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New trends in the search for alternative antifungal therapies

Nowe trendy w poszukiwaniu alternatywnych terapii przeciwgrzybiczych

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Abstract

The paper addresses the issue of fungal infections in the context of growing resistance to currently available antifungal agents and the development of new antimycotics. Fungal pathogens belonging to the genuses *Candida*, *Aspergillus*, *Pneumocystis* and *Cryptococcus* account for about 90% of all fungal infections. *Candida albicans* infections are a global clinical problem, and systemic candidiasis is considered one of the most severe fungal infections, with mortality rates of about 40% despite treatment. Currently, there are five classes of antimycotics available, of which only three (azoles, echinocandins and polyenes) are used for systemic infections. The limited variety of available therapies as well as their overuse in both therapy and prevention have contributed to the growing resistance among fungal pathogens. Many mechanisms of resistance to antimycotics have been identified. These include in particular: mutations in genes encoding target proteins, increase or decrease in target protein, protein pump activity, biofilm formation or activation of stress response. The growing incidence of fungal infections and the difficulty of their treatment have forced the search for alternative therapeutic agents with new mechanisms of action. Due to the eukaryotic nature of fungal cells, recent trends in literature imply that novel agents should specifically target virulence factors or stress response of the pathogen.

Keywords: *Candida albicans*, antifungal agents, virulence

Streszczenie

Niniejsza praca podejmuje tematykę zakażeń grzybiczych w kontekście narastającej oporności na dostępne leki przeciwgrzybicze oraz opracowywania nowych antymikotyków. Około 90% wszystkich zakażeń grzybiczych jest powodowanych przez grzyby należące do rodzajów: *Candida*, *Aspergillus*, *Pneumocystis* oraz *Cryptococcus*. Zakażenia o etiologii *Candida albicans* stanowią globalny problem kliniczny, a kandydozy układowe uznawane są za jedne z cięższych rodzajów grzybic, w których śmiertelność pacjentów wynosi około 40% – pomimo podjęcia leczenia. Jak dotąd dostępnych jest pięć klas antymikotyków, z których jedynie trzy (azole, echinokandyny oraz polieny) stosuje się w zwalczaniu zakażeń układowych. Mała różnorodność dostępnych leków, a także ich nadużywanie w terapii i profilaktyce przyczyniły się do narastania oporności wśród patogenów grzybiczych. Zidentyfikowano szereg mechanizmów oporności na antymikotyki. Obejmują one w szczególności: mutacje w genach kodujących białka docelowe, zwiększenie lub zmniejszenie ilości białka docelowego, działanie pomp białkowych, tworzenie biofilmu czy aktywację odpowiedzi stresowej. Wzrastająca częstotliwość występowania grzybic oraz trudność ich leczenia wymuszają poszukiwanie alternatywnych terapeutyków, o nowych mechanizmach działania. Ze względu na eukariotyczny charakter komórek grzybiczych najnowsze trendy w literaturze przedmiotu sugerują, aby mechanizm działania nowych leków ukierunkować specyficznie na czynniki zjadliwości lub na odpowiedź stresową patogenu.

Słowa kluczowe: *Candida albicans*, leki przeciwgrzybicze, wirulencja

INTRODUCTION

Advances in areas such as transplantology and oncology have increased the average life expectancy of patients[1]. However, the risk of opportunistic fungal infections is growing proportionally to medical progress as immunocompromised patients are particularly susceptible to such infections[2,3]. Some of the factors that promote fungal infections include immunosuppressants, HIV infection, neoplasms, prematurity, advanced age, acute leukaemia, broad-spectrum antibiotics, diabetes, extensive burns, long-term catheterisation, corticosteroid therapy, bone marrow or organ transplantation[1–4]. The incidence of infections depends on socioeconomic factors and geographical region. Fungal pathogens belonging to *Candida*, *Aspergillus*, *Pneumocystis* and *Cryptococcus* are the most commonly diagnosed cause of fungal infections[5,6]. Skin wounds (superficial candidiasis) and mucous membranes of internal organs (systemic candidiasis) are at particular risk of infection. Furthermore, the development of fungal biofilm on medical instruments, such as e.g. surgical prostheses, venous or urinary catheters, promotes colonisation and tissue invasion[7].

Invasive fungal infections are a global problem due to high mortality rates. This is associated with limited therapeutic possibilities and long-term diagnosis[2,4,5]. Therefore, better understanding of the mechanisms underlying fungal pathogenesis and resistance is deeded to identify new intracellular targets for novel antimycotics. The paper discusses the problem of invasive fungal infections, presents a brief characteristic of the available antifungals as well as outlines new trends in the search for alternative therapies.

