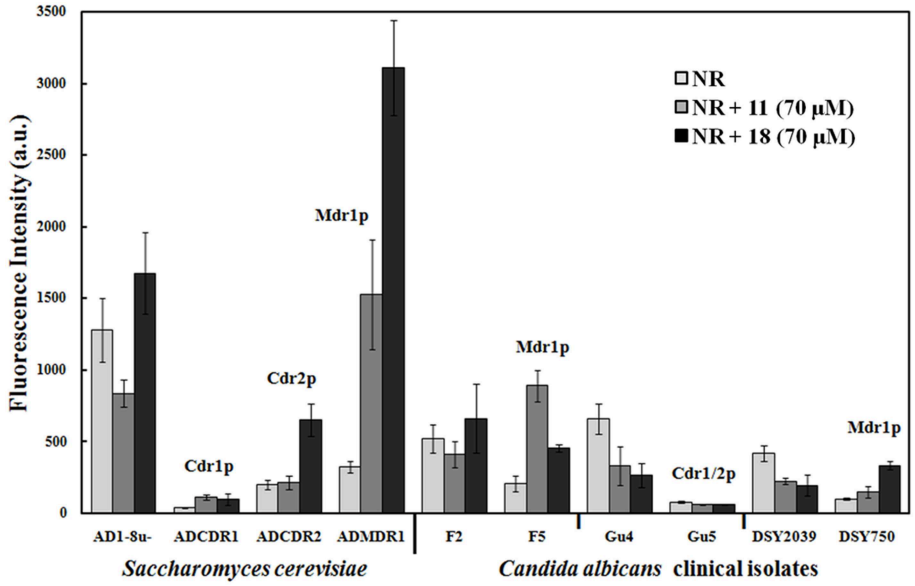


FIGURE 2 | Influence of selected chalcones on Nile Red accumulation in drug efflux pump-free and MDR yeast cells. Cells were incubated for 30 min with Nile Red and chalcones and then fluorescence was measured with a flow cytometer. Values are the means of three independent experiments. Bars represent SD.

Interestingly, in all sensitive strains, presence of **21** resulted in lower NR accumulation than presence of **18**.

Effect of Chalcones on Rhodamine 6G Efflux

In order to get more data characterizing chalcone derivatives as substrates of membrane multidrug transporters, their effect on efflux from yeast cells of another probe, Rhodamine 6G (R6G), which is a known substrate of Cdr1p/Cdr2p but not of the Mdr1p transporter, was investigated. *Saccharomyces cerevisiae* ADCDR1, ADCDR2 and control AD1-8u$^-$ cells were first de-energized in presence of 2-deoxy-D-glucose and 2,4-dinitrophenol, then loaded with R6G (final concentration 10 μM) along with a compound tested and subsequently activity of drug-effluxing ABC-type proteins was triggered by glucose addition. Concentration of the effluxed R6G was determined after 30 min in supernatants obtained after cell harvesting.

The ADCDR1 and ADCDR2 cells extruded five times more R6G than the AD1-8u$^-$ cells, thus confirming that this compound is indeed a substrate of the Cdr1p and Cdr2p drug transporters. Presence of compounds **6**, **18,** and **21** at 100 μM (∼ 40 μg/mL) did not change the amount of R6G released from AD1-8u$^-$ cells. Addition of **21** at 100 μM to the suspension of ADCDR1 or ADCDR2 cells inhibited R6G efflux only in about 5 ± 9% and 8 ± 4%, respectively, whereas presence of **6** and **18** at the same concentration resulted in 45 ± 10% and 38 ± 9% inhibition of the probe efflux from ADCDR2 and in 27 ± 8% and 33 ± 6% inhibition of R6G export from ADCDR1, compared to the untreated cells. These results corresponded well with those from the Nile Red assay and showed that compounds **6** and **18** may block to some extent the Cdr1p/Cdr2p-mediated efflux from recombinant *S. cerevisiae*, while presence of **21** had almost no effect on the activity of this drug transporter.

Influence of Chalcones on Cell Wall Structure

The effect of selected chalcones on morphology and ultrastructure of *C. albicans* cells was investigated using transmission electron microscopy (TEM). The morphological alterations observed in cells treated with **6, 11, 18,** or **21** at 10 μg/mL were documented by microphotographs and some of these photos are shown in **Figure 3**. The cross-section of untreated cells reveals a typical morphology with an intact cell wall and cytoplasmic membrane, separated by a low-density space (**Figure 3A**). Treatment of cells with compounds **6, 18,** and **21** did not cause any visible changes, as the cross-sections of chalcone-treated cells looked very similar to those of the untreated control (photos not shown). This is not surprising, since 10 μg/mL is well below the MIC value of these compounds (64 μg/mL). On the other hand, **11** induced significant morphological changes, which ranged from some discrete alterations to the total destruction of the outer layers of fungal cells (**Figures 3B–D**). A common alteration observed after treatment with compound **11** was a loss of a typical layered structure and discontinuity or even disappearance of the cytoplasmic membrane (**Figures 3C,D**). Other changes comprised appearance of the irregular cell surfaces, loss of cell-wall integrity and penetrating lesions of the wall with an apparent shedding of the cell components (**Figure 3C**).

Discussion

Three out of 27 chalcones studied in this work (**Figure 4**) appeared effective chemosensitizers, able to restore to large extent sensitivity to fluconazole of MDR *C. albicans* strains. Compound **21** effectively chemosensitized cells overexpressing the MFS-type Mdr1p, compound **6** did the same with cells

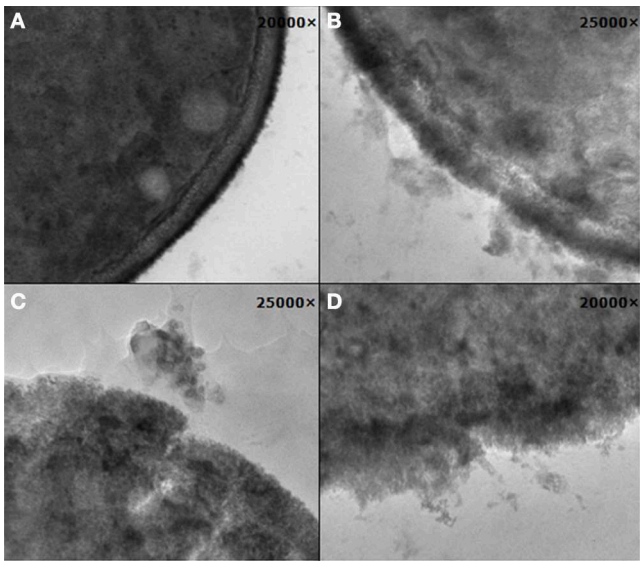


FIGURE 3 | Changes in the cell surface of *C. albicans* cells observed by transmission electron microscopy: (A) control cells; (B–D) cells treated with 11, 10 μg/mL, for 3, 6, and 9 h.

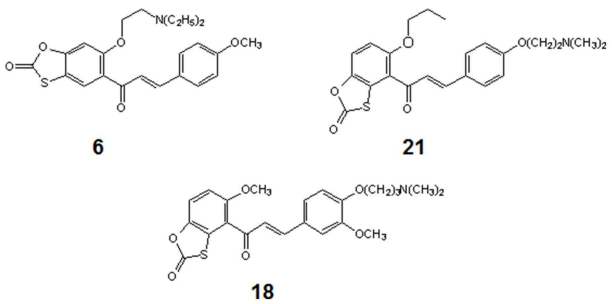


FIGURE 4 | Structures of chemosensitizers of MDR yeasts selected in this study.

FLC-resistant due to the activity of ABC-type drug transporters and derivative **18** partially reversed fluconazole resistance mediated by both types of yeast drug efflux pumps. This is worth mentioning that compounds **6**, **18,** and **21** demonstrated low *in vitro* mammalian toxicity against different cell lines in the tissue cultures (Konieczny et al., 2007a,b,c), what makes them promising candidates for clinical application as agents augmenting antifungal chemotherapy with FLC of infections caused by MDR *C. albicans*. On the other hand, the chemosensitizing potential of **11** seems questionable, since this compound exhibits a strong growth inhibitory and fungicidal effect at relatively low concentrations. In our previous studies we provided evidence for inhibition of chitin biosynthesis as a molecular basis of fungistatic effect of **11** and for inhibition of β(1→3)glucan synthase resulting in fungicidal action of this chalcone derivative (Łącka et al., 2011). The latter has been now confirmed by the loss of continuity of *C. albicans* cells and the appearance of the cell wall defects, followed by leakage of cell components, demonstrated by TEM upon the action of **11** at concentration well above its MIC and close to the MFC value. Destruction of *S. cerevisiae* cells treated with **11**, revealed by the results of experiments involving the diS-$C_3(3)$ fluorescent probe, provides another evidence confirming this hypothesis. Inhibition of chitin biosynthesis by **11** at concentrations close to its MIC (Łącka et al., 2011) seems to constitute a molecular basis for the observed synergism of **11** and FLC, similarly as it was shown previously for combination of the known inhibitor of chitin synthase nikkomycin and azole antifungals (Milewski et al., 1991).

It is not clear why the chemosensitizing potency of **6**, **18,** and **21** is much better than that of their other close structural analogs tested by us. The only characteristic common structural pattern observed here is presence of the 4′-dimethylaminoalkoxy substituent in ring B (compounds **11**, **16**, **18,** and **21**) that seems beneficial for the chemosensitizing efficacy (**18** and **21**) or high antifungal activity (**11**) of type 2 oxathiolone fused chalcones but this effect is abolished when the similar substituent is also present in the A ring (**16**).

