SciMedCentral

JSM Microbiology

Review Article

Update on the Fungal Biofilm Drug Resistance and its Alternative Treatment

Karina Siow Yen Ng How Tseung and Jingjun Zhao*

Department of Dermatology and Venereology, Tongji University School of Medicine, China

***Corresponding author**

Jingjun Zhao, Department of Dermatology and Venereology, Tongji Hospital, Tongji University School of Medicine, no.389, Xincun Road, Putuo District, 200065, Shanghai, China, Tel: 8613917696735; Email: zhaomyco@163.com

Submitted: 25 February 2016

Accepted: 13 March 2016

Published: 15 March 2016

Keywords

- *Aspergillus fumigates*
- Biofilm
- Antifungal resistance
- Combination therapy
- Traditional Chinese medicine

Abstract

The mortality rate caused by fungal infections has been drastically increasing over the past few decades. The population affected consists mostly of immuno-compromised patients such as organ transplant patients, human immunodeficiency virus (HIV) positive patients or those suffering from leukemia. The two most common causative pathogens for mortality within the population of these affected patients are *Candida albicans* (*C. albicans*) and *Aspergillus fumigatus* (*A. fumigatus*). Life threatening diseases, for example, invasive aspergillosis, are caused by the opportunistic filamentous mold, *A. fumigatus*. It has been demonstrated that antifungal agents were not able to completely eradicate the disease, or that high doses were required to do so. This eventually resulted in severe side effects and patients` resistance to the drug became a major issue. C. *albicans* and *A. fumigatus* were both found to form a multicellular complex known as biofilm. This structure has the ability to resist conventional doses of antifungal drugs thus making treatment of such fungal infections problematic. This paper aims in reviewing the numerous factors involved in the formation of biofilm and their contributions to the resistance against antifungals. An urgent need for new treatment protocols capable of eliminating biofilms and reduce recurrences are required, therefore alternative possibilities in treating biofilms are also discussed here. Combination of conventional drugs were brought to scientists' attention as a potential way of treating those difficult cases, however it comes with drawbacks. An alternative approach is to look into natural products, such as traditional Chinese medicine (TCM), which comes with multiple benefits and were demonstrated to possess antifungal properties. Although several reports showed TCMs having beneficial effects on C.*albicans* and dermatophytes, only a few papers reported its effects on *A. fumigatus*, and no studies were done on their biofilms. Research which consisted of combining standard antifungal agents with TCM have shown good synergism against *Candida* species and dermatophytes, further investigations for its application in the clinical setting need to be undertaken.

ABBREVIATIONS

C. albicans: Candida albicans; A. fumigatus: Aspergillus fumigates; TCM: Traditional Chinese Medicine; ECM:Extracellular Matrix; eDNA: Extracellular DNA; SOD: Superoxide Dismutases; DDC: Diethyldithiocarbamate; ABC: ATP Binding Cassette; MFS: Major Facilitator Superfamily; ROS: Reactive Oxygen Species; Hsp-90: Heat Shock Protein 90; MIC: Minimum Inhibitory Concentration; IC_{50}: 50% Inhibitory Concentration; CNS: Central Nervous System; FICI: Fractional Inhibitory Concentration Index; PSB: Pseudolaric acid B; BBR: Berberine Chloride; CFU: Colony Forming Units

INTRODUCTION

In the last few decades, infection due to fungi has risen drastically with one of the most common causative agent being *Aspergillus spp*, it comes second after *Candida albicans* (*C. Albicans*). *Aspergillus* species are an increasingly widespread opportunistic filament forming molds consisting of more than 180 species [1]. *Aspergillus fumigatus* (*A. fumigatus)* is the most common species which targets mainly the lungs forming a variety of life threatening systemic diseases such as invasive aspergillosis. Research shows that immunocompromised patients, such as organ transplant recipients, people who are human immunodeficiency virus (HIV) positive, those suffering from leukemia or those under long term therapy with glucocorticoids are mostly affected.

Mortality rate among these individuals has risen to 50% or higher over the past few years [2]. Despite recent advances in the development of new antifungal agents, clinical resistance is still a problem that is hard to resolve. A fascinating discovery showed pathogenic fungi capable of producing a multicellular

complex known as biofilm, which is able to resist conventional doses of antimicrobial drugs. This discovery has lead researchers to believe that these structures could make clinical treatment of infectious diseases increasingly problematic [3]. It was clearly established in the past, that bacterial biofilms predominate in nutrient-sufficient environments [4], and researchers were persuaded that fungal resistance to drugs was also associated with a biofilm formation [5].

