

REVIEW ARTICLE

Novel strategies to fight *Candida* species infection

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Abstract

In recent years, there has been a significant increase in the incidence of human fungal infections. The increase in cases of infection caused by *Candida* species, and the consequent excessive use of antimicrobials, has favored the emergence of resistance to conventional antifungal agents over the past decades. Consequently, *Candida* infections morbidity and mortality are also increasing. Therefore, new approaches are needed to improve the outcome of patients suffering from *Candida* infections, because it seems unlikely that the established standard treatments will drastically lower the morbidity of mucocutaneous *Candida* infections and the high mortality associated with invasive candidiasis. This review aims to present the last advances in the traditional antifungal therapy, and present an overview of novel strategies that are being explored for the treatment of *Candida* infections, with a special focus on combined antifungal agents, antifungal therapies with alternative compounds (plant extracts and essential oils), adjuvant immunotherapy, photodynamic therapy and laser therapy.

Keywords

Antifungal agents, immunotherapy, low-level laser therapy, natural compounds, photodynamic

History

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Introduction

The incidence and prevalence of invasive fungal infections caused by yeasts of the *Candida* genus is a major problem, especially in immunocompromised patients or those hospitalized with serious underlying diseases (Arendrup et al., 2005; Enoch et al., 2006; Espinel-Ingroff et al., 2009; Gahn et al., 2007; Ruhnke et al., 2011; Sims et al., 2005; Van de Veerdonk et al., 2010).

Candida species are frequent colonizers of the skin and mucous membranes of animals and their dissemination in nature is widespread (Ruhnke, 2006; Ruhnke et al., 2011). There are over 350 heterogeneous *Candida* species, but only a few have been implicated in human disease: *Candida albicans*, *Candida dubliniensis*, *Candida parapsilosis*, *Candida tropicalis*, *Candida glabrata*, *Candida kefyr* (*pseudotropicalis*), *Candida lusitanae*, *Candida krusei*, *Candida guilliermondii*, *Candida utilis*, *Candida lipolytica*, *Candida famata*, *Candida haemulonii* and *Candida rugosa* (Ruhnke, 2006; Ruhnke et al., 2011; Williams et al., 2011). Although *C. albicans* remains as the most common pathogen, non-*Candida albicans* *Candida* (NCAC) species are now increasingly associated with invasive candidiasis (Baran et al., 2001; Diekema et al., 2002; Macphall et al., 2002; Pappas et al., 2004; Pfaller et al., 1998; , Trick et al., 2002).

The spectrum of diseases caused by *Candida* consists of superficial and invasive *Candida* infections. These infections range from non-life-threatening mucocutaneous illnesses to

invasive processes that may involve virtually any organ (Blumber et al., 2001; Kojic & Darquouiche, 2004; Kuhn et al., 2002a; Michalopoulos et al., 2003; Munoz et al., 2000; Pappas et al., 2004; Ruhnke et al., 2011; Rüping et al., 2008; Tumvarello et al., 2007; Wey et al., 1989; Wenzel, 1995; Williams et al., 2011). A number of factors have been implicated in the significant increase of incidence of human fungal infections, but it is generally accepted that the main influences relate to the more widespread provision of new medical practices, such as immunosuppressive therapy and use of broad spectrum antibiotics, central venous catheters, parenteral nutrition, invasive surgical procedures and indwelling medical devices (e.g. dental implants, catheters, heart valves, vascular bypass grafts, ocular lenses, artificial joints and central nervous system shunts) which can act as substrates for biofilm formation (Blumber et al., 2001; Michalopoulos et al., 2003; Muñoz et al., 2000; Ruhnke et al., 2011; Rüping et al., 2008; Wenzel, 1995; Wey et al., 1989; Williams et al., 2011). Indeed, most manifestations of candidiasis are in fact associated with the formation of *Candida* biofilms on surfaces, and this phenotype is implicated with infection at both the mucosal and systemic sites (Jabra-Rizk et al., 2004).

Biofilms, represent the most prevalent type of microbial growth in nature, and consist in a complex surface-associated cell populations embedded in an extracellular matrix (ECM) that posses distinct phenotypes compared to their planktonic cell counterparts (Fanning & Mitchell, 2012). Biofilm cell communities are more resistant to antifungal drugs than planktonic cells and can create a source of persistent infection (Chandra et al., 2001). Contributing factors include biofilm structural complexity, presence of ECM, metabolic

heterogeneity intrinsic to biofilms and biofilm-associated up-regulation of efflux pump genes. The actual fold increase in resistance varies with both the drug and species (Chandra et al., 2001; Fanning & Mitchell, 2012).

Biofilm-associated *Candida* infections show uniform resistance to a wide spectrum of the currently available conventional antifungal agents, which implies that antimicrobial drugs that specifically target biofilm-associated infections are needed (Jabra-Rizk et al., 2004).

Epidemiology

Invasive candidiasis, mostly candidaemia, is a serious and potentially lethal disease. It is associated with a high global mortality rate, ranging from 36% to 63% in different patient groups (Gudlaugsson et al., 2003; Guery et al., 2009; Kullberg et al., 2011; Marchetti et al., 2004; Pappas et al., 2003; Tortorano et al., 2004; Van de Veerdonk et al., 2010; Wisplinghoff et al., 2004; Zaoutis et al., 2005), and represents a significant burden on the public health system in terms of patient management and healthcare costs (Kullberg et al., 2011; Tortorano et al., 2004). Of particular significance is the fact that approximately half of all *Candida* infections now occur in intensive-care units (ICUs; Kullberg et al., 2005, 2011; Vincent et al., 1998).