Results of experiments employing Rhodamine 6G and Nile Red showed that some chalcones studied effectively interfered with extrusion of the fluorescent probes by the ABC and/or MFS proteins. Compounds **18** and **6** inhibited the efflux of Nile Red by Cdr1p, Cdr2p, and Mdr1p, export of Rhodamine 6G by the Cdr1p and Cdr2p transporters and efflux of diS-$C_3(3)$ from the *S. cerevisiae* strain overexpressing *PDR5*. On the other hand, **21** did not affect the Rhodamine 6G and Nile Red efflux mediated by Cdr1p/Cdr2p efflux and poorly affected export of diS-$C_3(3)$ from *S. cerevisiae* strains overexpressing *PDR5* and/or *SNQ2*, while it effectively inhibited the efflux of Nile Red from strains overexpressing *MDR1*. An inhibitory effect of **6** on Pdr5p- and Snq2p-mediated efflux of the diS-$C_3(3)$ probe and a very slight inhibition of the Cdr1p/Cdr2p-derived ATPase activity under *in vitro* conditions indicates its possible inhibitory activity against different ABC-type yeast drug transporters, probably not resulting from interaction with the ATP-binding domains. Previously it was shown that 4-alkoxychalcones (structure different from that of compounds described in this study) bind to the ATP binding site and to the steroid binding site of mammalian ABC-type drug transporter P-glycoprote (Conseil et al., 1998). It is possible therefore that compound **6** may also bind to more than one site in the ABC-type yeast drug transporters.

The fact that some chalcones effectively prevented extrusion of particular fluorescent probes from MDR *C. albicans* cells and chemosensitized MDR cells to FLC but on the other hand, their intrinsic anticandidal activity against FLC-resistant MDR cells, was very similar or the same as against FLC-sensitive cells, may indicate that these compounds bind to the MDR proteins outside their substrate-binding sites and prevent binding of probes or fluconazole to these sites but are not effectively extruded by the drug efflux pumps. In summary, the observed effect of sensitization of resistant strains of *Candida albicans* to FLC in the presence of chalconic chemosensitizers, most likely results from a non-competitive inhibition of drug efflux proteins, especially those of the MFS-type, although this hypothesis should be further verified.

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The anti-*Candida* activity by Ancillary Proteins of an *Enterococcus faecium* strain

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An antimycotic activity toward seven strains of *Candida albicans* was demonstrated erstwhile by a wild-type *Enterococcus faecium* isolated from a penguin rookery of the Antarctic region. In the present study the antimicrobial principle was purified by ion exchange and gel permeation chromatography and further was analyzed by LC-ESI-MS/MS. In the purification steps, the dialyzed concentrate and ion exchange fractions inhibited *C. albicans* MTCC 3958, 183, and SC 5314. However, the gel filtration purified fractions inhibited MTCC 3958 and 183. The data obtained from the LC-ESI-MS/MS indicate that the antimicrobial activity of the anti-*Candida* protein produced by *E. faecium* is facilitated by Sag A/Bb for the binding of the indicator organism's cell membrane. Partial N-terminal sequence revealed 12 N-terminal amino acid residues and its analysis shown that it belongs to the LysM motif. The nucleotide sequence of PCR-amplified product could detect 574 nucleotides of the LysM gene responsible for binding to chitin of the cell wall of *Candida* sp.

Keywords: antimycotic peptides, anti-*Candida* protein, *Enterococcus faecium*/lactic acid bacteria (LAB), Lysin M, *Candida albicans*

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Introduction

Antimicrobial and antimycotic peptides are small cationic and amphipathic molecules, generally with fewer than 50 amino acids. These peptides are omnipresent and have been isolated from prokaryotes and eukaryotes in plants, bacteria, fungi, and animals (Zasloff, 2002; Bulet et al., 2004).

Amongst Lactic Acid Bacteria (LAB), members of the genus *Enterococcus* are widely distributed throughout nature as inhabitants of the gastrointestinal tract of humans and other animals and are also present in vegetables, plant materials and foods (Giraffa, 2002). Many LAB bacteriocins, particularly those produced by enterococci (enterocins), are characterized by their broad range of activity against many gram-positive bacteria (van Belkum and Stiles, 1995; Nes et al., 2007). Bacteriocins may also play an important role in maintaining bacterial community structures (Riley and Wertz, 2002) in specific ecological niche. They have also been proposed as probiotics for both the gastrointestinal and urogenital tracts (Redondo-Lopez et al., 1990). One of the main mechanisms used by the LAB to interfere with the colonization of pathogens and avoid proliferation of those potential pathogens is production of antimicrobial agents, such as organic acids, hydrogen peroxide and bacteriocins or related substances (Redondo-Lopez et al., 1990; Jack et al., 1995).

Though the vast majority of enterocins produced by *Enterococcus faecalis* and *Enterococcus faecium* are found active only against Gram-positive bacteria (Larsen, 1993), some exceptions with broad activity spectra have been described in recent years to show the

ability of bacteriocins to inhibit the Gram-negative microorganisms (De Kwaadsteniet et al., 2005; Line et al., 2008). Reports on antifungal prowess of *E. faecium* or *E. faecalis* are relatively rare. It was demonstrated in one of the previous investigations that the antifungal compounds such as phenyllactic acid and 4-hydrophenyllactic acid were produced by *Lactobacillus plantarum* (Lavermicocca et al., 2000). Besides this, bacteriocin-like substances and other compounds were produced by *L. pentosus*, *L. coryniformis*, and *E. faecalis* (Okkers et al., 1999; Magnusson and Schnurer, 2001; Lupetti et al., 2002).

Candida albicans is the major human fungal pathogen of immunocompromised patients (Lupetti et al., 2002). The nature of the resistance to a few drugs has been identified as related to altered transport, modification of an enzyme, and a change in membrane composition (Lupetti et al., 2002). Additionally, antimicrobial peptides are promising candidates for the treatment of fungal infections since they have both mechanisms of action distinct from available antifungal agents and the ability to regulate the host immune defense systems as well (Lupetti et al., 2002). Probiotic strains that can be used for the treatment of vulvo-vaginal infection should be able to produce metabolites that are fungistatic for *C. albicans* and *C. glabrata* (Strus et al., 2005).

Though an umpteen number of peptide bacteriocins from *Enterococci* have been purified and genetically characterized over the last several years, yet the anti-*Candida* peptide/proteins are less investigated from the *E. faecalis* and *E. faecium*. Due to the increasing frequency of fungal infections in immunocompromised patients and development of an ominous trend in the treatment failures amongst the candidiasis patients receiving long-term antifungal chemotherapy resulted from the rapidly acquiring multidrug resistance (MDR) amongst pathogenic *C. albicans* a strong and pressing need has been felt in finding alternative form of antifungal antibiotics (Matejuk et al., 2010).

In this study, the APR210, the anti-*Candida* factor producer strain that was earlier identified as *E. faecalis* based on the biochemical tests and fatty acid methyl ester (FAME) analysis got redisgnated as *E. faecium* based on rDNA sequence analysis (Shekh et al., 2011), biochemical tests (e.g., L-Arabinose+, Raffinose+, Melibiose and Sorbitol−), and PCR-amplification data obtained using the *E. faecium*-specific primers (Cheng et al., 1997) and the genomic DNA of the producer strain. In this study the anti-*Candida* protein produced by the wild type *E. faecium* was gel-filtration purified and the peptides responsible for the anti-*Candida* activity in the pooled active fractions was identified by LC-ESI-MS/MS Supplementary 1 (Image 1-a gel picture).

Results

We attempted to purify the antimicrobial protein by using crude proteins in the cell free supernatant. A three-step method was followed that included salting-out by ammonium sulfate fractionation, ion exchange and gel filtration chromatography. The fractions collected from each step of purification was checked for the antimicrobial activity by cut-well agar assay using three *C. albicans* strains that are different in drug-resistance pattern and are from different sources. The protein band that produced the anti-*Candida* activity in the zymogram assay was subjected to the LC-ESI-MS/MS analysis. The PCR-amplified product corresponding to the LysM domain gene was sequenced and analyzed.

Gel Filtration of the Antimycotic Protein

Dialyzed concentrate (after ammonium sulfate fractionation and dialysis) showed a clear zone of inhibition against *C. albicans* SC 5314 (**Figure 1A**) and MTCC 3958 (photo not shown). Prior to gel filtration, the ion exchange fractions were tested against *C. albicans* MTCC 3958, 183, and SC 5314. The ion exchange fractions showed clearly the antimicrobial activity against these three yeast strains. However, we have shown here the activity against SC 5314 only (**Figure 1B**). The Sephadex G-75 gel filtration chromatography showed that the recovery of antifungal activity was approximately 22-fold (Shekh and Roy, 2012) with a reduction of 45%. The chromatogram of fractions collected during gel filtration on Sephadex G-75 is shown in **Figure 2**. The

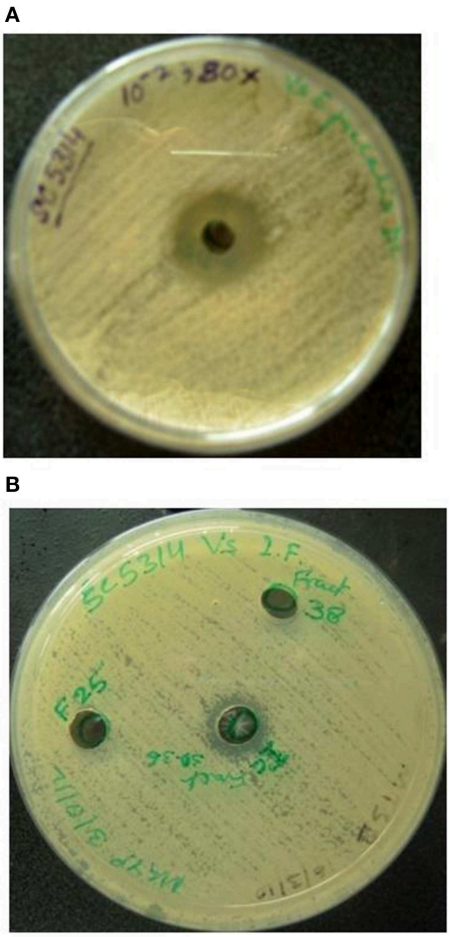

FIGURE 1 | (A) Dialyzed concentrate (after ammonium sulfate fractionation and dialysis) shows clear zone of inhibition against *C. albicans* SC 5314. F25 and F38 denote ion-exchange fractions showing no inhibition. **(B)** Anti-*Candida* activity of pooled (30–36) fractions (collected during DEAE-Sepharose anion exchange chromatography) against *C. albicans* SC 5314.

