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Susceptibility profile of biofilms of non-*albicans* *Candida* spp. to echinocandins

(Diploma Thesis)

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Citlivost biofilmů non-*albicans Candida* spp. k vybraným echinokandinům

(diplomová práce)

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Declaration

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Pavla Pešková

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Contents

Diploma Thesis assignment

The main aim will be to find susceptibility profiles of biofilms of *non-albicans Candida species - Candida krusei, Candida guilliermondii* a *Candida lusitaniae* to echinocandins. There will be tested caspofungin, micafungin a anidulafungin.

Clinical isolates will be gathered from the National Collection of Fungal Pathogens stationed at the Microbiology Department, Medical School, National and Kadodistrian University of Athens.

Experimental part will be expand with a review focused on echinocandins and *Candida* biofilms.

The thesis will be written in English.

Zadání diplomové práce

Cílem experimentální práce bude zjistit citlivost biofilmů non-*albicans Candida* druhů k vybraným echinokandinům. Budou testovány *Candida krusei, Candida guilliermondii* a *Candida lusitaniae.* Z echinokandinů budu testovány caspofungin, micafungin a anidulafungin.

Budou použity klinické izoláty pocházející z National Collection of Fungal Pathogens umístěné na oddělení mikrobiologie Medical School na National and Kadodistrian University of Athens.

Experimentální práce bude doplněna rešerší na téma echinokandiny a kvasinkové biofilmy.

Diplomová práce bude sepsaná v anglickém jazyce.

Abstract

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Faculty of Pharmacy in Hradec Kralové
Department of Biological and Medical Sciences
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Diploma Thesis: Susceptibility profile of biofilms of non-*albicans* *Candida* spp. to echinocandins

Yeasts of the genus *Candida* are one of the most frequent human fungal pathogens. Infections caused by them are related to a specific form of growth – biofilm (BF), which has increased their resistance to antifungal treatment. Since bloodstream infections caused by non-*albicans Candida* species are increasing, it is important to focus on their susceptibility characteristics.

The main aim of our experiment was to examine the susceptibility profiles of BF produced by rare non-*albicans Candida* species to echinocandins. We tested 3 species of the genus *Candida* – *C. lusitaniae, C. guilliermondii* and *C. krusei* and 3 different echinocandins – anidulafungin (AND), caspofungin (CAS) and micafungin (MFG). Echinocandins have unique mechanisms of action. They inhibit the function of the enzyme β-1,3-glucan synthase. Disruption of its function leads to inhibition of β-1,3-glucan production, damage of fungal cell wall and loss of viability of the cell.

In experimental part we used YNB medium and RPMI 1640 medium to grow *Candida* species BF and planktonic cells (PL). We incubated both BF and PL in 96-well microtiter polystyrene plates. Antifungal activity was assessed by the 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]2H-tetrazolium-5-carboxanilide (XTT) metabolic assay. Each drug concentration was processed in pentaplicate for each isolate.

Results indicate that MFG have the lowest MIC_{50} and that it is the most efficient drug to all tested species. MFG to BF formed by *C. krusei* (MIC_{50} 0.125 mg/L) was most efficient, followed by *C. guilliermondii* (MIC_{50} 2 mg/L) and less susceptible *C. lusitaniae* BF (MIC_{50} 16 mg/L). AND was most efficient

against *C. krusei* BF (MIC_{50} 0.125 mg/L), then *C. guilliermondii* (MIC_{50} 4 mg/L) and *C. lusitaniae* (MIC_{50} >256 mg/L). CAS was most efficient against *C. krusei* BF (MIC_{50} 1 mg/L), then against *C. guilliermondii* (MIC_{50} 32 mg/L) and *C. lusitaniae* (MIC_{50} 32 mg/L).

In the conclusion of our project we pronounced our findings stating that echinocandins seem to be efficient against non-*albicans Candida* biofilms in vitro. Biofilm was more resistant to echinocandins than planktonic cells.

Key words: echinocandins, biofilm, *Candida*, resistance, XTT

Abstrakt

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Diplomová práce: Citlivost biofilmů non-*albicans Candida* spp. k vybraným echinokandinům

Kvasinky rodu *Candida* jsou jedny z nejčastějších lidských houbových patogenů. Jimi zapříčiněné infekce jsou spojené s výskytem specifické formy růstu zvané biofilm (BF), která vykazuje zvýšenou rezistenci vůči antimykotické léčbě. Jelikož systémové infekce způsobené non-*albicans Candida* druhy se v posledních letech vyskytují stále častěji, je potřeba se zaměřit na zkoumání jejich citlivosti k různým antimykotikům.

Hlavním cílem naší práce bylo zjistit citlivost biofilmů (BF) non-*albicans Candida* druhů k vybraným echinokandinům. Testovali jsme 3 druhy z rodu *Candida* – *C. lusitaniae, C. guilliermondii* a *C. krusei* a 3 echinokandiny – anidulafungin (AND), caspofungin (CAS) and micafungin (MFG). Echinokandiny mají jedinečný funkční mechanismus. Inhibují funkci enzymu β-1,3-glukan syntetázy. Poškození jeho funkce vede ke ztrátě produkce β-1,3-glukanu, narušení buněčné stěny a ztráty životaschopnosti houbové buňky.

V experimentální části jsme jako růstové médium pro BF a planktonické buňky (PL) z rodu *Candida* použili YNB a RPMI 1640. PL i BF jsme inkubovali na 96-jamkových polystyrenových mikrotitračních destičkách. Antimykotická aktivita byla vyhodnocena pomocí 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]2H-tetrazolium-5-carboxanilide (XTT) testu. Každá koncentrace léčiva byla pro každý izolát hodnocena v pentaplikátu.

Výsledky ukázaly, že MFG měl nejvyšší antimykotickou aktivitu z testovaných echinokandinů. Nejcitlivější byl vůči MFG BF tvořený druhem *C. krusei* (MIC_{50} 0,125 mg/L), následoval druh *C. guilliermondii* (MIC_{50} 2 mg/L) a nejvíce rezistentní byl *C. lusitaniae* BF (MIC_{50} 16 mg/L). AND byl nejúčinnější proti BF *C. krusei* (MIC_{50} 0,125 mg/L), dále proti BF *C. guilliermondii* (MIC_{50}

4 mg/L) a nejméně účinný vůči BF *C. lusitaniae* (MIC_{50} >256 mg/L). CAS jevil největší účinnost proti BF *C. krusei* (MIC_{50} 1 mg/L), dále *C. guilliermondii* (MIC_{50} 32 mg/L) a *C. lusitaniae* (MIC_{50} 32 mg/L).