Of the *Candida* species isolated from humans, *C. albicans* is the most prevalent in both health and disease. Mycological studies have shown that candidaemia is most often caused by *C. albicans* (45–65%), but there has been a significant pathogen shift towards other *Candida* species over the past few years in some patient groups (Arendrup et al., 2008; Dupont et al., 2009; Kullberg et al., 2011; Tortorano et al., 2004; Trick et al., 2002). In particular, *C. glabrata* (15–30%), *C. tropicalis* (10–30%), *C. parapsilosis*, *C. krusei*, *C. lusitanae* and *C. guilliermondii* have been implicated in invasive candidiasis (Ruhnke et al., 2011; Tortorano et al., 2004; Viscoli et al., 1999).

A shift in the etiology of candidaemia has been documented in Slovakia and France, where the rate of NCAC species, especially *C. glabrata*, increased in 10 years from 0% to 46% (Krcmery & Kovacichová, 2000; Ruhnke et al., 2011; Sendid et al., 2006). In contrast, in Spain and Italy, *C. parapsilosis* is the predominant agent of candidaemia after *C. albicans* (Almirante et al., 2006; Bassetti et al., 2007; Ruhnke et al., 2011). A study in Denmark documented an increase in the incidence of candidaemia, from 2003 to 2004, with *C. glabrata* being second after *C. albicans* (Almirante et al., 2006; Bassetti et al., 2007; Ruhnke et al., 2011). These increased prevalence of NCAC species in disease could also reflect their inherently higher level of antifungal drug resistance (González et al., 2010; Williams et al., 2011) compared with *C. albicans*, as this would promote their persistence, possibly to the detriment of *C. albicans*, in mixed-species infections treated with traditional antifungal agents (Colombo et al., 1999; Kullberg et al., 2011; Rocco et al., 2000; Williams et al., 2011). The changing epidemiology has been partly attributed to selection of less sensitive *Candida* species, due to the widespread use of fluconazole as a prophylactic and therapeutic agent (Colombo et al., 1999; Kullberg et al., 2011; Rocco et al., 2000).

The relatively high rate of infection by *Candida* species in ICUs, the increasing prevalence of NCAC species, and the associated mortality suggest that new treatment approaches are required. Findings from numerous prospective and retrospective studies indicate that optimizing and reducing the delay of antifungal therapy reduces attributable mortality in patients with candidemia whereas inappropriate antifungal therapy is a significant predictor of mortality (Antoniadou et al., 2003; Bassetti et al., 2007; Blot et al., 2002; Dupont et al., 2009; Ibrahim et al., 2000; Morrell et al., 2005).

Standard treatment

The current standard treatment of *Candida* infections consists of antifungal agents licensed from four different groups: polyenes, azoles, echinocandins and nucleoside analogues (Caillot et al., 1994; Ruhnke et al., 2011; Van de Veerdonk et al., 2010).

Polyene antifungal agents (e.g. nystatin and amphotericin B) exert their fungicidal activity by causing porosity on the fungal cell membrane followed by interaction with the membrane ergosterol and subsequent loss of cytoplasmic content (Henry et al., 2000; Onyewu & Heitman, 2007; Sanglard et al., 2003; Williams et al., 2011). A second proposed mechanism of action involves a cascade of oxidation reactions and interactions with lipoproteins that impair membrane permeability through the release of free radicals (Barwicz et al., 1998; Brajtburg et al., 1990; Onyewu & Heitman, 2007).

Nystatin was the first antifungal agent reported and is available in topical creams and oral washes for treatment of cutaneous and mucocutaneous candidiasis. For its turn, Amphotericin B exhibits broad spectrum antifungal activity against a variety of pathogenic fungi (Andes, 2003; Onyewu & Heitman, 2007), but its clinical utility is limited by a severe nephrotoxicity (Luber et al., 1999; Onyewu & Heitman, 2007; Sawaya et al., 1995). Indeed, the affinity of Amphotericin B for cholesterol, the main sterol of mammalian cell membranes, is believed to cause alterations in the membrane permeability of renal tubules and vasculature, contributing to toxicity (Deray, 2002; Onyewu & Heitman, 2007). Furthermore, this antifungal has a poor oral and intramuscular absorption, and is consequently formulated as a micellar suspension solubilized in deocyclolate (D-AMB) or as a lipid formulation liposomal amphotericin B (L-AMB), amphotericin B lipid complex (ABLCL) or amphotericin B colloidal dispersion (ABCD; Caillot et al., 1994; Ruhnke et al., 2011).

Despite their wide use over several decades, the actual incidence of *Candida* resistance to polyenes is rare, but can sometimes arise through a reduction in the ergosterol content of the cell membrane (Sanglard et al., 2003; Williams et al., 2011).

Azole antifungal agents are fungistatic through interference with the fungal cytochrome P450-dependent enzyme lanosterol 14 α -demethylase (key enzyme in the biosynthesis of ergosterol), which leads to an accumulation of aberrant sterol intermediates and depletion of ergosterol in the fungal cell membrane (Bossche et al., 1987; Henry et al., 2000; Onyewu & Heitman, 2007; Williams et al., 2011).

The first azoles developed were the imidazoles (miconazole and ketoconazole) for the systemic treatment of human mycoses, followed by the first-generation triazoles (fluconazole and itraconazole; Onyewu & Heitman, 2007). The imidazoles can be used to treat invasive disease, but are most effective as topical therapies against cutaneous and mucocutaneous infections (Kyle & Dahl, 2004; Onyewu & Heitman, 2007). Both fluconazole and itraconazole are well absorbed through the gut, which means that oral administration is an effective mean of systemic delivery. Fluconazole has also a good safety profile when given systemically, with few side effects (Brammer et al., 1990; Onyewu & Heitman, 2007; Williams et al., 2011). The low-toxicity profile, favorable pharmacokinetics and general efficacy of fluconazole have made it the predominant prophylactic and primary treatment choice for invasive candidiasis for the past 20 years (Onyewu & Heitman, 2007). However, since the triazoles fluconazole and itraconazole have limitations related to their spectrum of antifungal activity and their tolerability, efforts to develop improved triazoles have led to the regulatory approval of voriconazole (approved by the FDA in 2002) and posaconazole (approved by the FDA in 2006) (Bink et al., 2011; Katragkou et al., 2008).