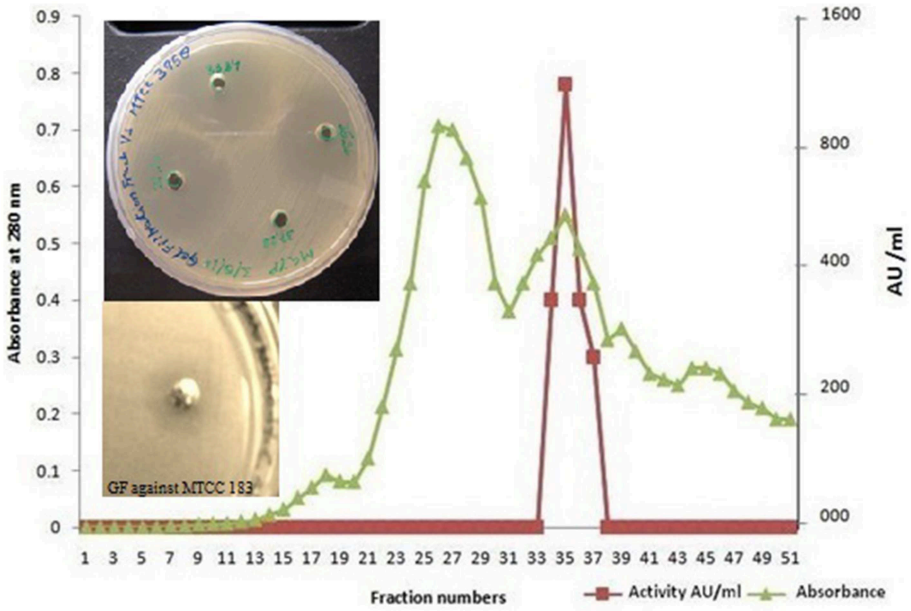

FIGURE 2 | Gel filtration elution profile of ACP on Sephadex G 75 column. Anti-*Candida* activity was detected in fractions 31–36. The pooled fractions showed a distinct anti-*Candida* activity against *C. albicans* MTCC 3958 and MTCC 183 in the inset. ■, represents AU ml^{-1}; ▲, represents absorbance at 280 nm.

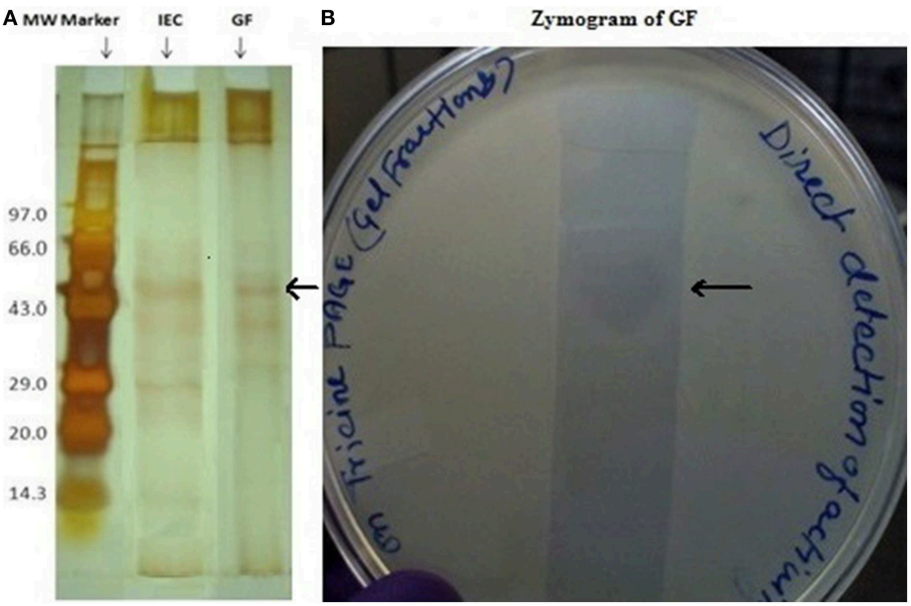

FIGURE 3 | (A) Tricine SDS-PAGE (10%) profile of ion exchange and gel filtration fractions having biological activity in zymogram. Lane1 (from left) shows the molecular weight marker, lane 3 pooled ion exchange fractions (IEF) and lane 4 gel filtration fractions (GF). The arrow shows the band that showed in-gel inhibition in a zymogram assay. **(B)** Zymogram (right side) of the gel-filtration purified fraction after concentration.

fractions were tested against *C. albicans* MTCC 3958, MTCC 183, and SC 5314. It was observed that fractions between 31 and 36 showed antimicrobial activity against MTCC 3958 and MTCC 183 (**Figure 2** inset), whereas no zone of inhibition was recorded against SC 5314. These active fractions were pooled for further processing. As observed in **Figure 3A**, 10% SDS-PAGE fractionation indicated the presence of a protein band from ion exchange and gel filtration fractions that showed antimicrobial activity in the zymogram assay of Tricine Native PAGE (**Figure 3B**). Minimum inhibitory concentration (MIC) values against MTCC 183 and 3958 were 133 and 267 μg/ml respectively as determined in the earlier study (Shekh and Roy, 2012).

PCR-Amplification from the N-Terminal Sequence

N-terminal amino acid analysis of the ion-exchange purified antimicrobial protein revealed the following partial sequence: NH2-DEVYTVKSGDSL (Shekh and Roy, 2012). Homology search was performed using NCBI BLAST (http://blast.ncbi.nlm.nih.gov/). The sequence DEVYTVKSGD displays high similarity (as evident from the *e*-values) to LysM domain containing protein of most of the *Enterococcus* species 29 amino acids residues away from the N-terminus of the LysM domain protein (sequences similarity and alignments are shown below). This sequence demonstrated identity with LysM domain proteins of six *E. faecium* strains.

The forward (34–50) and reverse (592–610) primers were designed based on the N-terminal and C-terminal sequence of the LysM domain respectively. The 574 bp amplicon (**Figure 4**) was obtained by PCR and was further sequenced and the sequence analysis revealed 94.7% similarity with the 574 bp LysM domain-encoding gene of the strain *E. faecium* DO chosen for the PCR amplification. The nucleotide sequence that encodes 12 amino acids (DEVYTVKSGDSL) previously found as a result of N-terminal sequencing was detected.

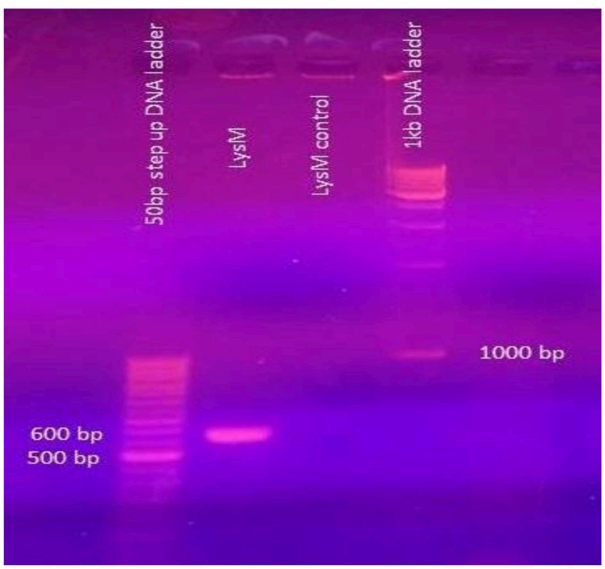

FIGURE 4 | From the left. Lane 1 50 bp step-up DNA ladder; Lane 2 574 bp amplified fragment of LysM gene; Lane 3. Control reaction showing no amplification, Lane 4 1 kbp DNA ladder.

Identification of the Protein by LC-ESI–MS/MS

The gel-filtration purified band that showed anti-*Candida* activity in zymogram assay (**Figure 3B**, right side plate), was subjected to ESI-MS/MS analysis. The resulting protein sequence was blasted against the NCBI database with the best overall match showing 30 % identity with the NlpC/P60 family protein Tax_Id=791161 protein of *E. faecium* [PC4.1]. Out of 509 amino acids of the NlpC/P60, 149 amino acids were detected by LC-MS/MS and the subsequent data analysis revealed that 30% sequence match with the NlpC/P60 family protein (**Figure 5**). Based on the LC-ESI-MS/MS, the peaks corresponding to (NQQADAQSQIDALESQVSEINTQAQDLLAK (**Figure 6C**), DIADLQER, VQAMTTMVK, and TSLAAEQATAEDKK) were obtained in the mass spectra.

The peptide sequence NQQADAQSQIDALESQVSEINTQAQDLLAK revealed the score 96.0 with an expected value of 3.4e-^{08} in LudwigNR database, tr|D4VYT1|NlpC/P60 family protein Tax_Id=791161 [*E. faecium* PC4.1] whereas the peptide sequence TSLAAEQATAEDKK scored 69 (hall mark of identity) with an expected value of 5e-^{05}. The individual peaks with corresponding amino acids are shown in **Figures 6A–C**.

Protein View

MASCOT Search Results: Protein View: D4VYT1
tr|D4VYT1|NlpC/P60 family protein Tax_Id=791161 [*E. faecium* PC4.1]

Database:	LudwigNR
Score:	251
Nominal mass (M_r):	53958
Calculated pI:	4.35

Protein sequence coverage 30%.