CANDIDA ALBICANS – AS A MODEL ORGANISM

Since many essential cellular processes in fungal cells proceed in a similar manner as in higher organisms, *Saccharomyces cerevisiae* and *Candida albicans* (*C. albicans*) can be successfully used as eukaryotic model organisms[8,9]. *C. albicans* is one of the most common and best-known opportunistic fungal pathogens. The completed sequence mapping and the availability of *C. albicans* genome allowed for extensive research to understand fungal pathogenesis. Furthermore, the last 20 years of research have brought about new data on drug resistance, biofilm formation, genome structure and dynamics as well as gene expression (including genes coding for virulence) depending on factors such as temperature, pH, growth pattern, the stage of infection or the use of antimycotics[8].

C. albicans is a part of natural microflora of the skin, gastrointestinal tract and genital/respiratory mucosa in mammals[10]. *C. albicans* is found in about 50–70% of the human population without causing any symptoms[1]. However, the fungus may induce superficial and systemic infections in immunocompromised patients[1,10]. These are usually endogenous infections. The first type of infections involves mucous membranes, skin and nails. In the case of systemic infections, the first stage involves blood infection with fungal cells (the so-called candidemia), which later spread to other organs of the host (systemic candidiasis). This may lead to single- or multiple-organ failure[2,3]. Literature data[1,5] indicate that *C. albicans* remains the main cause of in-hospital infections. Lower contribution is attributed to *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, *C. krusei*, and *C. dubliniensis*[6].

Poor health condition of the host (the predisposing factors discussed above) is the main predisposing factor for invasive *C. albicans* infections. Fungal virulence accounting for pathogenicity is an additional factor contributing to infection[10]. The most extensively explored *C. albicans* virulence factors include adhesion, the ability to penetrate host tissues, phenotypic variability, polymorphism, enzymatic activity, complex structure of the cellular wall, and growth in the form of biofilm, which is also known as biological membrane[7,10]. At the same time, the ability to form biofilm plays a key role in the pathogenesis, protection against the host's immune system as well as resistance of biofilm cells to the available antifungals[7].

ANTIMYCOTICS AVAILABLE FOR MEDICAL TREATMENT

Proper antifungal therapy is determined by proper identification of the pathogen and determining its susceptibility to the antifungal used as well as the half-life of the drug in the patient's body. Currently used antimycotics have been classified into 5 groups based on their mechanism of action: 1) azoles inhibiting the synthesis of ergosterol by blocking the activity of lanosterol demethylase; 2) polyenes interfering with the functioning of the cellular membrane; 3) echinocandins disrupting cellular wall biosynthesis by inhibiting β-1,3-glucan synthase; 4) fluorinated pyrimidine derivatives (5-FC) inhibiting DNA and/or RNA synthesis; 5) allylamines (terbinafine) inhibiting ergosterol biosynthesis by inhibiting squalene epoxidase and/or accumulation of toxic intermediates of sterol synthesis[6,11,12].

Azoles are first-choice prevention drugs for patients suspected of fungal infection[4]. Their mechanism of action involves inhibiting lanosterol 14α-demethylase (encoded by *ERG11*), which disturbs ergosterol synthesis, increasing 24α-methyl sterols in fungal cells[11]. Accumulation of toxic sterols in the cells inhibits fungal growth (fungistatic action). Fluconazole is the most commonly used agent in clinical practice due to its wide availability and tolerability[4,12]. The use of azoles in antifungal therapy has certain limitations due to their nephrotoxicity and interaction with other drugs, such as e.g. statin and corticosteroids[13]. Furthermore, the fungistatic activity of azoles has contributed to selection for resistant strains.

Polyenes, such as amphotericin B (AmB), bind to ergosterol, and thereby impair cell membrane function.

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Ergosterol is the predominant sterol in fungal plasma membranes and is essential for their integrity. Polyenes bind to ergosterol in the lipid bilayer[12,13], leading to pore formation in the membrane, which results in the loss of integrity and lysis. These phenomena cause cellular death[11]. The toxicity of all polyenes is due to their affinity to cholesterol, which is a human equivalent of ergosterol found in fungi[14]. Therefore, AmB therapy is associated with multiple adverse effects, such as neurotoxicity. However, most of these negative effects may be avoided by using liposomal formulations[4,12].

Echinocandins (caspofungin, anidulafungin, and micafungin), which interact with fungal cell wall, have been recently introduced in antifungal therapy. The mechanism of action of echinocandins is based on a non-competitive inhibition of β-1,3-glucan synthase[11,13,14]. This causes fibril dysfunction, resulting in the loss of fungal cell wall integrity[13]. Since there is no β-1,3-glucan equivalent in higher organisms, echinocandins show low toxicity[15].