Certain *Candida* species are inherently resistant to azole antifungals, and acquired resistance has also emerged in recent years (Williams et al., 2011). The latter has mostly emerged due to the inadvertent selection of resistant subpopulations during exposure to azoles (Onyewu & Heitman, 2007; White et al., 1998).

Echinocandins (caspofungin, micafungin and anidulafungin) are the most recent advances in antifungal drug development. These fungicidal compounds are semi-synthetic amphiphilic lipopeptides composed of a cyclic hexapeptide core linked to a variably configured lipid side chain (Onyewu & Heitman, 2007). Echinocandins have a unique mechanism of action that consists of the inhibition of the β -1,3-D-glucan synthase, a predominant polysaccharide component of the Ascomycete cell wall that maintains osmotic integrity and is involved in cell division and growth. These three echinocandins demonstrate a broad and similar spectrum of *in vitro* and *in vivo* activity against most *Candida* species (Douglas, 2001; Espinel-Ingroff et al., 2009; Onyewu & Heitman, 2007; Pfaller et al., 2008).

Caspofungin, anidulafungin and micafungin are all well tolerated without serious adverse effects (Morrison, 2006; Onyewu & Heitman, 2007). However, the large size of these compounds precludes oral formulations, and they are currently only available for intravenous administration (Onishi et al., 2000; Onyewu & Heitman, 2007). Each agent has achieved therapeutic efficacy comparable to previously established standard agents. In a study of adults with invasive candidiasis, caspofungin treatment was equivalent to, but better tolerated than amphotericin B (Mora-Duarte et al., 2002; Onyewu & Heitman, 2007). Currently, caspofungin is regarded as reasonable initial treatment for invasive *Candida* infections except for *C. parapsilosis*, which has demonstrated less *in vitro* sensitivity (Onyewu & Heitman, 2007; Saravolatz et al., 2003).

Echinocandin drugs demonstrate fungicidal activity and low incidence of resistance (Onyewu & Heitman, 2007). Nevertheless, as newer drugs, long-term studies are not yet

available, and significant resistance mechanisms may emerge in the future (Onyewu & Heitman, 2007).

The group of nucleoside analogues is solely composed of flucytosine. This antifungal agent is only used as a part of a combination regimen due to the rapid emergence of resistance against it (Francis & Walsh, 1992; Ruhnke et al., 2011; Viviani, 1995). This is the only systemically used antifungal for which therapeutic drug monitoring is established to avoid toxic effects (Francis & Walsh, 1992; Pasqualotto et al., 2007; Ruhnke et al., 2011).

In spite of the myriad of antifungal agents available, the frequency of treatment failure is considerable. Indeed, in the last few years, *Candida* species have evolved multiple molecular strategies to exert drug resistance. The increase in cases of infection caused by *Candida* strains, and the consequent excessive use of antimicrobials, has favored the emergence of resistance of these yeast species to conventional antifungal agents over the past decades (Onyewu & Heitman, 2007; White et al., 2002). Nowadays, new approaches are needed to improve the outcome of patients suffering from *Candida* infections, because it seems unlikely that the established standard treatment will drastically lower the morbidity of mucocutaneous *Candida* infections and the high mortality associated with invasive candidiasis (Van de Veerdonk et al., 2010).

Therefore, this review will discuss the last advances in the traditional antifungal therapy, and present an overview of novel strategies for the treatment of *Candida* infections, with a special focus on combined antifungal agents, antifungal therapies with alternative compounds (extracts of plants and isolated essential oils), adjuvant immunotherapy, photodynamic therapy and laser therapy.

***Candida* infection alternative therapy**

Combining antimicrobial agents therapy

In spite of the myriad of antifungal agents available, as mentioned above, the frequency of treatment failure is considerable, underscoring the necessity for new treatment strategies. *In vitro* experiments suggest that combinations of these agents might improve the antifungal efficacy (Baltch et al., 2008; Tobudic et al., 2010a; Van de Veerdonk et al., 2010). To note, for example, the synergistic effects obtained recently between newer and more traditional antifungal agents both *in vitro* and *in vivo* (Lewis & Kontoyiannis, 2001).

There are several predictable advantages in combinational antifungal therapy, which include a wider spectrum and potency of drug activity, faster antifungal effect, synergy, lower dosing of toxic drug and reduced risk of antifungal resistance. However, although the use of agents with different mechanisms of action is a hallmark in current medical therapies, the combinations have to be tested as they may be antagonistic or clinically indifferent with additive side effects (Lewis & Kontoyiannis, 2001).

There are several studies reporting positive results of synergistic combinations. Recently, Tobudic et al. (2010b) studied the *in vitro* activity and synergism of the combination of amphotericin B and either caspofungin or posaconazole against *C. albicans* biofilm cells. The combination of amphotericin B and posaconazole yielded synergism against

biofilms of various *C. albicans* isolates (Bink et al., 2011; Tobudic et al., 2010b). Caspofungin also demonstrated synergistic effects in combination with amphotericin B, which binds to membrane sterols. The mechanisms of synergic or additive effects for these agents are likely to be the inhibition of (1,3)- β -D-glucan formation by caspofungin leading to cell wall damage, that would allow amphotericin B easier access to the fungal cell membrane, where it binds to membrane ergosterol, resulting in pore formation and cell lysis (Franzot & Casadevall, 1997; Gahn et al., 2007).