Matched peptides shown in **Bold *red*.**

```
  1 MTLTAVASPI AAAADDFDSQ IQQQDQKIAD LKNQQADAQS QIDALESQVS
 51 EINTQAQDLL AKQDTLRQES AQLVKDIADL QERIEKREDT IQKQAREAQV
101 SNTSSNYIDA VLNADSLADA IGRVQAMTTM VKANNDLMEQ QKQDKKAVED
151 KKAENDAKLK ELAENQAALE SQKGDLLSKQ ADLNVLKTSL AAEQATAEDK
201 KADLNRQKAE AEAEQARIRE QQRLAEQARQ QAAQEKAEKE AREQAEAEAQ
251 ATQASSTAQS SASEESSAAQ SSTTEESSSA AQSSTTEEST TAPESSTTEE
301 STTAPESSTT EESTTAPESS TTEESTTVPE SSTTEESTTV PETSTEESTT
351 PAPTTPSTDQ SVDPGNSTGS NATNNTTNNT TNTTPTPTPS GSVNGAAIVA
401 EAYKYIGTPY VWGGKDPSGF DCSGFTRYVY LQVTGRDIGG WTVPQESAGT
451 KISVSQAKAG DLLFWGSPGG TYHVAIALGG GQYIHAPQPG ESVKVGSVQW
501 FAPDFAVSM
```

FIGURE 5 | Aligned amino acid sequence (in red, bold font) of the novel protein with sequences from the amino acid database of Mascot and LudwigNR.

Discussion

A number of species belonging to the genus *Enterococcus* have been reported to synthesize bacteriocins active against Gram-positive and negative bacteria. However, the reports on the anti-*Candida* activity of *Enterococcus* are rather rare.

In the present investigation, the anti-*Candida* substance was purified to a near homogeneity from the cell free supernatants via a three-step purification protocol. Sephadex G-75 gel filtration fractions containing nearly purified protein showed antimicrobial activity against *C. albican*s MTCC 3958 and MTCC 183 (**Figure 2**).

Based on the 12 amino acid residues from the N-terminal sequence determined in the previous study (Shekh and Roy, 2012), primers were designed and the PCR-amplified fragment of size 574 bp obtained (**Figure 4**) using the purified genomic DNA (as template) of the producer strain was sequenced; the nucleotide sequence was analyzed by BLAST using the NCBI search. The deduced amino acid sequence from the nucleotide sequence generated from the PCR amplified fragment (**Figure 4**) reveals the LysM motif that includes the DEVYTVKSGDSL. In a mutational analysis study conducted by Onaga and Tiara (2008), the LysM domain of PrChi-A was found to bind a chitin contributing significantly to the antifungal activity mediated by PrChi-A through their binding activity. The N-terminal sequence of PrChi-A was determined as DCTTYTVKSGDTCYAISQAN. This sequence is not homologous to the sequence of any plant chitinase, but very interestingly shares homology with the LysM of other proteins from several organisms (Onaga and Tiara, 2008). The nucleotide sequence encoding the LysM motif and the N-terminal sequence belonging to the LysM motif derived from the protein band that produced the zone of inhibition in zymogram (in-gel assay) in our study revealed striking similarity with the PrChi-A that exhibited antifungal activity (Onaga and Tiara, 2008). The shaded serine amino acid residue present adjacent to YTVKSGD is of same nature since threonine is the hydroxylated version of serine. However, the CYA amino acid residues in the N-terminal sequence of LysM of PrChi-A was not found in the translated sequence deriving from the nucleotide sequence of the PCR-amplified product; this CYA was also not found in the LysM motif sequence of *E. faecium* DO; however the pink shaded amino acid stretch ISQ matched with the LysM motif. LysM is a protein domain of about 45 amino acids found initially in several bacterial autolysin proteins (Joris et al., 1992). The domains are also known to bind N-acetylglucosamine (GlcNAc)-containing glycan molecules including peptidoglycan from several bacteria and chitin from fungi (Bateman and Bycroft, 2000; Buist et al., 2008; Iizasa et al., 2010; Petutschnig et al., 2010). In the plant kingdom, LysM domains are found in receptors for chitooligosaccharide and related compounds. The PrChi-A was reported to bind to chitin in the fungal cell wall mainly through LysM domains and then it degraded the chitin by hydrolytic action. This led to disruption of the fungal cell wall and fungal growth inhibition (Onaga and Tiara, 2008).

The LC-ESI-MS/MS generated the spectrum of separated peaks (**Figures 6A–C**) showing three abundant peaks, consistent with the molecular form of the purified protein. The resulting

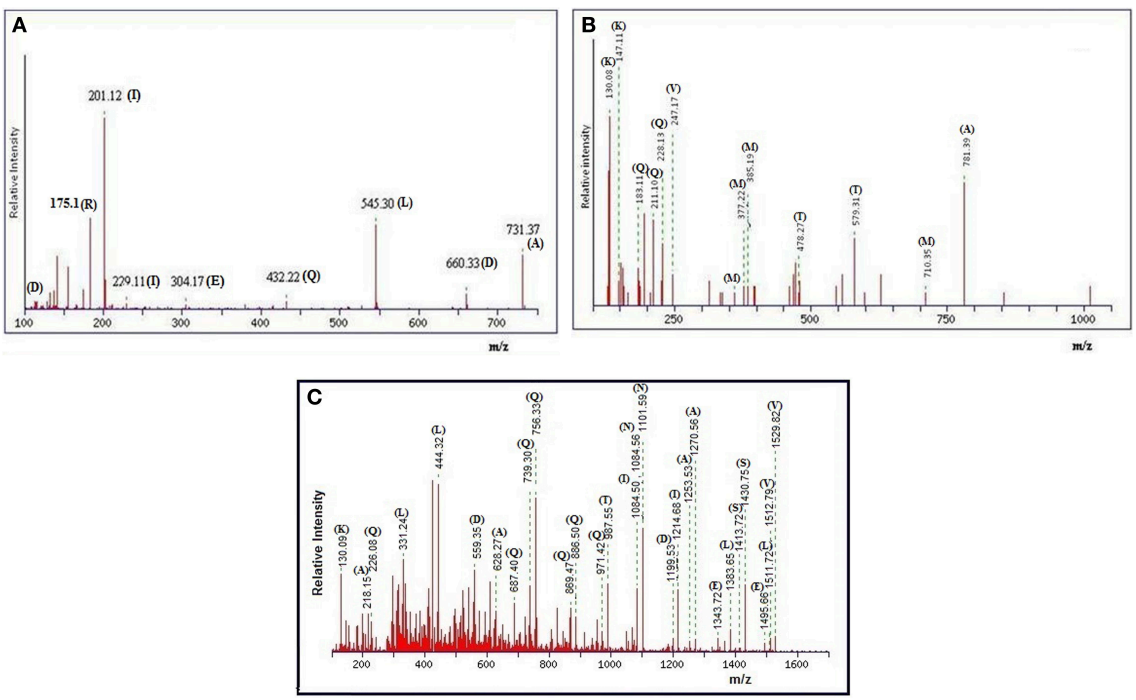

FIGURE 6 | (A) MALDI-TOF spectra of anti-*Candida* peptide DIADLQER, produced by *E. faecium*. **(B)** MALDI-TOF spectra of anti-*Candida* peptide VQAMTTMVK, produced by *E. faecium*. **(C)** MALDI-TOF spectra of anti-*Candida* peptide NQQADAQSQIDALESQVSEINTQAQDLLAK, produced by *E. faecium*.

protein sequence was blasted against the NCBI database with the best overall match showing 30 % identity with the NIpC/P60 family (Anantharaman and Aravind, 2003). In mass spectrometry analysis, the protein sequence matched upto 30% with NlpC/P60 family protein present in the MASCOT database (**Figures 6A–C**, **7A–C**). The **Figures 7A–C**, depict the dendrogram presentations of the multiple alignments of the deduced amino acid sequences of three peptides of NipC/P60 of *E.faecium* and other closely related strains. NlpC/P60 is a large family of cell-wall related cysteine peptidases that are broadly distributed in bacteria, archaea and eukaryotes (Anantharaman and Aravind, 2003). While their biochemical function seems to be conserved, the physiological roles of NlpC/P60 proteins are diverse, including cell separation, expansion, differentiation, cell-wall turnover, cell lysis, protein secretion and virus infection (Xu et al., 2010). Multiple proteins with NlpC/P60 domains are found in individual Gram-positive bacteria (Humann and Lenz, 2009). SagA of *E. faecium* belongs to this NIpC/P60 and is a secreted antigen which binds to the extracellular matrix proteins (Teng et al., 2003). Teng et al. (2003) reported the N-terminal sequencing of the fibrinogen- binding protein revealing a 20-amino-acid sequence, DFDSQIQQQDQKIADLKNQQ, identical to the predicted N-terminal sequence of the mature SagA (Teng et al., 2003). In our study, the significant peptides NQQADAQSQIDALESQVSEINTQAQDLLAK, DIADLQER, VQAMTTMVK, and TSLAAEQATAEDKK detected were matched with secreted antigen Sag A/SagBb proteins produced by the *E. faecium* strain. NlpC/P60 proteins are often fused to auxiliary domains, many of which are known cell-wall binding modules (e.g., LysM domain) (Xu et al., 2010). These auxiliary domains may be thought to function as targeting domains which localize their proteins to the cell wall (Xu et al., 2010). The species designation of *E. faecium* isolates was confirmed by amplification of specific DNA sequences by PCR. The results obtained in this study reveal a so far not described function for enterococcal LysM domain protein and taken together our findings clearly indicate the presence of this auxiliary domain in the form of LysM domain and NIpC/P60. However, the functional synergy between the NlpC/P60 domains and their auxiliary domains (Xu et al., 2010) needs to be investigated further to establish the rationale of our findings in the present study.