Pyrimidine analogues, such as 5-fluorocytosine (5-FC) and 5-fluorouracil (5-FU), are synthetic derivatives of one of the four nitrogenous bases found in DNA nucleotides, i.e. cytosine[14]. The mechanism of action of compounds belonging to this group involves inhibition of fungal RNA/DNA synthesis. Fungal cells metabolise 5-FC to fluorinated pyrimidines, which destabilise nucleic acids. Consequently, fungal cell growth is inhibited[12]. Despite multiple pharmacological benefits (good solubility and low toxicity), the use of 5-FC is increasingly limited in clinical practice. This is due to the common primary or acquired fungal resistance to this antimycotic. Therefore, 5-FC is used in combination with another antifungal, such as AmB or fluconazole, rather than as a monotherapy[14].

Allylamines have a minor role in the treatment of fungal infections as compounds belonging to this group (e.g. terbinafine) are used in the treatment of superficial skin infections[12]. They inhibit ergosterol synthesis by a P450 cytochrome enzyme-independent mechanism, by binding squalene epoxidase. Intracellular accumulation of large amounts of squalene leads to cellular disorganisation, increased membrane permeability and cell death[11].

The widespread use of antifungals has contributed to the development of effective defense mechanisms and the emergence of resistant strains. The primary mechanisms underlying resistance to the available therapies are presented in Tab. 1. In addition to the growing resistance of fungal pathogens to the drugs used, early diagnosis of the etiological factor in a given disease is another problem. Conventional diagnosis is still based on blood cultures, which are positive in only 50–70% of candidemia cases[4,16]. Furthermore, identification of the species and acquisition of data on drug susceptibility usually take a few to several days, which prevents early diagnosis and treatment initiation. Although microscopic evaluation is rapid and may prove helpful, its negative result does not exclude infection[4,16]. Therefore, the development of molecular diagnosis for an earlier initiation of appropriate antifungal therapy based on species-dependent susceptibility patterns is another goal in combating invasive fungal infections.

IN SEARCH FOR NOVEL THERAPIES

The growing resistance of *C. albicans* to available treatments points to the need for research to develop alternative therapies. Novel compounds should show high activity, broad spectrum of action, stability, insensitivity to salt concentrations (e.g. sodium chloride), and low toxicity[17,18]. In this context, attention was focused on natural peptides with antimicrobial properties. These compounds eliminate pathogens either directly or by stimulating pro-inflammatory reactions via activation of Toll-like receptors and pro-inflammatory cytokines[17,18]. Unfortunately, despite their promising potential, natural antimicrobial peptides show a number of unfavourable properties, such as instability, susceptibility to salt concentrations in the body or haemolytic activity[17]. These properties combined with high production costs redirect the search for new antimycotics to chemical synthesis.

Due to their physicochemical properties, tetrazoes are a promising starting group for drug design. Owing to their structural features, they easily interact with a variety of enzymes and/or receptors via weak linkages, such as hydrogen bonds. Furthermore, the tetrazole ring can be easily modified by adding various functional groups, which allows for obtaining a large group of compounds with a broad spectrum of biological activity[19]. Tetrazole derivatives are used in agriculture, medicine and biochemistry[20]. These compounds show, among other things, antihypertensive, anticancer, antifungal, antibacterial, anti-inflammatory and analgesic activity[19]. Tetrazoles disrupt membrane integrity by inhibiting ergosterol synthesis in fungal cells. These are mostly compounds with strong fungistatic activity, whereas only few of them exhibit fungicidal action[21].

Class of antimycotics	*C. albicans* resistance mechanism	References
Azoles	Outflow pump system – reduced uptake of exogenous azoles and/or elimination of azoles from the cell Overexpression/point mutation of *ERG11* coding for the target enzyme (lanosterol demethylase) Mutations in *ERG3* conferring tolerance to methylated sterols	(11–13)
Polyenes	A change or reduction in the content of ergosterol in cell membrane	(12,14)
Echinocandins	Mutations in *FKS* coding for a subunit of β-1,3-glucan synthase	(11,13)
Fluorine pyrimidine derivatives	Reduced cytosine deaminase or uracil phosphoribosyltransferase	(11,14)
Allylamines	Point mutation in *ERG1* coding for squalene epoxidase	(13,14)