Several studies support the combination of echinocandins with other antifungal drugs to treat *C. albicans* biofilms (Bink et al., 2011; Kuhn et al., 2002b; Pai, 2009). For example, synergy effects were found for micafungin and fluconazole, voriconazole or liposomal amphotericin B against *Candida* species (Espinel-Ingroff et al., 2009; Nishi et al., 2009; Olson et al., 2005). The triple combination of liposomal amphotericin B, micafungin and flucytosine (Bink et al., 2011; Pai, 2009), as well as the combination of flucytosine and voriconazole has also been shown to improve the treatment of *C. parapsilosis* infections (Bink et al., 2011; Pai et al., 2008).

Therefore, combinational therapy has been shown to enhance *Candida* eradication. For example, it was recently shown that the use of a human antibody fragment, efungumab, in combination with caspofungin enhanced the activity of this agent *in vivo* (Espinel-Ingroff et al., 2009; Hodgetts et al., 2008). Calcineurin inhibitors FK506 and cyclosporine A also demonstrated a synergistic effect in combination with fluconazole against *C. albicans* biofilms, both *in vitro* and *in vivo* studies. The synergism observed was mediated via direct inhibition of *C. albicans* calcineurin, which is known to contribute to fluconazole resistance in biofilms (Bink et al., 2011; Uppluri et al., 2008).

The combination of antibacterial agents with standard concentrations of antifungal agents against *C. albicans* biofilms has also been reported (Bink et al., 2011; Ku et al., 2010; Miceli et al., 2009). Indeed, Miceli et al (2009) determined that the use of doxycycline at specific concentrations improved the effect of both fluconazole and amphotericin B against *C. albicans* biofilms.

Due to the interesting results of combining antifungals with doxycycline, Ku et al. (2010) determined the activity of other antibacterial agents, including azithromycin, tigecycline and vancomycin against *C. albicans* biofilms. Tigecycline alone was the most active agent against *C. albicans* biofilms, but its combination with amphotericin B, caspofungin and fluconazole was not synergistic and in some cases even inhibited the effect of the antifungal agents (Bink et al., 2011; Ku et al., 2010). On the other hand, a synergism between fluconazole and minocycline was observed against fluconazole-resistant *C. albicans* clinical isolates. This synergism may have resulted from the enhancement of minocycline on fluconazole penetrating through the biofilm as well as interrupting the calcium balance instead of impacting on the uptake and efflux on fluconazole (Bink et al., 2011; Shi et al., 2010).

Although an interesting approach, it must be noted that the combination of high doses of antibacterial agents with standard concentrations of antifungal agents against

C. albicans biofilms may induce bacterial resistance (Bink et al., 2011).

The combination of different antifungal therapies has not yet demonstrated to result in additional efficacy for the treatment of invasive candidiasis in clinical trials (Van de Veerdonk et al., 2010). In fact, synergism and antagonism are *in vitro* concepts that are difficult to translate into clinical practice. Consequently, although the evidence of synergism of antifungal combinational therapy *in vitro* may represent a first step in establishing appropriate antifungal therapy (Bink et al., 2011), additional research is needed regarding the efficacy of these combinations in randomized clinical trials (Espinel-Ingroff et al., 2009).

Natural compounds as agents for antifungal therapy

Several new therapies are being developed based on medicinal plants, exploring the activity of their extracts, essential oils and fractions against microorganisms (Anibal et al., 2010). The use of natural products aimed the control of fungal diseases is considered as an interesting alternative to synthetic fungicides due to their lower negative impact, reduced cost and adverse reactions to plant preparations compared to modern conventional pharmaceuticals (Doddanna et al., 2013).

Medicinal plants have always played a major role in the development of medicine and public health (Doddanna et al., 2013). At least 80% of the world population is estimated to be still using traditional medicine, and plants represent a large source of bioactive compounds, which have resulted in the detection of a significant number of therapeutic properties (Anibal et al., 2010; Barros et al., 2010a; Guimarães et al., 2010; Pai et al., 2004; Supreetha et al., 2011). Indeed, approximately 50% of the new chemical molecules found between 2000 and 2006 have come from natural products (Anibal et al., 2010; Newman & Cragg, 2007). Finding naturally occurring compounds for use in pharmaceutical applications has become an interesting approach (Barros et al., 2010a; Carneiro et al., 2010; Guimarães et al., 2010).

However, the novel, complex and diverse chemical structures of the compounds obtained from the medicinal plants require a more thorough investigation prior to their use as novel antifungal agents. In this sense, the identification of the medicinal plant extracts active against resistant *Candida* clinical isolates and showing pathogen selectivity is important from a practical point of view (Anibal et al., 2010).

The antifungal compounds of plants are not well known, but the presence of flavonoids and terpenes and a certain degree of lipophilicity might determine toxicity by the interactions with the membrane constituents and their arrangement (Anibal et al., 2010; Tomás-Barberán et al., 1990). Such compounds can be obtained from wild plants and algae, which are rich in bioactive compounds (Guimarães et al., 2010; Silva et al., 2009), and have been reported to have a variety of biological effects, including anti-oxidant, anti-carcinogenic, anti-inflammatory and antimicrobial activities (Barros et al., 2010b; Tepe et al., 2004).

Many extracts of plants and isolated essential oils have demonstrated to exert biological activity, *in vivo* and *in vitro*, which has justified research on the characterization of their

antimicrobial activity (Agarwal et al., 2010; Anibal et al., 2010; Martínez et al., 1996).

The antimicrobial activity shown by plant oils is mainly due to a number of phenolic and terpenoid compounds, which have antibacterial or antifungal activity (Agarwal et al., 2010).