V závěru našeho experimentu jsme došli k přesvědčení, že echinokandiny působí antimykoticky na biofilmy non-*albicans Candida* druhů in vitro. Biofilm vykazoval vyšší rezistenci než planktonické buňky.

Klíčová slova: echinokandiny, biofilm, *Candida*, rezistence, XTT

1 Introduction

Yeasts of the genus *Candida* are one of the most frequent human fungal pathogens. They can occur on the skin and on the mucous membrane. Some may live on human body with no inconveniences. *Candida* may cause morbid states in cases of outbreak or in presence of high pathological species. They can be responsible either for initiation of superficial mycosis, which do not usually have serious course, or cause life-treating systematic infections in some cases. Most susceptible to these infections are patients with immunosuppressive and cytotoxic therapy, broad-spectrum antibiotics, patients suffering from HIV/AIDS and patients in another immunity suppressing states, to which the development of a systematic infection poses a most dangerous threat.

The occurrence of serious systematic fungal infections caused by the genus *Candida* is increasing nowadays along with their resistance to antimycotic drugs used for classic treatment – more and more often the resistance to azoles antimycotics can be found. The infection can evolve into a very serious state of candidaemia associated with high mortality especially in the risk group of immunocompromised patients. The state of candidaemia is very difficult to treat because once the infection pervades to bloodstream it can spread into liver, spleen and other vitally important organs. Surveys reveal that *C. albicans* is responsible for more than half of invasive candidaemias in Europe, but the frequency of non-*albicans* agents from the genus *Candida* seems to be increasing.

Most of *Candida* species create a specific form of growth called biofilm which is a structure of interconnected communities attached to the surface. Biofilm cells are embedded in a matrix of exopolymeric materials. The ability of this particular genus to create biofilm is one of the most important virulent factors leading to the increase of antifungal resistance. There is also a certain possibility that change of the spectrum of *Candida* species causing serious systematic infections observed in last years is influenced by a higher resistance of rare non-*albicans Candida* species biofilms.

The main aim of our project and my thesis was to find susceptibility profiles of biofilms produced by non-*albicans Candida* species to echinocandins.

We observed the activity of three different drugs from the group of echinocandins – anidulafungin, caspofungin and micafungin. Echinocandins are a relatively new group of drugs with a unique mechanism of action used for the treatment of systematic fungal infection. They block the enzyme β-1,3-glucan synthase. This enzyme is essential for the synthesis of β-1,3-glucan – the main component of the fungal cell wall. Disruption of its enzymatic function leads to inhibition of glucan production and loss of viability of fungal cell.

We were working with three species: *C. lusitaniae, C. guilliermondii* and *C. krusei.* The resistance of the *Candida* biofilm was evaluated in several aspects. We compared resistance of non-*albicans Candida* species to the same echinocandin amongst each other and searched for the highly effective drug for every species. Additionally we tested activity of echinocandins also against planktonic cells.

We used the XTT reduction assay for evaluation of the susceptibility to echinocandins.

2 The genus *Candida*

The genus *Candida* includes about a quarter of all yeast species (Schauer and Hanschke, 1999). Yeasts were observed firstly by Anton van Leeuwenhoek and they were not considered to be living organisms (Huxley, 1871). The first scientist who classified yeasts as living organisms was Louis Pasteur in 1857. Significant progress in their research has been done since then.

2.1 Taxonomy of the genus *Candida*

Candida is a genus of yeasts which belongs into the kingdom *Fungi*, the phylum A*scomycota*, the class S*accharomycetes* and the order S*accharomycetales*. This taxonomy classification was firstly described by Christine Marie Berkhout in 1923 (Barnett, 2004).

2.2 The yeast cell

Yeasts can exist in two morphological forms – as an unicellular spherical yeast or oval shaped pseudohyphae or as filamentous multicellular hyphae (Hugo et al., 2004).

They are eukaryotic organisms. Their cell contains a nucleus with a nuclear membrane encapsulating the DNA in comparison to prokaryotic cell (Hugo et al., 2004) [Figure 1].

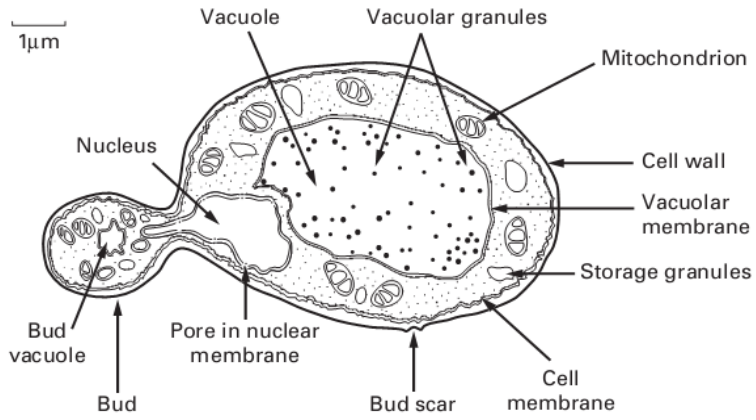

Figure 1: **Diagrammatic representation of the yeast cell**

Source: Hugo et al., 2004

In the nucleus is concentrated most of genetic material. The rest of genetic information can occur in the form of plasmids (Hugo et al., 2004).

The cell wall has a typical structure containing mainly glucan, mannan and chitin. The thickness of this structure is life cycle dependent, but it ranges between 100 to 300 nm (Hugo et al., 2004). Damage of this structure is the main target of most antifungals. Cell wall structure is more precisely described in chapter Echinocandins.

There is a periplasmatic space between the cell wall and cell membrane. It contains many proteins, molecules secreted from the membrane and other structures. It is also a location for numerous enzymes (Hugo et al., 2004).

The cell membrane is a phospolipid bilayer consisting of proteins as 1,3-β-D-glucan synthase - echinocandins target, phospolipids, lipids and sterols. The main sterol is ergosterol which is the target of amphotericin B (Hugo et al., 2004).

Other organelles of the yeast cell, in our case *Candida* species cells, are mitochondrions, endoplasmic reticulum, Golgi apparatus, ribosomes, vacuoles, peroxisomes and other vesicles (Hugo et al., 2004).