Before biofilm in pathogenic fungi became a major issue, there was a generalized perception that microorganisms existed as free floating (planktonic) organisms in liquid culture [6]. However, there were instances of increased resistance to antifungal agents which called into question the theory of this planktonic feature. *C. albicans* was the most popular fungal pathogen studied, and it was observed to adhere to a variety of biotic and abiotic surfaces, where they form an organized structure of sessile cells that differ immensely from their planktonic type. Such communities of cells are referred to as biofilms. The fact that there is a discrepancy in the in vitro MICs of planktonic and sessile form of *C. albicans* reinforce the premise that this fungi exists as biofilm and can possibly explain the lack of absolute correlation between clinical and in vitro resistance. Our focus in this review is to highlight that *A. fumigatus*, like *C. albicans*, can exist in the form of biofilm and this phenomenon contributes to the resistance against many antifungal agents. We also make recommendations for future work in order to improve the situation.

DISCUSSION AND CONCLUSION

Biofilms and their resistance to antifungal agents

Chronic *A. fumigatus* infection starts off by human beings inhaling the conidia from the surrounding air. Histology and microscopic examination of bronchopulmonary lavage samples from aspergillosis patients have revealed that *A. fumigatus* forms multiple intertwined hyphae gathered into a complex multicellular mycetoma structure, similar to *Candida* species [7]. Although the life cycle of *C. albicans* and *A. fumigatus* are significantly different [8], cellular differentiation and the ability to form filamentous growth are similar key aspects to their potential to produce biofilm. However, there were some controversies on the true definition of biofilms; where Chandrasekar PH et al. argued that adherence to plastic microscopic coverslips or polystyrene bottoms of microtiter plates do not assure the production of biofilm. Such as in one such case, where *Saccharomyces cerevisiae* can adhere to biotic or abiotic surfaces without producing a biofilm [3]. The presence of an extracellular matrix (ECM) along with other mechanisms such as efflux pump activity, persister cells, stress response, overexpression of drug targets and the general physiology of the cells all contribute in making *A. fumigatus* biofilm resistant to antifungal therapy. This is reflected in its unaffected growth at the standard dose of concentration of antifungal agents. At times, resistance can even reach up to 1000 fold higher than planktonic morphologies [10]. Therefore, conventional therapeutic approach of invasive aspergillosis is far from ideally eradicating fungal biofilms.

Chronic infection with *A. fumigatus* is dependent on the ability of its conidia to germinate and evolve into mycelia. This mycelial structure eventually invades the pulmonary epithelial and endothelial cells. In terms of kinetics, the development of *A. fumigatus* biofilms is divided into initial adherence at 4 hours of incubation, conidial germination at approximately 6 hours, and maturation and differentiation at 8 hours onwards[11,12]. The presence of ECM satisfies the simple definition of a biofilm, it is composed of galactomannan, α-1, 3 glucans, galactosaminogalactan, monosacharides, polyols, melanin, proteins [13]. ECM of microbial biofilms has also been shown to contain extracellular DNA(eDNA); eDNA is the key structural component of the ECM, which in turn is known to be a self-manufactured extracellular matrix essential for maturation of biofilm. Protecting against external stress, ECM binds to specific classes of antifungal agents and limits penetration of the drugs. Although little is known concerning the presence of eDNA in fungal biofilms, *C. albicans* biofilm's structural and architectural integrity were associated with an increase in eDNA as the biofilms matured [14]. It was demonstrated by Margarita Martins et al., where after being treated with DNase, *C.albicans* biofilm underwent a substantial inhibitory reaction. Researchers suggested that it might be due to the direct effect on the ECM, compromising its architectural structure and maintenance. They thus concluded that eDNA played an essential role in matured biofilms rather than young developing ones. Rajendran et al. were the first to report the presence of eDNA in *A.fumigatus*. In their study, they were able to demonstrate that as *A.fumigatus* biofilm matures, eDNA, a by-product of autolysis, increases. This is believed to be part of a mechanism of antifungal resistance when compared to planktonic cells. eDNA might be a genomic DNA released from *A.fumigatus* during biofilm maturation. The addition of DNase remarkably improved the antifungal effects of amphotericin B or caspofugin. This suggests that inhibiting eDNA production can offer a potential management therapy of *A. fumigatus* infections [15]. There were however no particular changes in biofilm when tested against the combination of azoles and DNase; the reason is that eDNA forms part of the ECM and is involved in biofilm stability rather than ergosterol biosynthesis pathway targeted by azoles.

Conidial seeding density is also essential in overall structural integrity of the biofilm and resistance to antifungal agents, a concentration of $1x10^5$ conidia/ml demonstrated the most robust filamentous structure, resistant to mechanical disruption [1]. The presence of persister cells is crucial in chronic fungal infection, they form a subpopulation of dormant cells which are highly tolerant to antifungal agent and they ensure the survival of biofilm even after extensive and long-term course of antifungal therapy, thus promoting recurrence. There is a hypothesis that persister cells are present in *C.albicans* biofilm even after treatment, this is explained by a biphasic killing pattern post treatment, where a portion of fungal cells are left unaffected after a dose-response activity is observe [16]. Generation of miconazole-resistant persisters in *C.albicans* biofilms are induced by Superoxide dismutases (SODs). Through their inhibition by the Cu/Zn-Sod inhibitor, N, N-diethyldithiocarbamate(DDC) persister cells were found to be less[17].