Agarwal et al. (2010) tested several plants oils, namely, clove (*Syzygium aromaticum*), tulsi (*Ocimum tenuiflorum*), ginger grass (*Cymbogon martini* va. *matinii*), tea tree (*Melaleuca alternifolia*), ocimum tenuiflorum (*Ocimum tenuiflorum*), castor (*Ricinus communis*), juniper (*Juniperus*), malkangani (*Celastrus paniculatus*), coconut (*Cocos nucifera*), peppermint (*Mentha piperita*), babchi (*Psoralea corylifolia*), mahua (*Madhuca longifolia*), ginger (*Zingiber officinale*), mustard (*Sinapis*), rose oil (*Rose*), jasmine (*Jasminum*), eucalyptus (*Eucalyptus*), lavender (*Lavandula*), linseed (*Linum usitatissimum*), neem (*Azadirachta indica*), chamomile (*Matricaria recutita*), sesame (*Sesamum indicum*), jyotishmati (*Celastrus paniculatus*), jojoba (*Simmondsia chinensis*), walnut (*Juglans regia*), almond (*Prunus dulcis*), khus (*Chrysopogon zizanioides*), wheatgerm (*Triticum aestivum*), chaulmoogra (*Hydnocarpus wightiana*) and cade oil (*Juniperus oxycedrus*). Eighteen of these selected plant oils were found to be effective, with peppermint, eucalyptus, ginger grass and clove oils acting as potent antifungal agents against *C. albicans* with better results than fluconazole. The significant antifungal activity of these oils suggests that they could serve as a source of compounds with therapeutic potential against *Candida*-related infections (Agarwal et al., 2010).

For its turn, Duarte et al. (2005) observed that the oil of *Achillea millefolium*, *Mikania glomerata* and *Stachys byzantina* have a strong activity against *C. albicans*, while *Aloysia triphylla*, *Anthemis nobilis*, *Cymbopogon martini*, *Cyperus articulatus*, *Cyperus rotundus*, *Lippia alba*, *Mentha arvensis* and *Mentha piperita* showed moderate activity (Anibal et al., 2010; Duarte et al., 2005).

The antimicrobial nature of the essential oils isolated from several *Lamiaceae* species has been attributed to the presence of various substances, mainly the phenolic monoterpenes thymol and carvacrol. The individual oil components were tested, and thymol was found to be the most active substance with the widest spectrum, followed by carvacrol, R-terpineol and terpinen-4-ol, whereas *p*-cymene was reported to be the least active (Dorman & Deans, 2000; Tepe et al., 2004). The results of Tepe et al. (2004) are in agreement with the latter case. However, the most active component was found to be carvacrol, rather than thymol, as already seen in previous studies (Cimanga et al., 2002; Cosentino et al., 1999, Dorman & Deans, 2000; Tepe et al., 2004).

Anibal et al. (2010) reported some studies where activity against strains of different *Candida* species were observed using the extracts of *Mentha piperita*, *Arrabidaea chica*, *Rosmarinus officinalis*, *Tabebuia avellaneda*, *Syzygium cumini*, *Punica granatum*, *Casearia sylvestris* and *Arctium lappa*. For its turn, Zhang et al. (2006a) observed that the fractions TTS-12 and TTS-15 of *Tribulus terrestris* had significant antifungal activities against the five yeasts tested: *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *Cryptococcus neoformans* (Anibal et al., 2010; Zhang et al., 2006a).

The casbane diterpene fraction extracted from *Croton nepataefolius* (an aromatic plant native of the Northeast of Brazil extensively used in folk medicine as a sedative and antispasmodic agent) showed antimicrobial activity against some microorganisms tested, namely, *C. albicans* and *C. tropicalis* (Carneiro et al., 2010).

Previous studies showed that the ethanolic extracts of some Italian and Ethiopian plants have antimycotic activities against *C. albicans* (Abdullah et al., 2013), as well as the ethanolic extract of ginger powder. In addition, Silva et al. (2001) investigated ethanol extracts from leaves of *Annona crassiflora* and *A. coriacea*, and the fruits of *Solanum lycocarpum* and *S. grandiflorum* against 52 strains of *C. albicans*, four strains of *C. tropicalis* and three strains of *C. krusei* clinical isolates. Among these, leaves of *A. crassiflora* had the greatest antifungal activity, being active against all the microorganisms tested (Silva et al., 2001).

Alves et al. (2014) evaluated the potential antifungal effect of phenolic compounds (gallic acid, catechin, luteolin and quercetin) identified from flowers of the Northeastern Portugal, against *Candida* planktonic and biofilm cells. In that study, Gallic acid presented the highest antifungal effect against different planktonic *Candida* species (*C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*) and catechin showed a similar effect only against *C. albicans* cells. In biofilm evaluation, gallic acid and quercetin demonstrated a slight effect against the different *Candida* species studied.

Barros et al. (2013b) performed a screening of the antifungal potential of a phenolic extract of *Cistus ladanifer* from Northeastern Portugal, against different *Candida* species. The results presented a strong inhibition of *C. albicans*, *C. glabrata* and *C. parapsilosis* growth (MIC < 0.05 mg/mL), and a moderate inhibition of *C. tropicalis* growth (MIC = 0.625 mg/mL; Barros et al., 2013b). The same authors studied the antifungal potential of a phenolic extract of *Castanea sativa*, *Filipendula ulmaria* and *Rosa micrantha* flowers from Northeastern Portugal, against the mentioned *Candida* species. Overall, extracts of *C. sativa*, *F. ulmaria* and *R. micrantha* revealed promising antifungal effects against *Candida* species, especially in the latter. The stronger effect showed by *R. micrantha* extracts against all *Candida* species and, particularly its fungicide effects in *C. glabrata*, might be related to the presence of flavonoids [e.g. (+)-catechin and procyanidin dimers and trimers] that were inexistent in the other samples (Barros et al., 2013a).

However, in spite of the promising results, these are only preliminary studies and the information available regarding active plants against *Candida* species has not yet resulted in effective formulations for human use. In particular, studies on the effect of these compounds against *Candida* biofilms have been scarce, and have indicated that high concentrations might be needed to obtain some effect on these structures (Alves et al., 2014).