Generally the eukaryotic cell is about 10 times bigger than prokaryotic, but shape and size are species characteristic. *Candida* species we tested in our study varied in both characteristics. We observed that *C. krusei* has oval cells with frequent pseudohyphae, *C. lusitaniae* has ovoid cells but pseudohyphae were not so abundant and *C. guilliermondii* has smaller circular cells often attached to pseudohyphae.

Yeasts can reproduce sexually and asexually. However asexual reproduction called budding is the most common type (Balasubramanian, 2004). Sexual reproduction can appear in stress conditions within some yeast species (Neiman, 2005). For *Candida* species the asexual reproduction by budding is typical.

2.3 The occurrence of *Candida* species caused bloodstream infections

The most frequent agent of human fungal infections is *C. albicans (*Hugo et al., 2004; Parkins et al., 2007). Out of other medically important species several can be named: *C. Parapsilosis, C. glabrata, C. tropicalis, C. krusei*, *C. lusitaniae, C. guilliermondii* and *C. dubiliensis*. Change in the profile of *Candida* species causing bloodstream infections was observed during last years – there is decrease of *C. albicans* and increase of other *Candida* species.

Wide international survey revealed that in Europe, Latin America, Canada and United States *C. albicans* is responsible for more than half of bloodstream infections (BSI). In Europe between years 1997 and 1999 *C. albicans* was responsible for 58 % cases of *Candida* BSIs. Another frequent pathogen was *C. parapsilosis* with 19 %. In general the profile was 55 % *C. albicans* and both *C. parapsilosis* and *C. guilliermondii* comprised 15 % of BSI isolates (Pfaller et al., 2001).

In another survey from United States from years 1998 – 2000 a rapid change of BSI caused by *C. albicans* was reported. In this survey *C. albicans* compried 45 % isolates and was followed by *C. glabrata* with 24 % and *C. parapsilosis* with 13 % (Hajjeh et al., 2004). The change in the occurrence was obvious when this survey was compared to the previous Pfaller survey. We could see there that in United States *C. albicans* was also most frequent, but its incidence was 55 %.

In more current European survey from years 2002 – 2003 it was observed that *C. albicans* was still the most frequent cause of BSI with 51 %. However its incidence slightly decreased. Second most common species was *C. parapsilosis* which incidence increased to 23 % and *C. glabrata* comprised 8 % of BSI isolates (Almirante et al., 2005).

The newest international survey from years 2008-2009, published in 2011, revealed changes in the profile of *Candida* species responsible for BSI. In this survey it was reported that 48,4 % cases of BSI are caused by *C. albicans,* 18.2 % *C. glabrata* and 17.1 % by *C. parapsilosis* (Pfaller, 2011).

Results of non-european survey from India are also interesting. There was reported a totally different spectrum of *Candida* species causing BSI. Out of all tested BSI isolates 38 % were containing *C. tropicalis*,

21 % *C. albicans* and *C. glabrata, C. krusei* and *C. lusitaniae* were each detected in 3 % of isolates (Hasan et al., 2007). Studies from other regions are illustrating the diversity in broad spectrum of *Candida* species causing infection.

However extensive international surveys document the current trend of increasing incidence of BSI caused by non-*albicans Candida* species.

3 Biofilm

Microorganisms are naturally living in cooperating communities like biofilms rather than staying in a state of planktonic free floating cells. Biofilm is a specific formation of microbial growth which is attached to a biotic or abiotic surface. Vast number of microorganisms is capable to organize into this form of growth which can develop in many environments.

The general theory of biofilm predominance was promulgated in 1978 in a paper by Costerton, Geesey and Cheng. Biofilm may contain fungal cells, bacterial cells or both. Cells create a community consisting of one species or it can occur as a mixed biofilm with more species living in a mutual profit (Jenkinson and Douglas, 2002). Cells in biofilm are encapsulated and connected together as well as to the surface with exopolymeric mass called matrix [Figure 2] (Costerton et al., 1987).

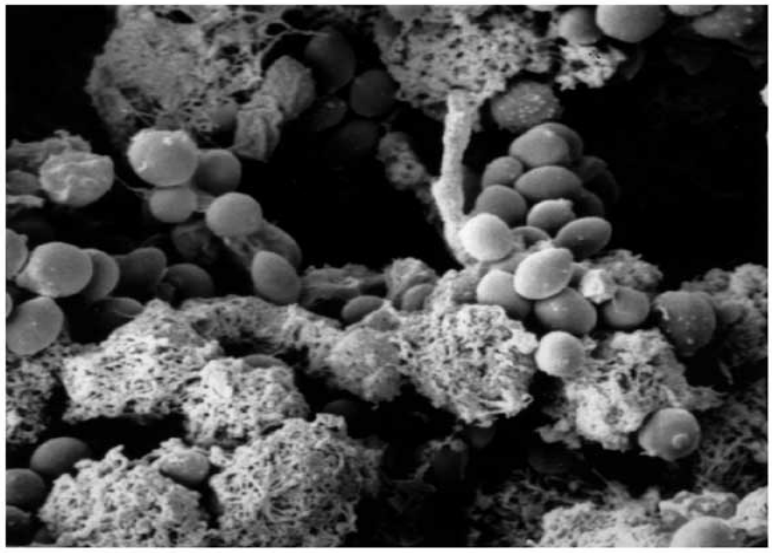

Figure 2: ***Candida* biofilm** Scanning electron micrograph of an infected catheter showing *C. albicans* biofilm

Source: Maki and Tambyah, 2001 *http://wwwnc.cdc.gov/eid/article/7/2/70-0342-f2.htm*

Biofilm can be attached to many kinds of living and non-living surfaces. It can develop in human body on biological surfaces like teeth, epithelium of respiratory system, heart valves, oral cavity and others. It is also able to adhere to surfaces of implanted materials and devices like venous or urinary

catheters, joint implants, stents or ocular glances. Implanted materials and devices are less often affected by fungal infection than bacterial. Nevertheless if fungal infections appear they are usually difficult to treat (Cox et al., 1988). The frequency of occurrence of biofilms on all kinds of surfaces has been a topic of many studies and reviews (Dolan, 2001; Douglas, 2003; Salamon et al., 2007; Prester et al., 2004; Raad, 1998; Tumbarello et al., 2007)

Specific cell organization into biofilm gains the microorganism many benefits. This survival strategy provides fungal and bacterial cells with the opportunity to metabolic cooperation, improvement of protection of the organism against the environment influences and increase in its resistance against the antimicrobial therapy and host defence.