Efflux pumps are transport proteins helping in the removal of toxic substrates from within the cells into the external environment, they help in maintaining homeostasis within the complex structure of biofilm. Increased efflux of the drug is

mediated by ATP binding cassette (ABC) and the major facilitator superfamily (MFS) transporters. Genetic elements encoding efflux pumps contribute in antifungal resistance. *A. fumigatus* was predicted to have at least 49 ABC family transporter and 278 MFS genes [18]. Analysis of biofilm resistance to voriconazole and efflux pump activity showed a consistent increase in efflux pump activity as biofilm matures from 8 hours to 24 hours [19]. *Candida* has at least 5 different drug resistance genes (CDR1 to CDR5) associated with drug efflux [20]. In previous reports, itraconazole resistance in *A. fumigatus* was due to overexpression of Atr1 clones, the gene being an ABC transporter drug resistant gene [21].

Ergosterol is the major sterol found in fungal membranes, its main function is to maintain fungal growth and is the main target for the three classes of antifungals commonly used. Overexpression of ergosterol increases the level of resistance towards drugs such as azoles or polyenes and all polyenes have high affinity for ergosterol. For example, in *Candida* species, the sterol binds with the hydrophilic ring of amphotericin B subsequently leading to formation of pores in the membrane, causing small ions such as potassium to leak out and disrupting the oxidative enzymes of the target cells, this action does not necessarily result to death [22]. There is evidence showing that the immediate killing of cells was independent of the interactions between ergosterol and amphotericin B but instead is a result of the polyene acting as an oxidizing agent with the formation of reactive oxygen species (ROS). ROS is an additional mode of action inducing apoptosis in fungal pathogens such as *A.fumigatus* and *C.albicans* [23]. Azoles, however, function by blocking ergosterol biosynthesis at the C-14 demethylation stage. Triazoles bind to lanosterol 14-α-demethylase (14-α-DM), and this leads to ergosterol depletion as lanosterol and other toxic 14-α methylated sterols keep accumulating. Azole resistance mechanism in *Candida* involves overexpression of the target 14-α-DM enzyme and possibly a downstream mutation in the ergosterol pathway [24]. Azole derivatives, with a reduced binding affinity for ERG11 gene product, 14-α-DM, is an important mechanism of azole resistance in yeasts and fungi. This reduction in affinity is caused by modification of 14-α-sterol demethylase (CYP51p). It was also demonstrated that in vivo and in vitro itraconazole resistant *A. fumigatus* strains have point mutations in CYP51A, a gene encoding CYP51p [25]. Over expression of CYP51A gene was also shown to be responsible for azole resistance in clinical isolates of *A. fumigatus*.

Other mechanisms involved in fungal biofilm resistance include heat shock protein 90 (Hsp90). Research on its implication with *C.albicans* demonstrated that it functions mainly by stabilizing phosphatase calcineurin and MaPk Mkc1 in planktonic cells. Inhibiting Hsp90 in planktonic *C.albicans* would cause a reduction in the levels of the above, however, none of these phenomena were observed in *C.albicans* biofilms. This led to the theory that Hsp90 regulates drug resistance through different mechanisms in biofilm. Inhibiting Hsp90 in *C.albicans* made the effect of azoles shift from ineffectual to highly efficacious in eradicating biofilms both in vitro and in vivo. When gendanamycin, an Hsp90 inhibitor was combined with fluconazole, MIC values were observed to be reduced by more than 30-fold. A reduction of almost 60% in matrix glucan level was observed with depletion of Hsp90, providing a link between Hsp90, glucan production and how they regulate biofilm drug resistance in *C.albicans.* Experiments were performed on *A.fumigatus* biofilms with a combination of azoles (fluconazole, voriconazole) or echinocandins (caspofungin, micafungin) and Hsp90 inhibitor, gendanamycin. The results showed good synergy between voriconazole or echinocandins and gendanamycin, but not with fluconazole. Upon observation with confocal scanning microscopy, multiple broken and burst hyphae were scattered all over the biofilm when tested with caspofungin and gendanamycin; whereas hyphae were defined as flat and ribbon-like upon addition of both voriconazole and gendanamycin. These results confirmed the hypothesis that inhibition of Hsp90 induces changes in the morphologies of *A.fumigatus* biofilms, and thus improving the effect of azoles and echinocandins against *A.fumigatus* biofilms [26]. Targetting Hsp90 may provide a positive therapeutic strategy for biofilm infections caused by both *C.albicans* and *A.fumigatus*.

Eilidh Mowat et al. demonstrated in a study that amphotericin B was the most effective against mature *A. fumigatus* biofilms [27]. However, Lass-Florl et al. reported that minimum inhibitory concentration (MIC) of amphotericin B higher than 2 μg/ml were highly associated with a fatal outcome [28]. Voriconazole was effective mostly in early stages of the biofilm, but showed poorer ability to counteract fully grown intertwined hyphae. Caspofungin was, on the other hand, consistently ineffective against all stages of biofilms [27]. Because of the above reasons leading to high resistance to common antifungal drugs, new and improved therapeutic strategies have to be established. An approach could be to look into natural products such as traditional Chinese medicine and its combination with other antifungals.