Tab. 1. Molecular mechanisms of C. albicans *resistance to antimycotics*

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The eukaryotic nature of fungal cells is a key problem in the development of new antimycotics. Due to the high similarity of the basic biochemical and biological processes occurring in eukaryotic cells, the new antifungal compounds cause adverse effects in mammalian cells[9]. Therefore, the latest trends in research on alternative antifungals imply focusing on virulence factors or fungal stress response[10,22].
In this context, sulfone derivatives are a promising group of biologically active compounds that exhibit e.g. anticancer, anti-inflammatory, herbicidal, antibacterial and antifungal properties[23–25]. Additionally, their fungicidal activity, which may be a starting point for the development of novel antimycotics based on sulfone group, is of great importance[24,25]. Recent studies have shown high antifungal activity of sulfone derivatives against reference strains and clinical isolates of *C. albicans*[23,24]. The activity of these compounds involved, among other things, chitin redistribution leading to the loss of cell wall and membrane integrity in yeast. Sulfones also interfere with morphogenesis and adhesive capabilities of *C. albicans*, which limits the development of biofilm[24]. Furthermore, they inhibit the activity of transcriptional factors *EFG1* and *CPH1*, which are involved in the formation of true hyphae, which allow for invasion of host tissues[23]. Due to the low *in vitro* and *in vivo* toxicity, the investigated sulfone derivatives are potential starting structures for optimising the production process of drugs used in antifungal therapy[23–25].

CALCINEURIN – A NEW TARGET FOR ANTIMYCOTICS

In recent years, the interest in transduction mechanisms mediated by the key secondary calcium transmitter has increased due to the involvement of stress response in adaptation and survival of fungal pathogens exposed to environmental stress[22]. Calcineurin, a calmodulin-activated enzymatic protein with serine/threonine phosphatase activity, is responsible for intracellular calcium homeostasis in *C. albicans* cells[22,26]. Calcineurin is a heterodimer consisting of a catalytic subunit A (encoded by *CNA1*) and a regulatory subunit B (encoded by *CNB1*). Increased cytosolic calcium ions induce calmodulin binding to the calcineurin A-subunit and blocking the self-inhibitory C-terminal domain, which leads to formation of an active calcineurin complex[27]. Furthermore, calcineurin is necessary for maintaining virulence and resistance to antimycotics in a number of pathogenic fungal species[22]. It was demonstrated that calcium ion regulation translates into, among other things, an increase in the form of true hyphae and decreased potency of drugs that inhibit ergosterol synthesis. The calcineurin signaling cascade mediates regulation of effectors, which maintain their ability to synthesise ergosterol and cellular wall (chitin, β-1,3-glucan) despite treatment with antimycotics[28]. Furthermore, the calcineurin pathway is involved in fungal programmed cell death[28,29]. Therefore, deletion of one of the genes coding for catalytic or regulatory subunit of calcineurin increases fungal susceptibility to stressors and significantly reduces *C. albicans* virulence[22].
The calcineurin pathway is also affected by heat shock protein 90 (Hsp90), which is responsible for folding and stabilisation of different regulatory proteins under stress conditions[29]. Depending on the substrate, Hsp90 binds to either inactive or active protein. This occurs via two mechanisms: a) Hsp90 binds to the substrate, maintaining it in an inactive form until the stimulatory-signal-mediated release of the substrate from the complex, allowing for folding completion; b) Hsp90 assists in the final folding, forming and/or maintenance of complexes only after substrate activation by the stimulatory signal[26]. Fungal calcineurin belongs to the first type of Hsp90 substrates, i.e. it is stabilised by Hsp90 in an inactive form, and its activation occurs upon the release from the calcineurin–Hsp90 complex[26]. By stabilising calcineurin, Hsp90 contributes to increased virulence of pathogenic strains (formation of invasive forms in the biofilm) and helps maintain cell wall integrity[13,26]. Inhibition of Hsp90 function blocks calcineurin activation, increasing the susceptibility of *C. albicans* to antifungals[26].
Therefore, calcineurin seems to be an attractive target for novel antimycotics. Unfortunately, the currently available calcineurin inhibitors, such as cyclosporine or tacrolimus, cannot be approved as antifungals due to their immunosuppressive activity. Therefore, it is necessary to search for new, more potent fungal calcineurin inhibitors which do not induce immunosuppression by non-specific binding to human calcineurin[22].

CONCLUSIONS

Fungal infections pose a threat to immunocompromised patients, and the choice of therapy is limited as only several classes of antifungals are currently available[11]. The unsatisfactory efficacy of the available therapies and widespread resistance of fungal pathogens contribute to the search for alternative therapeutic approaches. The majority of described *C. albicans* resistance mechanisms are a result of point mutations in enzymes targeted by antimycotics or in regulatory genes[12]. The latest trends imply targeting virulence or cellular pathways of fungal pathogens – as a monotherapy or in combination with other antimycotics[10]. In this context, inhibiting fungal stress response may be a breakthrough in combating systemic fungal infections. Therefore, a more thorough understanding of the calcineurin pathway and identification of domains specific for fungi are necessary for the development of new effective therapies targeting fungal pathogens[22].

Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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