Immunotherapeutic approaches

New knowledge on the mechanisms of host defense against *Candida* infections has led to the development of novel immunotherapeutic approaches to combat these pathogens. Current strategies being developed include vaccination,

therapeutic antibodies, recombinant cytokines and adoptive transfer of primed immune cells (Table 1; Van de Veerdonk et al., 2010).

Vaccination

Although vaccination against *Candida* infections is not yet clinically available, there are many vaccine approaches that seem to be promising, with positive results in animal tests, as those involving the use of diphtheria toxoid CRM197 conjugated with the algal antigen laminarin (Torosantucci et al., 2005; Van de Veerdonk et al., 2010); mannan protein conjugates (Han et al., 1999; Van de Veerdonk et al., 2010); adhesins Als1p and Als3p (Ibrahim et al., 2005, Spellberg et al., 2005, 2006; Van De Veerdonk et al., 2010); and live-attenuated *Candida* or low virulent CA2 strain (Van de Veerdonk et al., 2010). However, these strategies still need to be assessed for clinical efficacy and safety in humans. An especial challenge is the immunization of immunocompromised patients with *Candida* infections, since a successful vaccination is dependent on the appropriate host defense mechanisms to provide protection.

The current vaccines can be improved by the use of adjuvants. Recent knowledge into adjuvanticity indicates that it is possible to use adjuvants to shape the kind of adaptive immune response elicited by a vaccine, by inducing different cytokine profiles (Eisenbarth et al., 2008; Mata-Haro et al., 2007; McCluskie & Krieg, 2006). This can be used to specifically induce optimal defense against a pathogen. For example, the adjuvant should ideally facilitate a strong Th1 [e.g. alum, β -glucan (Leibundgut-Landmann et al., 2008; Lin et al., 2009; Van de Veerdonk et al., 2010)] and Th17 [e.g. mannans, dectin-2, β -glucan (Leibundgut-Landmann et al., 2008; Robinson et al., 2009; Van de Veerdonk et al., 2010)] response for disseminated and mucosal candidiasis, respectively.

Antibodies

Immunotherapy with anti-*Candida* antibodies is another recent alternative to treat or prevent *Candida* infections, which is particularly promising as a therapy for immunocompromised patients. These antibodies, induced artificially, have been tested in animal models and patients with candidiasis. Efungumab is an example of a human recombinant antibody that was developed against the fungal-specific heat shock protein 90 (Hsp90; Matthews, 1994). This antibody has been assessed in combination with amphotericin B (Pachl et al., 2006; Van de Veerdonk et al., 2010) and caspofungin (Hodgetts et al., 2008; Van de Veerdonk et al., 2010) for the treatment of candidiasis with positive outcomes (Hodgetts et al., 2008; Pachl et al., 2006; Van de Veerdonk et al., 2010).

Other approaches include the use of monoclonal antibodies (mAbs) and immune serum from mice vaccinated with *Candida*-mannan containing liposomes (Han & Cutler, 1995; Han et al., 2000; Van de Veerdonk et al., 2010), recombinant anti-mannan antibodies (Van de Veerdonk et al., 2010; Zhang et al., 2006b), protective antibodies induced by synthetic glycopeptide vaccines (Van de Veerdonk et al., 2010; Xin et al., 2008), anti- β -glucan antibodies induced by

Table 1. Advances and limitations of immunotherapeutic approaches, adapted from Van de Veerdonk et al. (2010).

Immune strategy	Advantages	Limitations	Agent	Therapeutic potential
Vaccination	Broad-spectrum activity Low risk for the development of resistance Long-term effects	Dependent on the competence of host immune status; most patients with disseminated candidiasis are immunocompromised Might also induce disease-enhancing Ab Relatively slow effects	Ribosomal cell fraction (D.561) Diphtheria toxoid CRM 197 conjugated with Lam Mannan protein conjugates Adhesins Als1p and Als3p	VVC VVC; disseminated candidiasis VVC; disseminated candidiasis Oropharyngeal candidiasis; disseminated candidiasis
Antibodies (Ab)	Can be directly fungicidal independent of host immune status Very specific (e.g. strain specific) Rapid effects Could be used as monotherapy Experience with efficacy and safety in patients Already on the market Rapid effects	May induce the development of anti-antibodies Potentially toxic	Als3p conjugated with alum Live attenuated <i>Candida</i> strain CA2 Recombinant Ab against heat shock protein 90 (Efungumab) Anti-mannan Ab Anti- β -glucan Ab Idiotypic antibodies (YKT neutralizing Ab KT4) GM-CSF	Disseminated candidiasis Disseminated candidiasis Invasive candidiasis VVC; disseminated candidiasis VVC; disseminated candidiasis VVC; disseminated candidiasis Refractory mucosal candidiasis; disseminated candidiasis
Cytokines	Experience with efficacy and safety in patients Already on the market Rapid effects	Mainly effective as adjunctive therapy Might influence the pharmacokinetics and efficacy of the combined antifungal drug Inflammation as a potential consequence	G-CSF IFN γ	Disseminated candidiasis Oropharyngeal (in HIV) candidiasis; disseminated candidiasis
Adoptive transfer of primer immune cells	Opportunity to develop immune cells before immunocompromised status	Risk for developing graft-versus-host disease after transplantation	Adoptive transfer of anti- <i>Candida</i> T cells or pulsed dendritic cells	Disseminated candidiasis

β -glucanconjugate vaccines (Pietrella et al., 2010; Van de Veerdonk et al., 2010) and idiotypic antibodies directed against KT4 mAbs, which neutralize the effect of the yeast killer toxin YKT (Polonelli et al., 1994; Van de Veerdonk et al., 2010).