3.2 Biofilm development

The manner of how bacteria and fungi form biofilm depends on the ecosystem and surface they inhabit. The development can be divided into several different stages. Nevertheless, I have decided to choose division into three main stages as optimal [Figure 3] (Characklis, 1981; Chandra et al, 2001b, Davies et al., 1996; O'Toole et al., 2000).

1. Attachment to the surface
2. Growth and maturation of the biofilm
3. Dispersal of cell from the biofilm

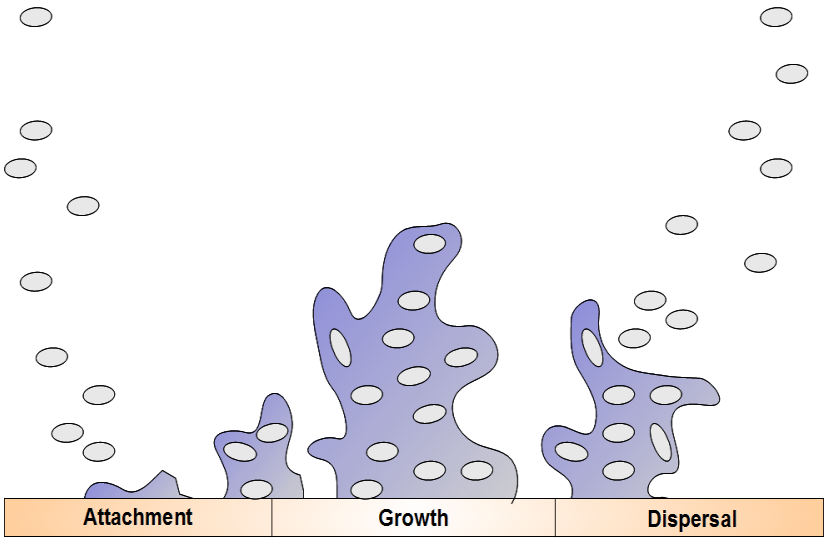

Figure 3: **Biofilm development** (Author: Pešková)

3.2.1 Biofilm attachment

The attachment of free floating cells to the wet surface is the first step of biofilm development. The solid-liquid interface as human tissue and body fluid provides an ideal environment for the development and growth of microbial biofilm. There are many characteristics which determine the most eligible substrate for formation of *Candida* biofilm as the roughness of the surface or its hydrophobicity (Quirynen and Bollen, 1995; Wimpenny and Colasanti, 1997; Nevzatoglu et al., 2007; Teughels et al., 1996).

Planktonic cells which are floating cells existing individually in their environment start to adhere to the surface, e.g. epithelium, endothelium, materials implanted to the body as catheters etc.

The attachment can be described in two phases. Initially it is a reversible process. After a few seconds or minutes the process becomes irreversible, if cells are not immediately separated from the surface. The adhesion is provided by weak electrostatics and London-van der Walls forces, interfacial tensions and covalent bonding. Irreversible changes in the adhesion are accomplished by production of extracellular polymers and participation of hydrolytic enzymes (Zobel, 1943; Costerton et al., 1981).

After the initial colonization the process of the growth and maturation of biofilm starts.

3.2.2 Biofilm growth

Growth and maturation of biofilm is a complex process. Firstly, we can observe small randomly distributed colonies of adhered cells on the surface. As the growth continues, these small colonies connect together and form a continuous layer of biofilm.

In early and intermediate stages of the biofilm development (attachment and growth) many genes of many specific molecules and also genes encoding structures enabling cell to cell communication called quorum sensing are expressed. It was discovered that *Candida albicans* quorum sensing molecules farnesol and tyrosol, which are responsible for a switch of yeast into filamentous state, can have a very complex effect on biofilm (Hornby, 2001; Alem et al., 2006). Certain amount of tyrosol is secreted in early stages and it is probably responsible for hyphal formation. Another *Candida* quorum sensing molecule

farnesol has an opposite effect and inhibits the development of filamentous forms (Ramage et al., 2001; Alem et al., 2006). However the research of biofilm quorum sensing is still in progress.

Once the biofilm is developed it stays on the place of its initial attachment, grows and changes its shape and size. Because cells need nutrients and water it has to be created channel systems for them to circulate free in the biofilm (Ramage 2001b, Ramage 2001d).

3.2.3 Biofilm dispersal

Dispersal or detachment is a terminal phase of the biofilm life cycle. As the biofilm grows and the mass increases, the fluid share stress, nutrient starvation and other factors are leading to division of biofilm cells into the environment (Trulear and Characklis, 1982; Hunt et al., 2004).

Planktonic cells can consequently leave the biofilm at this point, disperse and multiply. Afterwards biofilm acts as a nidus of incessant infection (Costeron et al., 1999). If such dispersal occurs repetitively in certain periods, it can become a reason of many chronic infections relapses.

3.3 Biofilm structure

Biofilm is a very variegated structure. Its heterogeneity is apparent both in space and in time. Fundamentally it can be divided into 2 diverse structures – the aggregation of cells and matrix - the extracellular polymeric substance (EPS).

Fully developed *Candida* biofilm contains a net of yeasts, hyphae and pseudohyphae (Hawser and Douglas, 1994). Hyphal forms have not been observed in liquid cultures or on agar surface. This finding leads to the assumption that contact with solid surface is requisite for development of this structure.

Extracellular polymeric substance can either attach cells to the surface and hold them together or it can be secreted into the environment. It occupies most (over 95 %) of the biofilm volume (Zhang, 1998). EPS contains water (up to 97 %) and many polymeric structures such as proteins, polysaccharides and extracellular DNA. However, it is primarily composed of polysaccharides. Some of them are neutral, but the majority is polyanionic. That is caused by the presence of either uronic acids or ketal-linked pyruvate and specific

groups as carboxyl, phosphate and sulphate groups (Sutherland, 1990; Al-Fatani and Dougas, 2006; Suterland, 2001). Polysaccharides form various structures in biofilm due to interactions with lipids, proteins, lectins and another polysaccharides. It was observed that micoorganisms with mutations involving an absence of genes encoding polysaccharides are unable to create real biofilm (Allison and Shuterland, 1987; Watnick and Kotler, 1999). However once they are parts of mixed biofilms it is possible for one species to live in a commensalist relationship with another species producing a sufficient amount of EPS (James et al., 1995).