Traditional Chinese medicine as an alternative antifungal agent?

Recently, research has shown great interest on natural products with potential antifungal properties. Traditional Chinese medicines (TCMs) or their extracts have gathered a lot of attention due to their easy availability and minimal side effects. More than 300 herbs have been known to have “pesticidal” activities, some even have antifungal potential and have been used in the clinical world for centuries [29]. Although studies on the effect of TCM on *A. fumigatus* are very scarce, there is a large quantity of research based on *Candida* spp. and how TCM inhibits their growth in vitro. In an attempt to screen 56 widely used dried Chinese medical plants for their antifungal properties against *A. fumigatus, C. albicans, Geotrichum candidum and Rhodoturula rubra*, Blaszczyk and his team demonstrated that Flos Carthami (*Carthamus tinctorius L.*) was specifically effective against *A. fumigatus* with a growth inhibitory area of diameter 7cm after 4 days. R. et rh. Rhei (*Rheum palmatum L.*) also proved effective against *A. fumigatus* as well as *C. albicans*[30]. Another study that included *A. fumigatus* as their test pathogens demonstrated that chrysophanol, an isolated compound of *Rheum emodi* rhizomes had significant antifungal actions on *A. fumigatus* with an MIC of 50 μg/ml [31].

With an increase in immunocompromised patients, it was shown that non-*albicans candida* such as *Candida krusei* or *Candida glabrata* contributed to more and more infections. C. Seneviratne et al., reported for the first time the antifungal effect

of crude extracts of *Rhizoma Coptidis* and *Cortex phellodendri Chinesis* against *C. krusei* and *C. glabrata* with MIC values of 50µg/ml and 100 µg/ml respectively. Berberine is the alkaloid found in *Rhizoma Coptidis* that contributed largely in its antifungal activities [32]. Their results concurred with previous works on the fact that berberine hydrochloride showed weak inhibition against *C. albicans* but instead demonstrated significant effect on *C. krusei* as well as *C. glabrata*. In another study where 40 TCMs were investigated against 8 superficial fungal strains, they demonstrated that *Melaphis chinensis*, *Polygonum cuspidatum*, *Punica granatum* and *Schisandra chinensis* were the 4 TCMs to inhibit fungal growth with MIC value of 50µg/ml; the most susceptible fungi being *trichophyton violaceum* and *tricophyton tonsurans* [33]. They also hypothesized that variance in MICs compared to previous studies might be because of the usage of different parts of the plants thus inducing different pharmacological effects. For instance, *Melia azedarach* showed no changes in the growth of *Trichophyton rubrum* and *Microsporum gypseum* whereas in previous reports, the MIC value was significantly low (16µg/ml) [34]. *Punica granatum*, however, showed similar inhibitory results on all 8 fungi tested with MIC of 50µg/ml compared to reports on dermatophytes, *A. niger, C. albicans* and even *Cryptococcus* ssp [35].

Berberine was found effective against *Trichophyton mentagrophytes, Trichophyton rubrum, Microsporum canis* and *Microsporum gypseum* with a 50% inhibitory concentration (IC_{50}) of 2.1 to 26.6 µg/ml. Berberine can be found in many herbs reputed to possess antifungal capabilities, and hence it might be a direct candidate for future therapeutic treatments. Berberine is also one of the main alkaloids of *Mahonia aquifolium*, however in a study undertaken by Volleková et al., they demonstrated that among the protoberberines of *M. aquifolium* (berberine, jatrorrhizine, palmative and columbamine), jatrorrhizine also had significant inhibitory actions especially against dermatophytes such as *Trichophyton* and *Microsporum* with MIC values of 62.5 to 125 µg/ml or 250 to 500 µg/ml for *Candida* spp [36]. The antifungal effects of jatrorrhizine on dermatophytes were almost the same as that of fluconazole and biconazole. It has been previously reported that the mechanism of action of protoberberines, berberine and palmative, against *C. albicans* was believed to be caused by the inactivation of sterol 24-methyl transferase (24-SMT) and chitin synthase, both important enzymes in ergosterol and chitin biosynthesis [37]. Jatrorrhizine also showed similar antifungal properties, therefore, is believed to have similar mode of actions. However, according to the structural model of jatrorrhizine, it possesses an additional polar 2- methoxy-3-hydroxy substitution compared to berberine and palmative. This free hydroxy group, together with inactivation of 24- SMT and chitin synthase, are reasons to consider jatrorrhizine as a potential target for future antifungal therapy.