Cytokine therapy

Cytokines can be used to modulate the immunological response during infections. The granulocyte-macrophage colony-stimulating factor (GM-CSF) has been investigated for this purpose. GM-CSF enhances phagocytosis and the release of reactive oxygen species (ROS) by polymorphonuclear leucocytes (PMNs), prolongs the survival of neutrophils by inhibiting the programmed cell death (Brach et al., 1992; Van de Veerdonk et al., 2010), upregulates the expression of dectin-1 on macrophages (Willment et al., 2003) and upregulates chitinase promoting fungicidal activity by cleaving the chitin present in the inner cell wall of *C. albicans* (Van de Veerdonk et al., 2010; Van Eijk et al., 2005). The administration of GM-CSF to both humans and mice significantly reduced the fungal outgrowth (Gaviria et al., 1999; Kullberg et al., 1999; Van de Veerdonk et al., 2010). Furthermore, its use as an adjuvant to antifungal therapy has led to clinical and mycological improvements (Kullberg et al., 2004; Shahar et al., 1999; Van de Veerdonk et al., 2010; Vazquez et al., 1998).

Interferon gamma ($\text{IFN}\gamma$), produced by T and NK cells, increases macrophage cytotoxicity and kills intracellular pathogens (Hübel et al., 2002; Van de Veerdonk et al., 2010). $\text{IFN}\gamma$ therapy is most effective in patients with granulomatous disease, reducing the incidence of infections, with several studies indicating its benefit for the treatment of *Candida* infections (Bodasing et al., 2002; Dignani et al., 2005; Van de Veerdonk et al., 2010). However, the particular effector functions are debatable. Some works indicate an increase of the anti-*Candida* activity of the macrophages (Baltch et al., 2005; Brummer et al., 1985, 1991; Redmond et al., 1993; Van de Veerdonk et al., 2010), others report no effect on macrophage activity (Marcil et al., 2002; Van de Veerdonk et al., 2010), and some works report an increased capacity of PMNs to kill *Candida*.

Adoptive transfer of primed immune cells

Antigen primed dendritic cells (DCs) can be used as another approach for antifungal immunotherapy. For example, by *ex vivo* priming of dendritic cells with antigens that induce specific cytokine profiles, followed by infusion in the patient (Bacci et al., 2002; Van de Veerdonk et al., 2010), it is possible to angle the adaptive immune response towards anti-*Candida* effector functions (Bozza et al., 2004; Van de Veerdonk et al., 2010). Indeed, it has been shown that DC vaccination induces Th1-dependent antifungal protection in mice that received allogeneic bone marrow transplants (Bozza et al., 2003; Van de Veerdonk et al., 2010). Furthermore, adoptive transfer of anti-*Candida* T cells has been proposed as potential immunotherapy in patients with candidiasis after hematopoietic stem cell transplantation (Tramsen et al., 2007; Van de Veerdonk et al., 2010). These T cells do not appear to be affected by cryopreservation

(Tramsen et al., 2007), so it might be possible to generate anti-*Candida* T cells before the patient reaches an immunocompromised status, and adoptively transfer these cells during infection when the patients are immunocompromised (Van de Veerdonk et al., 2010).

Low-level laser therapy

Laser light has been evaluated, both *in vitro* and *in vivo*, as an alternative antimicrobial treatment (Schwarz et al., 2005, 2006; Sennhenn-Kirchner et al., 2009; Soukos et al., 2003). Low-level laser therapy (LLLT) uses low doses of visible light of appropriate wavelength to activate existing biological chromophores in cells, leading to the generation of ROS (Marques et al., 2004; Souza et al., 2010). The LLLT sensitization depends on parameters related to the laser such as wavelength, power density or light intensity that reaches to the tissue and the energy density that is responsible for the desired radiation effect (De Souza et al., 2006; Wilson & Mia, 1993).

The significant antimicrobial potential of LLLT has been documented *in vitro*, on diverse microbial species, and *in vivo*, in the treatment of gingivitis, periodontitis and other oral diseases (Basso et al., 2011; Lino et al., 2011; Moritz et al., 1998; Nussbaum et al., 2003), with most studies focusing on bacteria and few reporting its effect on fungi (De Souza et al., 2006; Donnelly et al., 2007; Ward et al., 1996). The antimicrobial mechanism of LLLT on bacteria seems to be related to thermal effects and photo-disruption (Basso et al., 2011; Maver-Biscanin et al., 2004). LLLT may not cause immediate cell death, but it causes sublethal damages, such as destruction of the cell wall and accumulation of denatured proteins in the bacterial cytoplasm (Basso et al., 2011).

The scarce works using LLLT against fungi involved *Candida* species, and the LLLT sources used included a near infrared indium gallium arsenide phosphide (InGaAsP) diode laser prototype (LASERTable; 780 ± 3 nm wavelength, 0.4 W power output; Basso et al., 2011), a gallium–aluminum–arsenide (GaAlAs) laser (660 nm wavelength, 0.035 W power output, 0.38 cm^2 illuminated area; Souza et al., 2010), an erbium:yttrium–aluminium–garnet (Er:YAG) laser (2940 nm wavelength) and a diode laser (810 nm wavelength; Sennhenn-Kirchner et al., 2009).

Although some studies have reported the fungicide effect (reductions in log CFU/ml) of LLLT alone (Maver-Biscanin et al., 2005; Souza et al., 2010), most *in vitro* works demonstrate that the use of a photosensitizer associated with the laser is very effective against bacteria, yeasts, virus and parasites (De Souza et al., 2006; Sarkar & Wilson, 1993; Wainwright, 1998; Wilson & Mia, 1993).

Despite the promising results demonstrated by this new approach for the treatment of *Candida* infection, much research still need to be done in the area to consolidate the existing findings.