3.4 Comparison of *Candida* species biofilms

The ability to form biofilm is a general attribute of *Candida* species. Nevertheless there are differences in the biofilm architecture, resistance, rate of growth, matrix (EPS) composition and substrate demands among species. It was observed that *C. krusei* create weaker biofilm than *C. albicans* (Tournu and Van Dijk, 2012). This conclusion was confirmed by our pre-experiment evaluating the ability to create biofilm of species *C. krusei, C. lusitaniae* and *C. guilliermondii* in comparison with standard *C. albicans* strain M-61. It became apparent that *C. krusei* produce less biofilm (47 – 73 % compare to 100 % growth of M-61) than *C. lusitaniae* (more than 100 % compare to M-61) and *C. guilliermondii* (also more than 100 % compare to M-61). Our pre-experiment also confirmed the need of longer 72 hours incubation time of *C. krusei* strains to get biofilm of comparable quality to biofilms of C. albicans*, C. guilliermondii* and *C. lusitaniae* extracted after 48 hours long incubation. Slower growth of *C. krusei* biofilm has been observed before (Hasan et al., 2007).

3.5 Biofilm model systems

Many kinds of in vitro model systems can be used for observation of *Candida* biofilms and their characteristics, e.g. acrylic strips, catheter discs, cylindrical cellulose filters, perfused biofilm fermenters or microtiter plates (Ramage, 2001; Hawser and Douglas, 1994; Chandra et al., 2001; Baillie and Douglas, 1998; Baillie and Douglas, 1998b). In our study the last mentioned model system – the 96-well microtiter plate was used.

Growth or the fungal cell damage can be evaluated by two basic

methods – XTT assay and other colorimetric methods or [H^3] leucine inter-corporation. Both methods give a great correlation with biofilm dry weight (Hawser and Douglas, 1994).

Another issue necessary to be considered is the fact that in vivo the biofilm is influenced by many environment factors – specific composition of nutrient in the substrate, certain temperature level and dynamic liquid flow. The provision of dynamic condition is essential especially for *Candida* species biofilms, because they produce a significantly larger mass in dynamic than in static conditions (Hawser et al., 1998b). However, all mentioned conditions can be provided for the 96-well microtiter plate model in laboratory by using the right growth medium, guarantee of temperature stability and by using the rocker to provide gentle shaking.

The 96-well microtiter plate model is very useful for testing of antifungal susceptibility of biofilm especially in combination with an evaluation colorimetric method such as XTT reduction assay. This method is easy to use, rapid, relatively accurate and reproducible for number of *Candida* species isolates (Ramage, 2001). The possibility to use this model for high-throughput screening and the fact that there is no need for special and expensive equipment make it indisputably a great choice for clinical and experimental laboratories.

4 Resistance of *Candida* species biofilms

4.1 Biofilm resistance

It is well known fact that biofilms are more resistant to antifungal drugs than planktonic cells. The amount of classic antifungal treatment drugs (amphotericin B, fluconazole, flucytosine, Itraconazole, ketoconazole and combination of amphotericin B + flucytosine and Fluconazole + flucytosine) used to decrease the metabolic activity of *C. albicans* to 50 % biofilm growth on PVC discs is five to eight time higher than the amount for planktonic cells (Hawser and Douglas, 1995). This increase in resistance was proved in coincidental studies also for biofilms growth on other surfaces such as polystyrene, silicone elastomer, polyurethane or cellulose (Ramage et al., 2001b; Chandra et al, 2001b; Lewis et at., 2002; Baillie and Douglas, 1999).

Resistance causes many problems in chemotherapy of *Candida* and other fungal and bacterial infections related with biofilm formations. It has been noticed that the susceptibility of biofilm is not only proved to be low against agents from polyenes, allylamines, azoles and pyrimidine analogues but also against echinocandins in these formations of growth (Kuhn et al., 2002). In any case it seems that echinocandins are one of most effective drugs in the therapy of *Candida* infections.

4.1.1 Common mechanisms of biofilm resistance

Microorganisms in biofilm can use many different mechanisms to increase their resistance against antifungal treatment and the host immunity.

All mechanisms of resistance have not been completely revealed yet, but possible mechanisms which can be involved in this process are: restricted or decreased penetration of drugs into biofilm, changes in phenotype as a result of low growth rate and nutrition limitation and surface induced expression of genes increasing resistance.

Restricted penetration

Restricted penetration of drug could be one of factors responsible for biofilm resistance. The production of matrix which provides adhesion to the surface and surrounds cells could decrease the cell surface exposed to drugs or the concentration of drug in the biofilm and delay the penetration.

However, many studies have demonstrated that matrix is not the main cause of biofilm resistance. There has been an assumption that the penetration can be delayed, but not significantly enough to be considered as main mechanism of resistance (Steward, 1996). The impact of matrix presence was also compared in systems when *Candida* biofilms were grown in static and dynamic conditions. Biofilms grown in dynamic conditions under gentle shaking produce more matrix. In this comparison no significant difference was found, but in separate studies with biofilms grown in flow conditions it was observed that resuspended cells with less matrix were more sensitive to amphotericin B (Baillie and Douglas, 2000; Baillie and Douglas, 1998). On that account the possibility that matrix can play a minor part in the biofilm drug resistance has to be considered.

Changes in phenotype as a result of low growth rate and nutrition limitation

Slow growth rate caused by limited supplying of the biofilm by nutrients from the environment can be another factor decreasing the susceptibility of biofilm to therapy. There are evidences that low biofilm growth is not the only cause of the biofilm resistance, because increased resistance to antifungals at all grow rates has been observed (Baillie and Douglas, 1998b).

Surface induced expression of genes increasing resistance

The adhesion of free floating cells to the surfaces is an initial step of the biofilm development. At this moment cells start to be a part of the biofilm, switch their phenotype and activate expression and repression of many genes. Upregulation of genes encoding multidrug efflux pumps can increase the resistance. *C. albicans* has two different types of efflux pumps - ATP-binding cassette transporters and major facilitators which are encoded by genes their expression increases during biofilm formation (Ramage et al., 2002). Nevertheless the fact that mutants with single or double deletion mutation

in that study were highly susceptible to the treatment signalizes that biofilm resistance is a multi-factorial process.

From other genetically determined mechanisms of biofilm resistance the alternation of a target enzyme which seems to be extremely important especially for the antifungal group echinocandins can be mentioned.

4.2 Resistance to echinocandins

Fungal pathogens use many different mechanisms to increase their resistance against antifungal therapy. These mechanisms can differently manifest in each species or they can differently influence resistance even in strains of the same species. In biofilms, which are generally difficult to treat, it is very important to define mechanisms of their drug resistance and so enable the choice and development of effective antimycotic agents.