Another promising alternative would be pseudolaric acid B, extracted from "tujingpi" or Cortex pseudolaricis; they have been known for their antifertility, cytotoxic, antigiogenic and antifungal activities. Pseudolaric acid B was observed to be effective against six different species of *Candida* namely, *albicans, glabrata, krusei, tropicalis, dubliniensis* and *parapsilosis* with MIC values ranging from 16 to 128 µg/ml. These values were approximately similar to that of fluconazole. It has also been pointed out that pseudolaric acid B had clear, non-trailing endpoints, demonstrating the fungicidal instead of fungistatic characteristics of this plant extract, against *Candida* species [38]. Pseudolaric acid B could be a potential answer to the problematic drug resistance where non-*albicans candida*s have become infection-causing pathogens, especially when *C. krusei* is naturally resistant to fluconazole.

Ginseng as a well-known herb, is widely used in herbal medicines across East Asia. Ginseng stem-leaf saponins, which is often chosen over Ginseng roots due to its lower cost and abundance, contain active ingredients with similar pharmacological functions. Many saponins have antifungal properties and provide protection against potential pathogens [39]. The mechanism of saponin fungitoxicity is believed to originate from its ability to disrupt cellular membranes by binding with membrane sterols and causing loss of membrane integrity [40]. Ginseng stem-leaf saponins comprise of ginsenosides, polysacharides, triterpenoids, and flavonoids. Many of its medicinal effects are mostly attributed to ginsenosides; it also has multiple pharmacological actions affecting the central nervous system (CNS), cardiovascular system, growth-metabolism system and immune system, as well as anti-fatigue, anti-hyperglycemic, anti-obesity, anti-cancer, anti-oxidant and anti-aging activities. Ginseng stem-leaf saponins was shown to also have some antifungal effects on planktonic *C. albicans* [41]. In a study done by Woo Sang Sung et al. they demonstrated this effect with Korean Red Ginseng saponins. During in vitro drug susceptibility testing, the MIC value for ginsenosides ranged from 50 to 100 µg/ml, showing antifungal activity against human pathogenic fungal strains. However, it showed less potent activity than amphotericin B which acted as a control with an MIC of 5µg/ml on all fungal strains [42].

Although TCMs have been proven effective on *Candida* spp and dermatophytes, very few is known about its action on *A. fumigatus*, let alone on biofilms. Further investigations are required for screening of TCMs against *Aspergillus* spp. and more importantly, the safety of these compounds must be established before clinical use.

COMBINATION THERAPY

What are the options?

As discussed above, there is a clear need to develop new strategies to overcome resistance of *A. fumigatus* biofilms. Discovery of new antifungal agents to be used in clinical settings has been lagging behind; although recently, efforts in drug combination have been made to overcome resistance to antifungal agents.

Since amphotericin B stays the preferred choice of antifungal therapy despite its high risk of nephrotoxicity, recent studies have been undertaken to lessen its drawbacks, in the meanwhile, reducing the effective dose of amphotericin B to avoid toxicity in renal tissues remains the main priority. A possibility is to accentuate its anti-biofilm activity by combining this polyene with another drug. About 47% of transplant patients used a combinational antifungal therapy course in hope of expanding the antifungal spectrum [43]. In a study on the treatment of cryptococcal meningitis [44], the combination of flucytosine and amphotericin B resulted in faster sterilization of the

cerebrospinal fluid compared to treatment with amphotericin B alone. In this study, recurrences were reduced, however, high cost and worrisome side effects limit the use of combinations of common antifungal drugs. In another study, renal dysfunction was induced by amphotericin B, leading to rapid accumulation of flucytosine and raising the patient's risk of hematological toxicity [45]. Hence, it is important to master the mechanism of each class of antifungal agents to avoid negative interactions or complications.

Controversies over the interaction of azoles-polyenes have been discussed in a few articles. Azoles function as inhibitors of ergosterol synthesis, however, polyenes need ergosterol to function. Azoles damaging part of fungus is essential for the activity of polyenes, making these two antifungal agents antagonistic. Another proposed mechanism for this interaction was that amphotericin B interferes with the cell membrane-associated protease, enabling the passage of itraconazole into the cell [46]. It is however difficult to replicate the amphotericin B-azole interaction in vitro because the two drugs possess different time course actions; amphotericin B has rapid fungicidal activity and tends to obscure the effect of slower azoles [47] whereas amphotericin B shows good synergy with echinocandin, caspofungin [48]. In an experiment undertaken by Weixia Liu and his team, they showed that the combination of caspofungin and amphotericin B had a synergistic inhibitory activity against 8 out of 11 *A. fumigatus* biofilms, with fractional inhibitory concentration index (FICI) of below 0.5 [10].