Photodynamic therapy

Photodynamic therapy (PDT) has been successfully applied to the treatment of malignant neoplasia, particularly among head and neck related tumors. Nowadays, the use of PDT to treat

other pathologies such as systemic rheumatoid arthritis, fungal and bacterial infections has been investigated (De Souza et al., 2006; Teichert et al., 2002; Usacheva et al., 2001; Williams et al., 2003).

PDT is based on the activation of photosensitizers added to cells and microorganisms, by an appropriate wavelength of light in the presence of oxygen, promoting a phototoxic response of the cells, usually via oxidative damage (Dougherty et al., 1998; Mima et al., 2010). Indeed, these photosensitizer products are cytotoxic to the target cell, killing the microorganism by causing disarrangements in the cell wall and DNA damage (Romanova et al., 2003; Souza et al., 2010). This effect leads to alterations in the mitochondrial gradient of ionic concentration, interfering in the respiratory chain, inhibiting or stimulating ATP synthesis and at the same time producing singlet oxygen, toxic to the cells (Bortoletto et al., 2004).

The PDT sensitization depends on the parameters related to the concentration, time of incubation and type of photosensitizer, as well as the physiological state of the microorganisms, time of exposure and energy density of the laser (De Souza et al., 2006; Souza et al., 2010; Teichert et al., 2002; Wilson & Mia, 1993).

Since most microbial species possess only specific wavelength ranges of endogenous photosensitive components, the use of an exogenous photosensitizer able to absorb light and to initiate the formation of free radicals expands the range of photonics interventions (Souza et al., 2010; Wilson et al., 1992). In this respect, the action of different photosensitizers, mainly phenothiazine dyes, porphyrins and phthalocyanines, has been investigated. Many studies have demonstrated the efficacy of phenothiazine dyes such as methylene blue and toluidine blue in PDT for the reduction of fungi (De Souza et al., 2006; Giroldo et al., 2009; Souza et al., 2010; Teichert et al., 2002). Another substance that can be used as a photosensitizer is malachite green, a cationic dye of the triarylmethane family (which also includes crystal violet).

PDT using the mentioned photosensitizers promotes an effective action in the reduction of the number of *Candida* cells, especially *C. albicans*. Indeed, some studies confirm these results (De Souza et al., 2006; Giroldo et al., 2009; Prates et al., 2007; Souza et al., 2010; Wilson & Mia, 1993). Giroldo et al. (2009) studied the photosensitization of *C. albicans* using the combination of the photosensitizer methylene blue with a diode laser (684 nm and 28 J/cm²) and obtained a significant growth decrease. For its turn, de Souza et al. (2006) studied the photosensitization of different *Candida* species with methylene blue (0.1 mg/ml) and InGaAlP laser light (685 nm and 28 J/cm²) and found a reduction in the numbers of CFU/ml of 91.6% of *C. krusei*, 88.6% of *C. albicans*, 84.8% of *C. dubliniensis* and 82.3% of *C. tropicalis*. In a study by Wilson & Mia (1993) a reduction in the numbers of CFU/ml of 77% for *C. albicans*, 65% for *C. tropicalis*, 63% for *C. stellatoidea* and 40% for *C. kefyr* was reported when these species were subjected to toluidine blue combined with helium–neon (He–Ne) laser light (632.8 nm and 66.36 J/cm²). Other study with *C. albicans* sensitized *in vitro* by toluidine blue, thionin and crystal violet associated with He–Ne laser, showed

a higher microbial reduction with toluidine blue (Wilson & Mia, 1993). This photosensitizer was also the most effective in a study by Souza et al. (2010) against different *Candida* species, where methylene blue, malachite green and low-power laser irradiation alone were also tested.

It is important to mention that divergent results have been obtained on studies concerning the application of photosensitizers alone in the absence of laser irradiation. While some show no significant difference when compared with the control groups, others demonstrate the presence of antifungal activity (Calzavara-Pinton et al., 2005; Souza et al., 2010; Usacheva et al., 2001; Wainwright & Crossley, 2002). These divergences reported in the literature might be due to the lack of pre-defined parameters for the use of PDT, a fact that impairs a reliable comparison between the results obtained in different studies.

In consequence of the use of non-specific oxidizing agents, organisms resistant to conventional antifungal agents may be successfully killed by PDT and the development of resistance to this therapy seems unlikely, making this a very promising therapy (Mima et al., 2010). However, evidently, the knowledge about the application of PDT and also LLLT to *Candida* species is even scarcer and demands for more and reliable studies in the area.

Concluding remarks

Candida infections account for a high burden of morbidity and mortality. Prolonged usage of antifungal agents to treat infections caused by *Candida* species has led to the emergence of resistance to conventional drugs. Considering this, new therapeutic approaches are urgently needed to improve the outcome of the patients, as the currently available treatment options have not reduced the mortality and morbidity associated with *Candida* infections over the recent years.

Possible solutions to improve the efficacy of the treatment would be the use of combined antifungal drugs and the exploration of alternatives compounds as antifungal agents (extracts of plants and isolated essential oils). One of the most promissory sources for the research of new agents is actually found in plants, which have compounds with antimicrobial properties that are only now beginning to be studied. The investigation of these active principles may be a potential area that must be explored.

Other solution would be the use of immunotherapy, which aims at improving host defense against *Candida*. The increase in understanding the mechanisms that underlie the pathogenesis of *Candida* infection could bring closer the development of efficient and feasible immunotherapeutic strategies.

Another approach discussed in the present review relates to the use of PDT and LLLT. Although, the significant fungicide potential of LLLT and PDT has been documented and proved in several studies it is evident that the knowledge about the application of these therapies to *Candida* species is even scarcer and demands for more and reliable studies in the area.

Therefore, although several methodologies have been developing in the last years to treat *Candida* infections, there is still much to do.

Declaration of interest

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