Factors which are responsible for increase in *Candida* species resistance can be various. In previous chapter general mechanisms that biofilm may use to increase its resistance are mentioned. It is known that there exist specific mechanisms which biofilm develops as a protection against certain drugs and which can be found also in planktonic cells. For example reduced drug import into the cell, modification of drug degradation in the cell, alternation of the target enzyme, changes in enzymatic pathways or increased drug efflux to name some.

Although echinocandins are considered to be effective drugs, there have been reported cases of to them resistant *Candida* infections (Miller et al., 2006; Hakki et al., 2006). Possible mechanisms of resistance are still being studied. Anyway it seems that increase amount of efflux transporters is not the main factor, because after their genes overexpression caspofungin reacts only with a minimal increase in MIC (Schuetzer-Muehlbauer et al., 2003). Modulation of echinocandin susceptibility can be caused by genes involved in chitin synthesis, protein mannosilation, PKC dependent cell pathways and FKS genes regulation. Nevertheless the modulation of susceptibility is considered moderate and these mechanisms can be impacts of natural drug tolerance and reasons of paradoxical effect (Stevens et al., 2005; Perlin, 2007).

4.2.2 Mutation of FKS1 gene - main mechanism of resistance

It was discovered that Fks1p, the major subunit of β-1,3-D-glucan synthase, is the main target of echinocandins (Douglas, 1994) and changes in this structure represent important mechanisms of *Candida* species leading to decreasing of susceptibility to echinocandins (Kurtz et al., 1996; Douglas, 1997; Balashov et al., 2006).

There were tested clinical isolates from patients whose therapy failed or had only a low response. It was observed an amino acid substitution at Ser645 at HS1 of the subunit Fks1p (Park et al., 2005). As HS1 is designated a „hot spot 1“, a region of Fks1p that confers reduced susceptibility to caspofungin. The change causes that the glucan synthase is about 1000 times less susceptible to the treatment (Park et al., 2005). Changes in HS1 region have been observed also for other resistant *Candida* species including *C. krusei* (Kahn et al., 2007; Garcia-Effron et al., 2010). However rare mutations of genes encoding Fks proteins are, their occurrence increase (Pfaller et al., 2011b).

4.2.3 Another mechanisms reducing susceptibility to echinocandins

Another mechanism which causes increase of echinocandin antifungal activity can be the activation of compensatory pathways like PKC cell integrity pathway which leads to elevation of chitin level in the cell (Reinoso-Martin et al., 2003). It was also proved that the chaperone protein Hsp90 which is associated with structures of many proteins and interacts with protein phosphate calcineurin is accordingly integrated in processes regulating resistance to echinocandins (Singh et al., 2009).

5 Echinocandins

There are five basic classes of antifungal drugs for treatment of life-threatening fungal infections – polyenes, allylamines, azoles, pyrimidine analogues and echinocandins. The last mentioned group of antifungals and their activity against *Candida* species is the subject of our experiment.

Echinocandins are a relatively new group of antifungal drugs active against most species from the genus *Candida* and also some other fungi. Clinically important is especially their activity against the genus *Candida* and *Aspergillus*.

Target of echinocandins - enzyme β-1,3-D-glucan synthase is responsible for synthesis of polysaccharid β-1,3-D-glucan which is one of main polymers of the fungal cell wall. The fungal cell wall is a rigid structure which contains mostly glucans. Apart from β-1,3-D-glucan, β-1,4-D-glucan and β-1,6-D-glucan, the cell wall composes of chitin, galactomannan, mannan and other glycoproteins [Figure 4] (Klis, de Groot and Hellingwerf, 2001; Douglas, 2001). Nevertheless the major representation in its structure has β-1,3-D-glucan which constitutes from 30 to 60 % of the cell wall of the genus *Candida* (Denning, 2003).

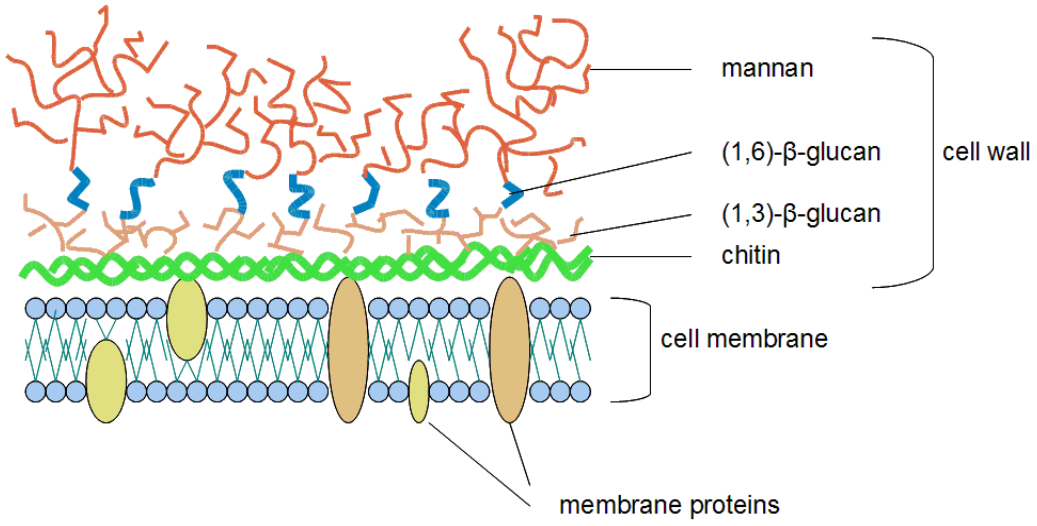

Figure 4: **Structure of the fungal cell wall** (Author: Pešková)

Numerous advantages of echinocandins are the reason why they have become one of the first line drugs in the treatment of *Candida* systemic infections (Pappas et al., 2004). Advantages of the therapy with echinocandins are their broad spectrum of activity, especially in the genus *Candida*, activity against azole-resistant *Candida* strains, low toxicity, long half-life allowing once a day dosing, the fact that the dose does not depend on age, gender or race and that they are almost no interactions with other substances on cytochrome P450 (Sakaeda et al., 2010; Damle et al., 2008; Colburn et al., 2004; Groll and Walsh, 2001; Cancidas PI, 2010; Mycamine PI, 2011; Eraxis PI, 2010).