Extracellular *E. faecium* Sag A/Bb protein that is antigenic in nature is apparently essential for growth and shows broad-spectrum binding to extracellular matrix (ECM) proteins forming oligomers (Humann and Lenz, 2009) whereas the secreted protein sspA or sspB produced by *Streptoccoccus sp.* was reported to adhere to collagen type I and *C. albicans* (Teng et al., 2003). Insertion inactivation experiments have shown that both sspA and sspB genes are necessary for the binding of *S. gordonii* cells to *C. albicans* (Holmes et al., 1996). The SspB protein, when expressed on the surface of *E. faecalis*, confers upon the enterococcal cells the ability to bind *C. albicans* (Teng et al., 2003). In the same study conducted by Teng et al. (2003) the gene in clones d1–27 and d2–29 named Sag A for major secreted antigen was found in all 11 *E. faecium* strains from different communities and/or from different geographic sources (Teng et al., 2003).

The results obtained in the present work are in partial agreement with the earlier works. The identified peptide sequences derived from the LC-ESI-MS/MS match partly with the secreted antigen A/Bb that might impart binding ability toward the extracellular matrix present on the cell surface of *C. albicans* and the bacteriocin secretion accessory proteins might play an important role in the antimicrobial activity of the producer strain. The SagA (often called P60), P54 from *E. faecium* and SagBb secreted by *Enterococcus hirae* (Teng et al., unpublished data; Muller et al., 2006) have been reported to be associated with the cell wall biosynthesis; however these secreted proteins have the likelihood in mediating cell wall hydrolysis to indicate that apart from the binding to the cell surface of *C. albicans*, Sag A/Bb might play a role in antimicrobial activity indirectly.

Methods

Bacterial Strains, Growth Conditions, and Media

The producer test strain was routinely propagated in TGYE (tryptone, 5 g l^{-1}; glucose, 1 g l^{-1}; yeast extract, 3 g l^{-1}, pH 7.2 ± 0.4) medium and was grown at 14 ± 0.5°C. The producer strain *E. faecium* and three indicator strains (*C. albicans* MTCC 3958, MTCC 183, and SC 5314) used in the present study were maintained in glycerol stock at −70°C (Shekh et al., 2011) and subcultured as and when required.

Gel-Filtration of ACP

The anti-*Candida* protein (ACP) was partially purified from supernatant of cultures of *E. faecium* using ammonium sulfate fractionation and ion exchange chromatography (Shekh and Roy, 2012) which was followed by gel filtration chromatography. The dialyzed sample was loaded onto a DEAE-Sepharose Fast flow column (GE Healthcare) equilibrated with 20 mmol sodium phosphate buffer, pH 8.0. The fractions were eluted using a linear gradient of 0–0.30 M NaCl (Shekh and Roy, 2012). The ion exchange fractions exhibiting the anti-*Candida* activity were pooled and further purified by gel permeation chromatography. Three grams of Sephadex G-75 were soaked in 200 ml of sterile distilled water and washed three times for removing fine particles, and then dissolved in 20 ml of sterile distilled water and poured in a 1 × 50 cm column. Void volume was determined by passing blue dextran (2000 kDa) through the column. The pooled ACP fractions (2.0 ml) were loaded onto the gel filtration column. The above mentioned buffer was used to elute the fractions each of 1.5 ml; those fractions were collected at a flow rate of 65 ml h^{-1} and read at 280 nm using UV-Visible spectrophotometer (Shimadzu). Antimicrobial assay for all fractions was performed against the freshly grown *C. albicans* MTCC 3958, MTCC 183 and SC 5314 by using cut-well agar assay (Shekh et al., 2011). The active fractions were concentrated by U-tube concentrator. That facilitated the removal of small molecules and also any salts present. The concentrated samples (100 μL) were added into a well of diameter 7.0 mm made in a freshly prepared MGYP agar plate which was seeded with freshly grown (diluted to 10^6–10^7 cells/ml) *C. albicans*. After 48 h of luxurious growth of *C. albicans* at 37°C, the plates were inspected for the zone of inhibition and the zone diameter was measured in terms of millimeter.

FIGURE 7 | Continued

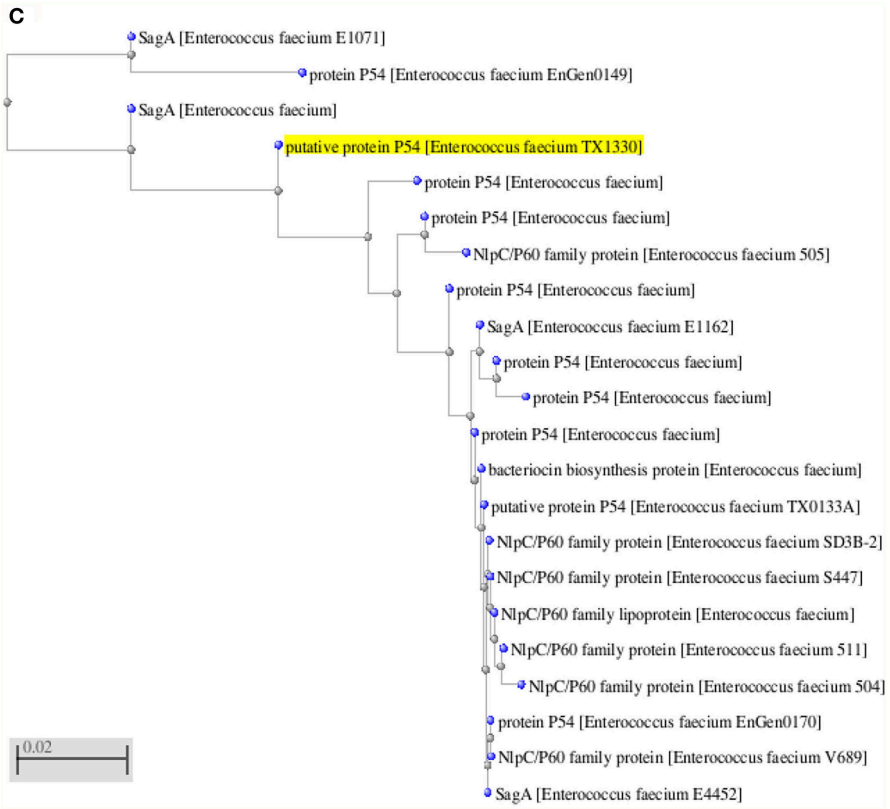

FIGURE 7 | (A) Dendrogram presentation of the multiple alignment of the deduced amino acid sequence of NQQ. LAK of NlpC/P60 from Enterococcus sp. and other closely related strains. **(B)** Dendrogram presentation of the multiple alignment of the deduced amino acid sequence of VQAMTTMVK of NlpC/P60 from Enterococcus sp. and other closely related strains. **(C)** Dendrogram presentation of the multiple alignment of the deduced amino acid sequence of DIADLQER of NlpC/P60 from Enterococcus sp. and other closely related strains.

Tricine SDS-PAGE

Samples were separated on a one dimensional SDS-PAGE. Slabs of 10% polyacrylamide (acrylamide/bisacrylamide, 30:0.8) with a 5% stacking gel were electrophoresed at 100 mV until the bromophenol dye reached the bottom of the gel. The gel was viewed after silver staining.

Direct Detection of Biological Activity on Tricine PAGE

Tricine Native-PAGE (10%) (Schagger and Von Jagow, 1987) followed by a gel overlay was performed with active pooled fractions from gel filtration. After electrophoresis for 2 h at 20 mA, 2 duplicate gels were cut. One of the gels was silver stained. The other gel was fixed in 20% (v/v) isopropanol and 10% (v/v) acetic acid for 30 min, rinsed with 500 ml of MilliQ water for 1 h, and placed aseptically on an MGYP plate. To identify the active peptide band, the Tricine gel containing pooled active fraction was overlaid by freshly grown *C. albicans* MTCC 3958. After the agar solidified, the plate was incubated at 37°C for 48–72 h until *C. albicans* grew uniformly over the plate or an inhibition zone was observed.

N-Terminal Amino Acid Sequence Analyses

The protein band that showed inhibition in the in-gel assay in Tricine-Native PAGE was further subjected to N-terminal amino acid sequencing using an ABI Procise 494 protein sequencer (Applied Biosystems), Iowa State University, US (Shekh and Roy, 2012).

PCR Amplification and Nucleotide Sequencing

Based on the N-terminal sequence (Joris et al., 1992) of the LysM domain of the *E. faecium*, forward and reverse primers were designed. The primers used were: forward primer 5′ ACT TTT GCT GCT GGT GC 3′ and reverse primer 5′ TTA GTA CCA GCC GTT TGC 3′; a 25 µl of reaction consisted of 100 ng of purified genomic DNA of the producer strain, forward and reverse primers (30 picomoles each) and 200 µM of dNTPs each, and 1.5 units of Taq polymerase. The PCR conditions consisted of an initial denaturation step at 94°C for 90 s, followed by 35 cycles of denaturation 60 s at 94°C, annealing at 50 s at 48°C, and 50 s at 72°C. The final extension step was at 72°C for 3 min. The nucleotide sequence was determined on both strands by using Big Dye Terminator chemistry and ABI 3500 × L Genetic Analyzer. The sequence data was evaluated

on the basis of sequence homology to GenBank entities using BLASTN and was analyzed using the open reading frame finder of NCBI.

Mass Spectrometry of the Gel-Filtration Purified Protein

The gel filtration purified fractions that contained anti-*Candida* principle were pooled and resolved using the SDS-PAGE. The band in the stained gel was precisely cut and used for LC-ESI-MS/MS (Proteomics International, Australia). Protein samples were trypsin digested and peptides extracted according to standard techniques (Xu et al., 2010). Peptides were analyzed by electrospray ionization mass spectrometry using the Ultimate 3000 nano HPLC system [Dionex] coupled to a 4000 Q TRAP mass spectrometer [Applied Biosystems]. Tryptic peptides were loaded onto a C18 PepMap100, 3 μm [LC Packings] and separated with a linear gradient of water/acetonitrile/0.1% formic acid (v/v). Spectra were analyzed to identify proteins of interest using Mascot sequence matching software [Matrix Science] with Ludwig NR database. Spectra were obtained for the major peptide ions in MS mode and sequence data obtained when the spectrometers automatically reverted to MS/MS mode. These spectra were then compared with databases (MASCOT and LudwigNR) to provide hits that could identify matching or similar sequences. The amino acid sequences were identified using the tool provided by the National Center for Biotechnology Information (NCBI) and ORF Finder tool (www.bioinformatics.org/sms/orf=find.html) for the sequences obtained. The translated ORFs were compared to known sequences deposited in the non-redundant protein databases (www.ncbi.nlm.nih.gov) using the BLAST program (Shekh and Roy, 2012). Multiple alignments were performed with the CLUSTAL W proGram (Altschul et al., 1993) Supplementary Material Images 2–4.