The focus of new research has been based on the combination of antifungals with non-antifungals. This research focus has helped reduce the high costs and serious side effects that come along with combination of antifungal drugs. Baicalein, a popular Chinese herb showed growth inhibitory actions on *C. albicans* when combined with Amphotericin B [49]. Pseudolaric acid B (PSB) with fluconazole also demonstrated significant synergism when tested against fluconazole-resistant *C. albicans*; with an MIC value reduced by almost 64-fold [38,50]. Anti-biofilm mechanism of these TCMs is believed to include reduction in drug efflux or alteration in sterol biosynthesis [51]. Berberine, as mentioned above showed weak activity against *C. albicans*. In a study where berberine chloride (BBR) was combined with fluconazole to test against fluconazole resistant isolates of *C. albicans*, they noticed a significant reduction in MIC of either of the individual agent with MIC_{80} of fluconazole dropping from ≥64μg/ml to ≤ 0.125-2 μg/ml whereas the MIC of BBR was reduced by at least 4 fold. 100% of the isolates showed synergism in terms of MIC_{80} with median FICI of 0.034 [52]. They concluded that the combination of these two drugs could be a good option to treat fluconazole resistant *C. albicans* in vitro. However, the mechanism behind this interaction is still not completely understood, they hypothesized that it may be due to the inhibition of sterol 24- methyl transferase by BBR, together with active efflux of the azole with overexpression of CDR, MDR1, or FLU1 and also alterations of target enzymes. Due to poor permeability across the fungal membrane, amphotericin B must be administered at a high dosage making the rise in side effects problematic; combining it to berberine was considered relatively safe considering that its fatal dose had to exceed 23 mg/kg of body weight in mice [53]. The study of Han et al. was the first to investigate the combination effect of berberine and amphotericin B on *C. albicans* infected mice. He used the enumeration of colony forming units (CFU) to determine growth inhibition and demonstrated that berberine alone was able to inhibit growth of *C. albicans* by approximately 60% compares to its control culture. Combining 50μg/ml berberine with 0.5μg/ml amphotericin B reduced CFU from $1.211x\ 10^8$ CFU/ml to $1.1\ x\ 10^4$ CFU/ml, doubling the concentration of amphotericin B resulted to >90% CFU reduction. In vivo synergistic effect was observed when infected mice were given a mixture of the 2 drugs with a survival rate of 23 days longer. This rate of survival was similar to mice given 4 times the average amphotericin B dosage.

CONCLUSION

Medical scientists came to the conclusion that fungal infections caused by the formation of biofilms are one of the many diseases whose pathogenesis and progression are becoming more complicated. A Single drug may not be effective enough or may be hampered by severe side effects or resistances to the drug. There is therefore a serious need of new treatment protocol, with minimum side effects, to combat these life threatening diseases and reduce recurrences. Combination of antifungal agents with TCM has proven to be relatively effective against planktonic dermatophytes and *Candida* species but rare are the reports on *A. fumigatus*. Further investigations need to be done to understand the mechanism of action of TCMs and how they can inhibit the growth of *A. fumigatus* as well as its biofilms.

REFERENCES

1. Mowat E, Butcher J, Lang S, Williams C, Ramage G. Development of a simple model for studying the effects of antifungal agents on multicellular communities of Aspergillus fumigatus. J Med Microbiol. 2007; 56: 1205-1212.
2. Karthaus M. Guideline based treatment of invasive aspergillosis. Mycoses. 2010; 53: 36-43.
3. Chandrasekar PH, Manavathu EK. Do Aspergillus species produce biofilm? Future Microbiol. 2008; 3: 19-21.
4. Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM. Microbial biofilms. Annu Rev Microbiol. 1995; 49: 711-745.
5. Ramage G, Vandewalle K, Wickes BL, López-Ribot JL. Characteristics of biofilm formation by Candida albicans. Rev Iberoam Micol. 2001; 18: 163-170.
6. Donlan RM1. Biofilms: microbial life on surfaces. Emerg Infect Dis. 2002; 8: 881-890.
7. Ramage G, Saville SP, Thomas DP, López-Ribot JL. Candida biofilms: an update. Eukaryot Cell. 2005; 4: 633-638.
8. Mowat E, Williams C, Jones B, McChlery S, Ramage G. The characteristics of Aspergillus fumigatus mycetoma development: is this a biofilm? Med Mycol. 2009; 47: 120-126.
9. Ramage G, Rajendran R, Sherry L, Williams C. Fungal biofilm resistance. Int J Microbiol. 2012; 2012: 528521.
10. Liu W, Li L, Sun Y, Chen W, Wan Z, Li R, et al. Interaction of the echinocandin caspofungin with amphotericin B or voriconazole against Aspergillus biofilms in vitro. Antimicrob Agents Chemother. 2012; 56: 6414-6416.
11. Kaur S, Singh S. Biofilm formation by Aspergillus fumigatus. Med Mycol. 2014; 52: 2-9.
12. Ramage G, Rajendran R, Gutierrez-Correa M, Jones B, Williams

C. Aspergillus biofilms: clinical and industrial significance. FEMS Microbiol Lett. 2011; 324: 89-97.

13. Beauvais A, Schmidt C, Guadagnini S, Roux P, Perret E, Henry C, Paris S. An extracellular matrix glues together the aerial-grown hyphae of Aspergillus fumigatus. Cell Microbiol. 2007; 9: 1588-1600.