3.1 Development of echinocandins

Echinocandins are chemically modified microbial products. The history of their development began in 1970s. The first agent with antifungal activity chemically related to echinocandins was a pneumocandin discovered in 1974 in Germany. Name of this compound was echinocandin B and it was a product of *Aspergillus nidulans* (Nyfeler and Keller-Schierlein, 1974). Even though echinocandin B has not been clinically used ever since, it has become a cornerstone for a new line of antifungal drugs - echinocandins.

The first semisynthetic echinocandin approved by U.S. Food and Drug Administration (FDA) was caspofungin, developed by Merc & Co., Inc. The approval by the European Medicine Agency (EMEA) was received in the same year with a trade name Mycamine (Mycamine: EPAR – Summary for the public). Caspofungin is a modified pneumocandin B_0 which is a fermentation product of *Glarea lozoyensis (*Letscher-Bru and Herbrecht, 2003).

Caspofungin was followed by micafungin which was invented by the drug company Astellas Pharma US, Inc. and approved by FDA in 2005 (Fujie, 2007) and EMEA in 2008 as Cancidas (Cancidas: EPAR – Summary for the public). Micafungin was prepared by a change in the structure of hexapeptide FR901370 of *Coleophoma empedra*.

The newest approved drug from the group of echinocandins is anidulafungin. It was originally developed by Vicuron Pharmaceuticals, but it was later acquired by Pfizer, Inc. which gained the approval of FDA in 2006. It was approved by EMEA in 2007 with a trade name Eclata (Ecalta:

EPAR – Summary for the public). Anidulafungin was developed from echinocandin B_0 produced by *Aspergillus nidulans.*

All echinocandins are distributed in a form of a powder for infusion. Anidulafungin and caspofungin are provided as lyophylised powders for reconstitution before infusion. Micafungin is manufactured as a powder ready for reconstitution (Denning, 2003; Kurtz and Rex, 2001; Carver 2004, Murdoch and Plosker, 2004).

3.2 Chemical characteristic of echinocandins

Currently clinically used echinocandins have a structure of semisynthetic pneumocandins. They are lipopeptides with a large molecule with molecular weight about 1200. We can distinguish two distinct parts of their structure - large amphiphilic cyclic hexapeptide and long N-linked acyl chain of fatty acid attached to its skeleton [Figure 5,6 and 7].

The composition and size of the side-chain is the characteristic of each echinocandin molecule. In the past the side-chain was considered to be a cause of haemolytic activity of early echinocandins. Later expedient modifications of this part of the molecule led to synthesis of safe compounds without haemolytic activity (Klein and Li, 1999).

The molecule of anidulafungin has an alkoxytriphenyl side-chain. Its molecular weight is 1140.24. Caspofungin has a simple side-chain and molecular weight 1093.31. Micafungin has a complex aromatic side-chain containing 3,5-diphenyl-substituted isoxazole and molecular weight 1270.28.

The molecular formula of anidulafugin is $C_{58}H_{73}N_7O_{17}$, caspofungin-acetate formula is $C_{52}H_{88}N_{10}O_{15} \cdot 2\ C_2H_4O_2$ and micafungin sodium formula is $C_{56}H_{70}N_9NaO_{23}S$.

Different structural characteristics of molecules determine their diverse solubility. Caspofungin (in form of acetate salt) and micafungin (in form of sodium salt) are both free soluble in water, whereas anidulafungin is not.

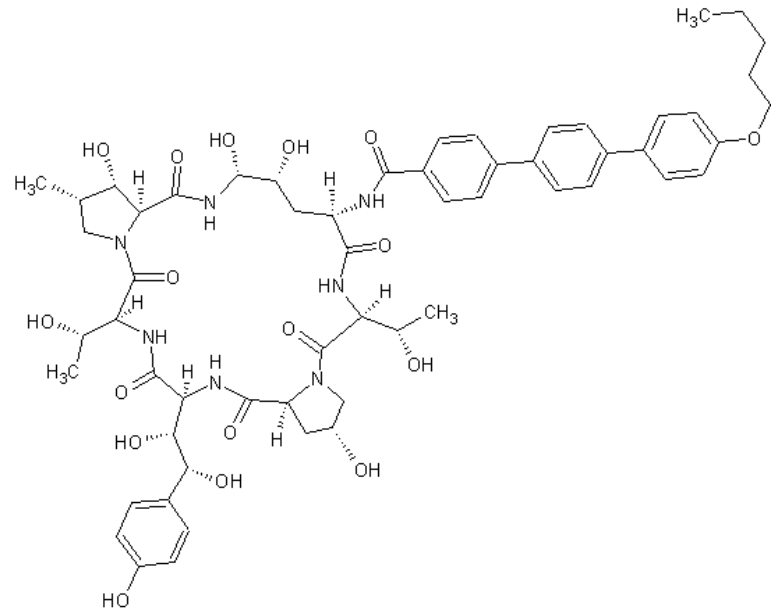

Figure 5: **Structure of anidulafungin** (Author: Pešková)

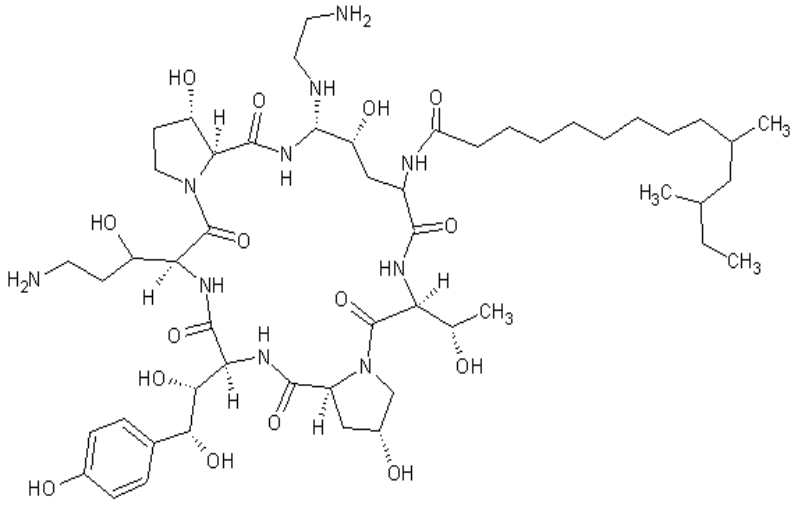

Figure 6: **Structure of caspofungin** (Author: Pešková)