Acknowledgments

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00339/abstract

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Detection of inhibitors of *Candida albicans* Cdr transporters using a diS-C_3(3) fluorescence

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Candida albicans is a major cause of opportunistic and life-threatening, systemic fungal infections. Hence new antifungal agents, as well as new methods to treat fungal infections, are still needed. The application of inhibitors of drug-efflux pumps may increase the susceptibility of *C. albicans* to drugs. We developed a new fluorescence method that allows the *in vivo* activity evaluation of compounds inhibiting of *C. albicans* transporters. We show that the potentiometric dye 3,3′-dipropylthiacarbocyanine iodide diS-C_3(3) is pumped out by both Cdr1 and Cdr2 transporters. The fluorescence labeling with diS-C_3(3) enables a real-time observation of the activity of *C. albicans* Cdr1 and Cdr2 transporters. We demonstrate that enniatin A and beauvericin show different specificities toward these transporters. Enniatin A inhibits diS-C_3(3) efflux by Cdr1 while beauvericin inhibits both Cdr1p and Cdr2p.

Keywords: *Candida albicans*, ABC transporters, inhibitors, diS-C_3(3), enniatin A, beauvericin

INTRODUCTION

The mechanism of resistance in yeasts to antifungal drugs is different depending on the mode of action of the antifungals (Spampinato and Leonardi, 2013). The drug-efflux system represented by plasma membrane transporters is one of four mechanisms of multidrug resistance in *Candida albicans* (Sanglard et al., 2009).

Three efflux pumps situated in the *C. albicans* plasma membrane are responsible for decreasing the intracellular concentration of antifungals. These pumps are encoded by *Candida* drug resistance (*CDR1* and *CDR2*) and multidrug resistance (*MDR1*) genes and they differ in the source of energy used for their activity and in the specificity to the antifungals molecules (Cannon et al., 2009).

One of the strategies used to identify the mechanism and function of the *C. albicans* efflux pumps, and to screen for their substrates and inhibitors, is the preparation of the collection of *C. albicans* mutants with deletions of the *CDR1*, *CDR2*, and *MDR1* genes, which are used for investigating the molecular mechanisms governing the regulation of multidrug transporter genes (Coste et al., 2004, 2009). Studies of the multidrug resistance process have provided important knowledge about efflux pump gene regulation, their substrates and inhibitors, sources of energy, and transport mechanism.

Another strategy for testing drugs' inhibition of the efflux pumps is to study their heterologous expression in the non-pathogenic yeast *Saccharomyces cerevisiae* (Cannon et al., 2009). Tanabe et al. (2011) cloned 28 chimeric constructs between *C. albicans* Cdr1p (CaCdr1p) and Cdr2p (CaCdr2p) into *S. cerevisiae*, showing that most of the transmembrane spans and the nuclear binding domains (NBDs) are inhibitor binding sites or affect substrate efflux. Although *S. cerevisiae* is a frequently chosen yeast organism for expression and investigation of *C. albicans* efflux pumps, it is important to consider the differences in the metabolism of the two microorganisms (Rodaki et al., 2009; Calahorra et al., 2012). Besides the obvious differences between the two species, heterologous expression affects several other intracellular interactions responsible for resistance to drugs. For example, tested transporter expression level, and its interplay with other proteins and regulations systems, could be completely different.

Thus, it is important to develop methods that may enable real-time observation of transporter activity fluctuations in response to environmental factors in wild, not modified strains. To this day the most popular method to measure activity of transporters is using rhodamine 6G or rhodamine 123 (Clark et al., 1996) or nile red as pump subtrates (Ivnitski-Steele et al., 2010). But methods and knowledge about the activity of the pumps in real time is scarce. Therefore, our purpose was to develop such a method and to validate it by using collections of isogenic strains with deletions of *CDR1*, *CDR2*, and *MRD1* genes and by testing transporters inhibitors.

One of the most potent inhibitors of MDR transporters are group of enniatins, cyclic hexadepsipeptides produced by *Fusarium spp.* Those mycotoxins have ionophoric properties but it was shown that enniatin can interact with *S. cerevisiae* Pdr5p (Hiraga et al., 2005) and *C. albicans* Cdr1p (Holmes et al., 2008) and inhabit their activity. Other compound from this family, beauvericin was observed to act synergistically with miconazole (Fukuda et al., 2004) and ketoconazole (Zhang et al., 2007) also suggesting its involvement in ATP binding cassette (ABC) transporters inhibition.

Hendrych et al. (2009) developed a novel screening method which uses potentiometric fluorescent probe diS-C_3(3) that measures the kinetics and potency of inhibitors of the *S. cerevisiae*

multidrug resistance pumps. In this work, we show for the first time in *C. albicans* that diS-$C_3(3)$ is pumped out of the cell by both Cdr1p and Cdr2p. We set up the method for testing new drugs and transporters inhibitors, and we also demonstrated that enniatin A and beauvericin are effective inhibitors of Cdr1p and both Cdr1p and Cdr2p, respectively.

MATERIALS AND METHODS

STRAINS AND GROWTH MEDIA

The *C. albicans* strains used in this study (**Table 1**) were generous gifts from D. Sanglard (Lausanne, Switzerland; Sanglard et al., 1995, 1997; Sanglard and Ischer, 1996). All strains were grown at 28°C on YPD medium with 2% glucose, 1% Bacto peptone (Difco), and 1% yeast extract (Difco) and they were shaken at 120 rpm, as described herein. Solid medium was supplemented with 1.5% agar.

SAMPLE PREPARATION

Cells were prepared according to Gásková et al. (1998) with modifications. Stationary cultures were prepared by growing strains at 28°C for 24 h. A volume of 150 μl of stationary culture was added to 20 ml of fresh YPD medium, incubated for 10 h at 28°C, and was shaken at 120 rpm. The cells were harvested by centrifuging at 110 × *g* for 3 min, washed twice with deionized water, resuspended in citrate-phosphate (CP) buffer (pH 6.0) at $OD_{600} = 0.1$ or $OD_{600} = 0.4$ (±10%), and kept on ice.

DiS-$C_3(3)$ UPTAKE INTO CELLS

Aliquots of cell suspensions in CP buffer (3 ml, $OD_{600} = 0.1$; 1.02×10^6 cfu) were labeled with diS-$C_3(3)$ (Sigma) at a final concentration of 5×10^{-8} M at room temperature. Fluorescence spectra were measured every 4 min for 120 min, with gentle stirring before each measurement, with a Fluorescence Spectrophotometer (HITACHI F-4500) equipped with a xenon lamp. The excitation wavelength was 531 nm and the fluorescence range was 560–590 nm. Scattered light was eliminated by an amber glass filter with a cutoff wavelength of 540 nm. Where indicated herein, 2% glucose was added after 60 min and enniatin A (2 μg/ml) (Sigma) and beauvericin (2 and 0.1 μg/ml) (Cayman) was added after 80 min. All experiments were repeated at least three times and means with standard deviation were used as staining curve.

Table 1 | Collection of *C. albicans* strains used in this study.

Strain	Genotype	Reference
CAF 2-1	*ura3Δ::imm434/URA3*	Fonzi and Irwin (1993)
DSY 448	*cdr1Δ::hisG-URA3-hisG/cdr1Δ::hisG*	Sanglard and Ischer (1996)
DSY 465	*mdr1Δ::hisG-URA3-hisG/mdr1Δ::hisG*	Sanglard and Ischer (1996)
DSY 653	*cdr2Δ::hisG-URA3-hisG/cdr2Δ::hisG*	Sanglard et al. (1997)
DSY 654	*cdr1Δ::hisG/cdr1Δ::hisG cdr2Δ::hisG-URA3-hisG/cdr2Δ::hisG*	Sanglard et al. (1997)
DSY 1050	*cdr1Δ::hisG/cdr1Δ::hisG cdr2Δ::hisG/cdr2Δ::hisG mdr1Δ::hisG-URA3-hisG/mdr1Δ::hisG*	Mukherjee and Chandra (2003)

DISK DIFFUSION ASSAY

Candida cells were suspended in deionized water (McFarland standard No. 0.5) and were streaked on YPG agar plates. Tested antifungal agents at concentrations described herein were applied to sterile OXOID Antimicrobial Susceptibility Test Disks, which were then placed on the agar. Culture growth was assessed after a 48 h incubation at 28°C. In disk diffusion assays concentrations below the one that gave inhibitory effect for a given compound was used 1/2 MIC (minimal inhibitory concentration)for fluconazole determined independently for each strain.

CONFOCAL MICROSCOPY

Cell suspensions in CP buffer (5 ml, $OD_{600} = 0.4$) were stained with 2×10^{-7} M diS-$C_3(3)$ probe for 30 and 150 min, with 2% glucose added after 60 min and enniatin A (2 μg/ml) and beauvericin (2–40 μg/ml) added after 80 min. Aliquots of cell suspensions were pelleted by centrifuging, washed in deionized water, and 4 μl of samples were viewed with Leica TCS SP8 X confocal microscope.