14. Martins M, Uppuluri P, Thomas DP, Cleary IA, Henriques M, Lopez-Ribot JL, Oliveira R. Presence of extracellular DNA in the Candida albicans biofilm matrix and its contribution to biofilms. Mycopathologia. 2010; 169: 323-331.

15. Rajendran R, Williams C, Lappin DF, Millington O, Martins M, Ramage G. Extracellular DNA release acts as an antifungal resistance mechanism in mature Aspergillus fumigatus biofilms. Eukaryot Cell. 2013; 12: 420-429.

16. LaFleur MD, Kumamoto CA, Lewis K. Candida albicans biofilms produce antifungal-tolerant persister cells. Antimicrob Agents Chemother. 2006; 50: 3839-3846.

17. Bink A, Vandenbosch D, Coenye T, Nelis H, Cammue BP, Thevissen K. Superoxide dismutases are involved in Candida albicans biofilm persistence against miconazole. Antimicrob Agents Chemother. 2011; 55: 4033-4037.

18. Nierman WC, Pain A, Anderson MJ, Wortman JR, Kim HS, Arroyo J, et al. Genomic sequence of the pathogenic and allergenic filamentous fungus Aspergillus fumigatus. Nature. 2005; 438: 1151-1156.

19. Rajendran R, Mowat E, McCulloch E, Lappin DF, Jones B, Lang S, et al. Azole resistance of Aspergillus fumigatus biofilms is partly associated with efflux pump activity. Antimicrob Agents Chemother. 2011; 55: 2092-2097.

20. Sanglard D, Ischer F, Calabrese D, Micheli M, Bille J. Multiple resistance mechanisms to azole antifungals in yeast clinical isolates. Drug Resist Updat. 1998; 1: 255-265.

21. Slaven JW, Anderson MJ, Sanglard D, Dixon GK, Bille J, Roberts IS, et al. Increased expression of a novel Aspergillus fumigatus ABC transporter gene, atrF, in the presence of itraconazole in an itraconazole resistant clinical isolate. Fungal Genet Biol. 2002; 36: 199-206.

22. Broughton MC, Bard M, Lees ND. Polyene resistance in ergosterol producing strains of Candida albicans. Mycoses. 1991; 34: 75-83.

23. Brajtburg J, Powderly WG, Kobayashi GS, Medoff G. Amphotericin B: current understanding of mechanisms of action. Antimicrob Agents Chemother. 1990; 34: 183-188.

24. Chamilos G, Kontoyiannis DP. Update on antifungal drug resistance mechanisms of Aspergillus fumigatus. Drug Resist Updat. 2005; 8: 344-358.

25. Mellado E, Garcia-Effron G, Alcazar-Fuoli L, Cuenca-Estrella M, Rodriguez-Tudela JL. Substitutions at methionine 220 in the 14alpha-sterol demethylase (Cyp51A) of Aspergillus fumigatus are responsible for resistance in vitro to azole antifungal drugs. Antimicrob Agents Chemother. 2004; 48: 2747-2750.

26. Robbins N, Uppuluri P, Nett J, Rajendran R, Ramage G, Lopez-Ribot JL, Andes D. Hsp90 governs dispersion and drug resistance of fungal biofilms. PLoS Pathog. 2011; 7: e1002257.

27. Mowat E, Lang S, Williams C, McCulloch E, Jones B, Ramage G. Phase-dependent antifungal activity against Aspergillus fumigatus developing multicellular filamentous biofilms. J Antimicrob Chemother. 2008; 62: 1281-1284.

28. Lass-Flörl C, Kofler G, Kropshofer G, Hermans J, Kreczy A, Dierich MP, Niederwieser D. In-vitro testing of susceptibility to amphotericin B is a reliable predictor of clinical outcome in invasive aspergillosis. J Antimicrob Chemother. 1998; 42: 497-502.

29. Liu X, Han Y, Peng K, Liu Y, Li J, Liu H. Effect of traditional Chinese medicinal herbs on Candida spp. from patients with HIV/AIDS. Adv Dent Res. 2011; 23: 56-60.

30. Blaszczyk T, Krzyzanowska J, Lamer-Zarawska E. Screening for antimycotic properties of 56 traditional Chinese drugs. Phytother Res. 2000; 14: 210-212.

31. Agarwal SK, Singh SS, Verma S, Kumar S. Antifungal activity of anthraquinone derivatives from Rheum emodi. J Ethnopharmacol. 2000; 72: 43-46.

32. Seneviratne CJ, Wong RW, Samaranayake LP. Potent anti-microbial activity of traditional Chinese medicine herbs against Candida species. Mycoses. 2008; 51: 30-34.

33. Yang F, Ding S, Liu W, Liu J, Zhang W, Zhao Q, Ma X. Antifungal activity of 40 TCMs used individually and in combination for treatment of superficial fungal infections. J Ethnopharmacol. 2015; 163: 88-93.

34. Orhan IE, Guner E, Ozcelik B, Senol FS, Caglar SS, Emecen G, et al. Assessment of antimicrobial, insecticidal and genotoxic effects of Melia azedarach L. (chinaberry) naturalized in Anatolia. Int J Food Sci Nutr. 2012; 63: 560-565.

35. Ponnusamy K, Petchiammal C, Mohankumar R, Hopper W. In vitro antifungal activity of indirubin isolated from a South Indian ethnomedicinal plant Wrightia tinctoria R. Br. J Ethnopharmacol. 2010; 132: 349-354.

36. Volleková A, Kosťálová D, Kettmann V, Tóth J. Antifungal activity of Mahonia aquifolium extract and its major protoberberine alkaloids. Phytother Res. 2003; 17: 834-837.

37. Park KS, Kang KC, Kim JH, Adams DJ, Johng TN, Paik YK. Differential inhibitory effects of protoberberines on sterol and chitin biosyntheses in Candida albicans. J Antimicrob Chemother. 1999; 43: 667-674.

38. Yan Z, Hua H, Xu Y, Samaranayake LP. Potent Antifungal Activity of Pure Compounds from Traditional Chinese Medicine Extracts against Six Oral Candida Species and the Synergy with Fluconazole against Azole-Resistant Candida albicans. Evid Based Complement Alternat Med. 2012; 2012: 106583.

39. Papadopoulou K, Melton RE, Leggett M, Daniels MJ, Osbourn AE. Compromised disease resistance in saponin-deficient plants. Proc Natl Acad Sci U S A. 1999; 96: 12923-12928.

40. Zhao X, Gao J, Song C, Fang Q, Wang N, Zhao T, Liu D. Fungal sensitivity to and enzymatic deglycosylation of ginsenosides. Phytochemistry. 2012; 78: 65-71.

41. Jiang M, Huang X, Shen L, Zhou F, Tu W, Shi W. Antifungal sensitivity of two Chinese traditional drugs against Candida in vitro. Chin J Mycol. 2011; 6: 26-30.

42. Sung WS, Lee DG. In vitro candidacidal action of Korean red ginseng saponins against Candida albicans. Biol Pharm Bull. 2008; 31: 139-142.

43. Hatipoglu N, Hatipoglu H. Combination antifungal therapy for invasive fungal infections in children and adults. Expert Rev Anti Infect Ther. 2013; 11: 523-535.

44. Larsen RA, Bauer M, Thomas AM, Graybill JR. Amphotericin B and fluconazole, a potent combination therapy for cryptococcal meningitis. Antimicrob Agents Chemother. 2004; 48: 985-991.

45. Groll AH, Gea-Banacloche JC, Glasmacher A, Just-Nuebling G, Maschmeyer G, Walsh TJ. Clinical pharmacology of antifungal compounds. Infect Dis Clin North Am. 2003; 17: 159-191.

46. Moore CB, Sayers N, Mosquera J, Slaven J, Denning DW. Antifungal drug resistance in Aspergillus. J Infect. 2000; 41: 203-220.

47. Steinbach WJ, Stevens DA, Denning DW. Combination and sequential antifungal therapy for invasive aspergillosis: review of published in vitro and in vivo interactions and 6281 clinical cases from 1966 to 2001. Clin Infect Dis. 2003; 37: S188-224.

48. Arikan S, Lozano-Chiu M, Paetznick V, Rex JH. In vitro synergy of caspofungin and amphotericin B against Aspergillus and Fusarium spp. Antimicrob Agents Chemother. 2002; 46: 245-247.

49. Fu Z, Lu H, Zhu Z, Yan L, Jiang Y, Cao Y. Combination of baicalein and Amphotericin B accelerates Candida albicans apoptosis. Biol Pharm Bull. 2011; 34: 214-218.

50. Guo N, Ling G, Liang X, Jin J, Fan J, Qiu J, Song Y. In vitro synergy of pseudolaric acid B and fluconazole against clinical isolates of Candida albicans. Mycoses. 2011; 54: 400-406.

51. Liu S, Hou Y, Chen X, Gao Y, Li H, Sun S. Combination of fluconazole with non-antifungal agents: a promising approach to cope with resistant Candida albicans infections and insight into new antifungal agent discovery. Int J Antimicrob Agents. 2014; 43: 395-402.

52. Quan H, Cao YY, Xu Z, Zhao JX, Gao PH, Qin XF, et al. Potent in vitro synergism of fluconazole and berberine chloride against clinical isolates of Candida albicans resistant to fluconazole. Antimicrob Agents Chemother. 2006; 50: 1096-1099.

53. Han Y, Lee JH. Berberine synergy with amphotericin B against disseminated candidiasis in mice. Biol Pharm Bull. 2005; 28: 541-544.

Cite this article

Ng How Tseung KSY, Zhao J (2016) Update on the Fungal Biofilm Drug Resistance and its Alternative Treatment. JSM Microbiology 4(1): 1027.