RESULTS

FLUORESCENT PROBE diS-$C_3(3)$ IS A SUBSTRATE FOR *C. albicans* Cdr1 AND Cdr2 TRANSPORTERS

Previous studies of *S. cerevisiae* have shown that the fluorescent probe diS-$C_3(3)$ is a substrate for the pleiotropic drug resistance (PDR) pumps, namely the Pdr5p and Snq2p pumps (Hendrych et al., 2009). The observed fluorescence results from both passive membrane-potential-dependent probe uptake and active probe extrusion by ABC pumps. The final maximum fluorescence wavelength (λmax) corresponds to the concentration equilibrium of the probe. Since the λmax of free probe in solution is about 10 nm lower than that of probe bound inside the cell, higher concentration of the probe within the cell results in higher λmax (red shift). The magnitude of this red shift decreases when the probe accumulation in the cell is lowered by the action of probe-expelling pumps; the extent of this lowering thus reflects relative activity of the transporters. To determine whether diS-$C_3(3)$ is suitable for measuring transporter activity in *C. albicans* and to determine which pumps are responsible for its export, we monitored fluorescence in a collection of strains which expressed all pumps (wild type, WT), or which lacked the Cdr1, Cdr2, or Mdr1 pump (**Table 1**; **Figures 1A,B**). A maximum red shift was measured in a strain with simultaneous deletion of both Cdr1p and Cdr2p and a strain lacking all three transporters–Cdr1, Cdr2, and Mdr1. Mutants lacking Cdr1p or Cdr2p stained more intensely than the parent strain (**Figure 1A**). This means that diS-$C_3(3)$ is actively expelled from *C. albicans* cells and serves as the substrate for the two Cdr transporters but not for Mdr1.

In the strain lacking Cdr2p the final λmax was higher, and thus the intracellular concentration of the probe was higher than in strain lacking Cdr1p. This indicates that under these conditions Cdr2p plays a larger role in lowering of the probe concentration in stained cells than Cdr1p.

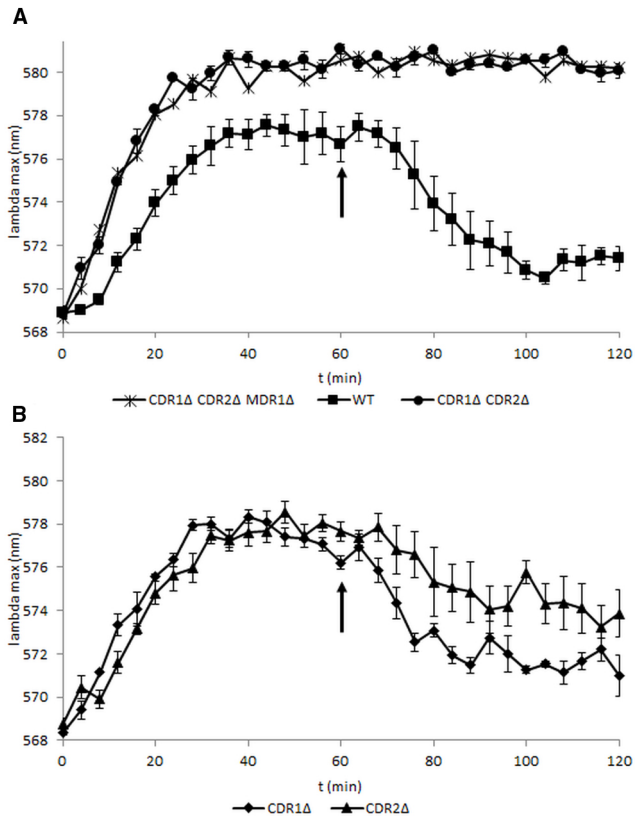

FIGURE 1 | Fluorescence of diS-C_3(3) probe in strains with deleted transporters and wild-type strain. Probe was added at the beginning of the experiment and 2% glucose was added at the 60 min point. Fluorescence in the strains was measured every 4 min. The probe is exported by the ABC transporters Cdr2 and, after addition of glucose, Cdr1—but not by the MFS family transporter Mdr1 ($N = 3$–4).

ENNIATIN A AND BEAUVERICIN INHIBIT TRANSPORTERS WITH DIFFERENT SPECIFICITY

Fluorescent probes enabling the measurement of transporter activity in real time are valuable tools for screening new pump inhibitors. We tested the influence of a known inhibitor of Cdr1, enniatin A, on the transporter activity measured by fluorescence (**Figures 2A,B**). Addition of enniatin A to yeast cells resulted in a red shift of the fluorescence maximum of the probe in strains expressing Cdr1p, showing that the inhibitor is specific for Cdr1p and does not affect the activity of Cdr2p (**Figure 2B**).

After validation of the method with enniatin A, we tested the specificity of a new *C. albicans* CDR pump inhibitor, beauvericin (**Figure 3**). This inhibitor has been found to increase cell sensitivity to miconazole (Fukuda et al., 2004). But, to our knowledge, its specificity toward *C. albicans* transporters has never been tested.

In contrast to enniatin A, which affects the activity of only Cdr1p (**Figure 2**), beauvericin inhibited the activity of both Cdr1p and Cdr2p (**Figure 3**). As shown by diS-C_3(3) efflux, Cdr1p was more sensitive to beauvericin than Cdr2p.

To observe the activity of enniatin A and beauvericin as inhibitors of ABC transporters in real time, we monitored the accumulation of diS-C_3(3) in *C. albicans* strains which express all pumps or lack Cdr1 and Cdr2 pumps using the confocal microscopy (**Figure 4**). We observed similar results to those obtained with the fluorimeter. The strain without Cdr1p pumped diS-C_3(3) out the cell faster than the strain without Cdr2p. In strain without Cdr2p the fluorescence is visible after 30 min while in strain without Cdr1 diS-C_3(3) is mainly present outside the cell (**Figure 4**). This confirms that Cdr2p plays a larger role in lowering of the probe concentration from the cells than Cdr1p.

After application of beauvericin the probe accumulated in both the *CDR1Δ* and *CDR2Δ* strains, unlike enniatin A in which activity as an inhibitor of probe efflux was observed only in the *CDR2Δ* strain (**Figure 4**). This result confirms our observation that beauvericin inhibited the activity of both Cdr1p and Cdr2p.

Inhibition of *C. albicans* transporters observed by using the fluorescent probe diS-C_3(3) should enable the screening for new drugs. We performed disk diffusion chemosensitization assays: paper disks containing fluconazole, alone, inhibitors (enniatin A, beauvericin), or combination of both were placed on plates seeded with *C. albicans* (**Figure 5**). The concentration of fluconazole was matched to the strain sensitivity so that it did not generate a growth inhibition zone. In case of strains expressing Cdr1p (*C. albicans* WT, *C. albicans MDR1Δ* or *C. albicans CDR2Δ*) inhibition zones were observed after addition of enniatin A or beauvericin together with fluconazole. This effect was not observed when the strains without Cdr1p were used. The combination of enniatin A with fluconazole increased the sensitivity of the strains in the same way shown by the fluorescence measurements (**Figure 2**). Beauvericin did inhibit probe export by both Cdr1 and Cdr2 (**Figure 2**), but it increased the sensitivity of the strain without Cdr2, not the strain without Cdr1 to fluconazole (**Figure 5**). This show further differences in the specificity of the inhibitors against diS-C_3(3) and fluconazole.

DISCUSSION

Multidrug resistance is a feature that causes serious medical problems associated with an increasing prevalence and diversity of fungal infections. This process was at first studied in the non-pathogenic yeast *S. cerevisiae* (Kolaczkowska and Goffeau, 1999; Rogers et al., 2001). But recently, investigators are focusing on pathogenic fungi such as *C. albicans*, *Candida glabrata*, or *Candida parapsilosis* (Morschhäuser, 2010).

The fluorescent probe diS-C_3(3) has been found to be a useful tool to estimate and to continuously follow changes of the plasma membrane potential (PMP) of whole *S. cerevisiae* cells (Gášková et al., 1999), as well as to measure the kinetics of PDR pumps (Hendrych et al., 2009).

Our results indicate that diS-C_3(3) may be useful in measuring the activity of PDR transporters in *C. albicans* as well. The diS-C_3(3) probe is a substrate of Cdr1p and Cdr2p, but not Mdr1p. Previous investigations suggest that high aromatic, molecular branching compounds are substrates for Cdr1p, probably because of interactions with a large number of aromatic residues at an active site of the transporter (Puri et al., 2010). The possibility of observing the activity of efflux pumps in real time could provide a new tool for obtaining the answers to as yet unresolved-questions like the speed of changes in pump activity in response to environmental factors (e.g., substrates or inhibitors).

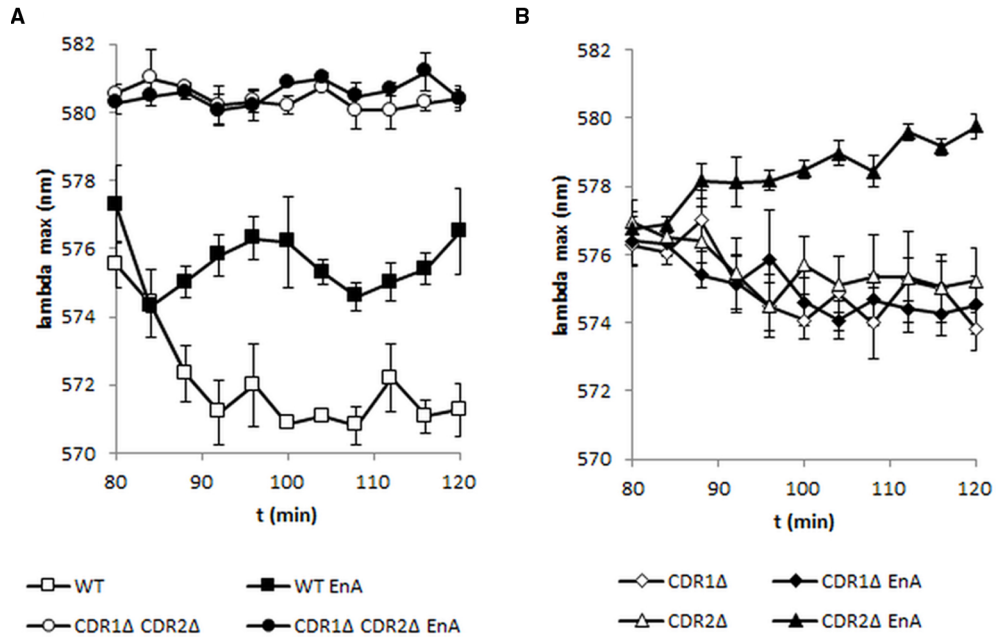

FIGURE 2 | Inhibition of ABC transporter Cdr1p by enniatin A. DiS-C_3(3) was added at the beginning of the experiment and fluorescence was measured every 4 min in strains with or without transporters; filled symbols indicate enniatin A addition (2 μg/ml) at 80 min, 2% glucose was added to the sample at 60 min ($N = 3$–4).

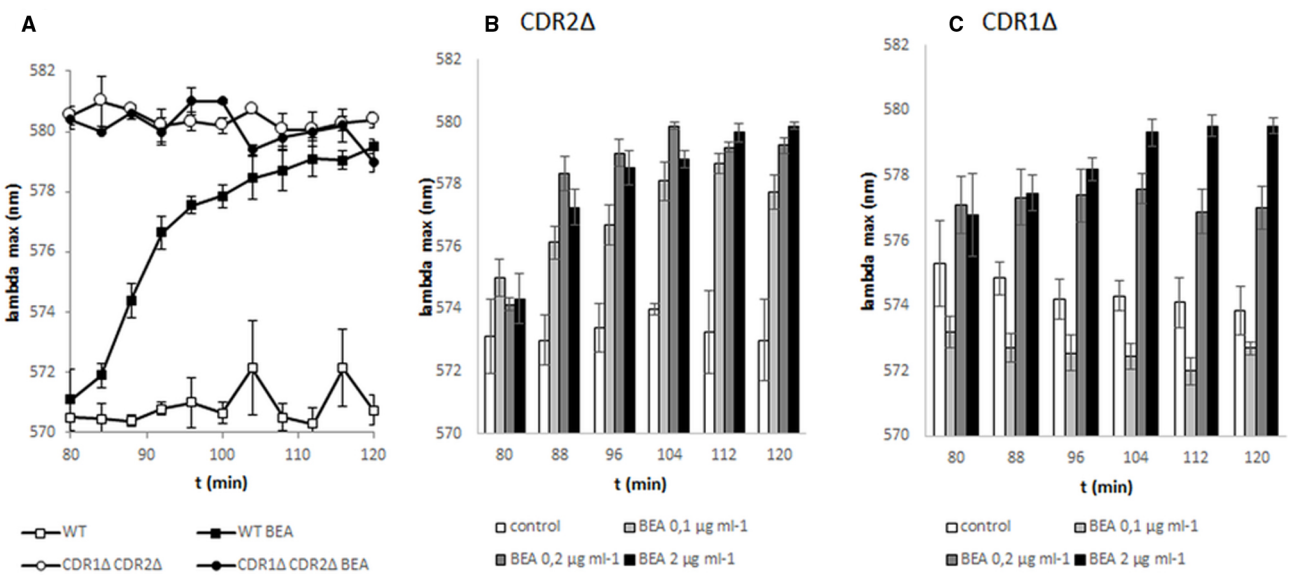

FIGURE 3 | Inhibition of ABC transporters Cdr1p and Cdr2p by beauvericin. DiS-C_3(3) was added at the beginning of the experiment and fluorescence was measured every 4 min. Two percent glucose was added to the sample at 60 min and beauvericin at 80 min. **(A)** Influence of 1 μg/ml beauvericin on diS-C_3(3) staining in *C. albicans* WT and CDR1ΔCDR2Δ strains. **(B)** Dose-dependent inhibition of diS-C_3(3) staining by beauvericin on *C. albicans* CDR1Δ strain. Lower lambda max indicates more probe transported out of the cells. **(C)** Dose-dependent inhibition of diS-C_3(3) staining by beauvericin on *C. albicans* CDR2Δ strain.

DiS-C_3(3) easily passes through the plasma membrane and accumulates in the cells in response to membrane potential (Gášková et al., 1999). The staining of *C. albicans* strains by diS-C_3(3) is approximately twice as slow as that of *S. cerevisiae* (**Figures 1A,B**; Hendrych et al., 2009). The reason for this difference in the rate of staining could be a lower PMP in *C. albicans* cells relative to *S. cerevisiae* cells. In the *S. cerevisiae* US 50–18C strain, with an overexpression of major pumps Pdr5p, Snq2p, and

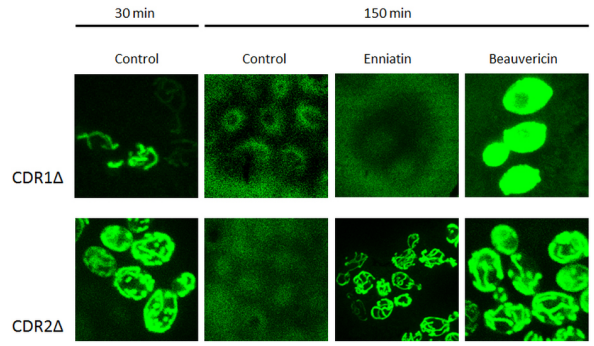

FIGURE 4 | Inhibition of diS-C_3(3) export by enniatin A and beauvericin. Staining was observed by confocal microscopy at 30 min (probe loaded into cells) and 150 min (full effect of the inhibitors).

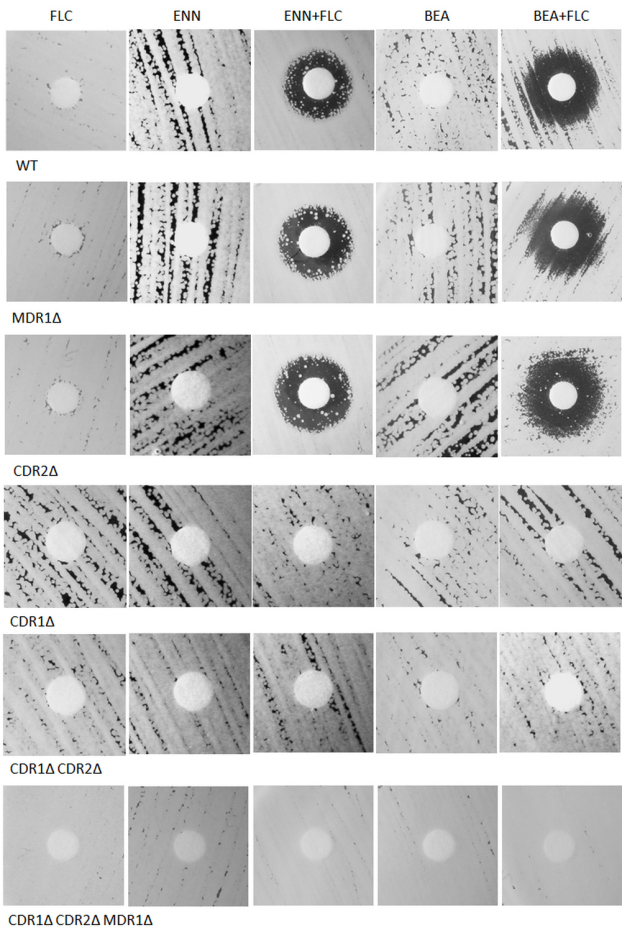

FIGURE 5 | Disk diffusion chemosensitization assays. Paper disks containing inhibitors (2 μg of enniatin A; 10 μg beauvericin; 1.25 μg fluconazole for WT, MDR1Δ, and CDR2Δ; 0.15625 μg fluconazole for the three remaining strains) were placed on the surface of agarose plates seeded with yeast and the plates were incubated at 28 °C for 48 h.

Yor1p, the cell ATP level varies depending on the growth phase and activity of PDR pumps (Krasowska et al., 2010). This is not the case in *C. albicans*.

The efflux of diS-C_3(3) from *Candida* cells was inhibited by the depsipeptides, enniatin A, and beauvericin (**Figures 2 and 3**). Hiraga et al. (2005) suggested that enniatin A is a potent and specific inhibitor for Pdr5p, and Holmes et al. (2008) complemented these data by showing that enniatin A functions as an inhibitor of Cdr1p. Beauvericin was been found to function as an inhibitor of miconazole efflux from *C. albicans* (Fukuda et al., 2004). Enniatin A and beauvericin are ionophores that enhance the permeability of the cell membranes for ions (Tonshin et al., 2010). As shown here by the inhibition of diS-C_3(3) efflux, both enniatin A and beauvericin interact with ABC transporters (**Figure 3**). But beauvericin, in contrast to enniatin A, shows a different synergism in case of fluconazole susceptibility (**Figure 5**). Similar differences in inhibitor activity were observed for curcumin (Sharma et al., 2009), the modulatory effect of which was restricted to rhodamine 6G or miconazole while it had no effect on the efflux of fluconazole. Indeed, most of inhibitors like enniatin A (Holmes et al., 2008), FK506 (Niimi et al., 2004), or curcumin (Sharma and Prasad, 2011) inhibit only Cdr1p. It seems that only tetrandrine blocks all Cdr1, Cdr2, and Mdr1 pumps (Zhang et al., 2009). To our knowledge, our report is the first to show that beauvericin is an inhibitor of not only Cdr1p but also of Cdr2p. The response of transporter activity was fast (visible after 12 min when examining gene transcription, as well as in cell staining) yet about 40 min were necessary to reach full activity.

CONCLUSION

In this paper we show that the carbocyanine dye diS-C_3(3) is employed in monitoring of real time activity of *C. albicans* ABC transporters Cdr1 and Cdr2. This method can be used as a powerful tools in the fight against multidrug resistance. Furthermore we present that two depsipeptides: enniatin A and beauvericin act as inhibitors of Cdr1p or Cdr1p and Cdr2p, respectively.

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