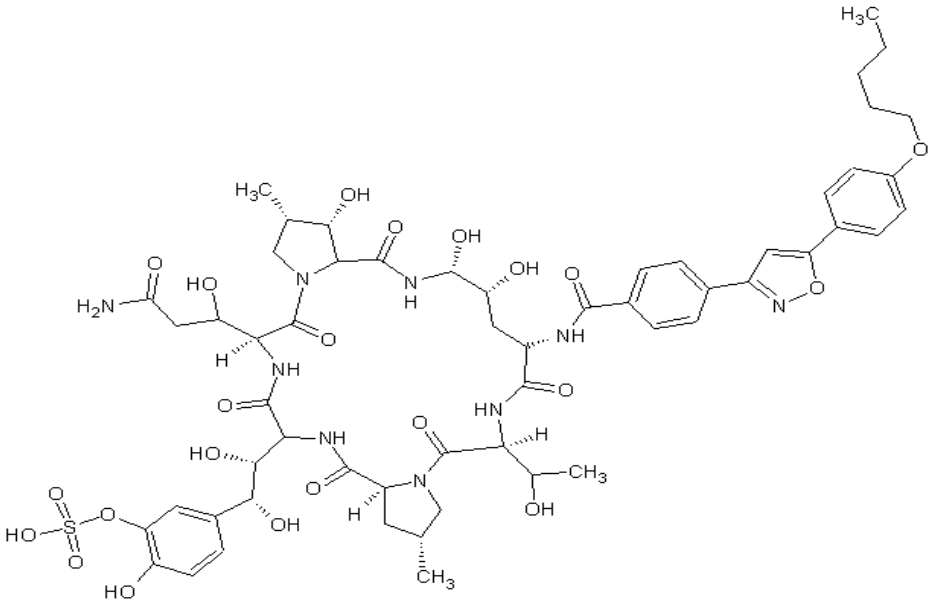

Figure 7: **Structure of micafungin** (Author: Pešková)

3.3 Mechanism of action of echinocandins

Mechanism of action of echinocandins is based on non-competitive and concentration-dependent inhibition of enzyme β-1,3-D-glucan synthase. This unique enzyme of fungi, which does not occur in human cells, is responsible for synthesis of essential carbohydrate component of the fungal cell wall – β-glucan. It is a polymer of glucose and it can occur in three main forms: as β-1,3-D-glucan, β-1,6-D-glucan and β-1,4-D-glucan. These polymers give the shape and mechanical strength to the cell wall and keep the osmotic stability. Changes in the amount of glucan, especially of β-1,3-D-glucan, are leading to the osmotic instability and the cell lysis.

Glucan synthase is an enzymatic complex bound to the cell membrane composed of 2 subunits. This enzyme and other compounds of related regulatory pathways have been observed in depth in many studies with *Candida* and other fungal species (Douglas, 1997; Mazur and Baginsky, 1996; Beauvais et al., 2001).

One of two compounds of glucan synthase is a catalytic subunit Fksp

which is a protein integrated in the cell membrane. It binds the molecule of echinocandin and a molecule of the intracellular UDP-glucose. The second compound is a regulatory subunit Rho1p, which is a small GTP-binding protein. The complex is polymerizing UDP-glucose to glucan and supplying the cell wall with new molecules of glucan [Figure 8] (Kang and Cabib, 1986; Mol et al., 1994; Shematek and Cabib, 1979; Arellano et al., 1996).

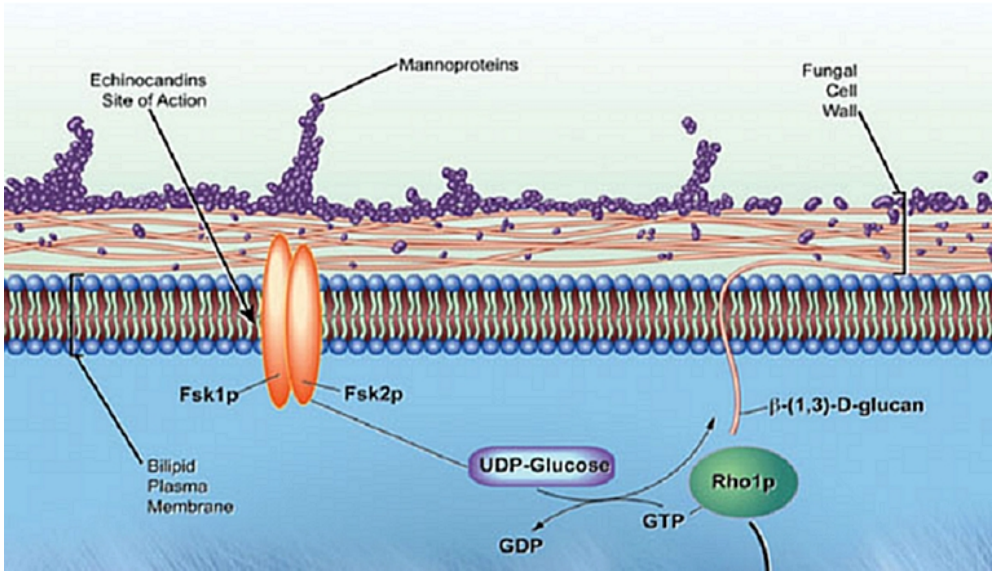

Figure 8: **β-1,3-D-glucan synthase enzymatic system** The picture is illustrating the role of β-1,3-D-glucan synthase in the synteshesis of β-1,3-glucan

Copied from: Safdar, 2009: Fungal Cytoskeleton Dysfunction or Immune Activation Triggered by β-Glucan Synthase Inhibitors

There were found two main forms of the catalytic protein Fksp called Fks1p and Fks2p. It was discovered that the gene encoding Fks2p (gene FKS2) is highly identical to the gene encoding Fks1p (gene FKS1) (Mazur et al., 1995). FKS1 is a cell-cycle dependent gene and its transcription leads to remodulation of the cell wall. FKS1 plays main role during the vegetative growth whereas FKS2 is important for the sporulation. Genes encoding these catalytic proteins of β-1,3-D-glucan synthase were first observed in *Saccharomyces cerevisiae* (Douglas et al., 1994). Later the presence of these two genes in other yeast species was researched. It was discovered that *C. albicans* catalytic subunit of 1,3-β-D-glucan synthase is encoded by similar genes as Fks1p and Fks2p

in *Saccharomyces cerevisiae* (Douglas et al., 1997).

However, the issue of where exactly echinocandins bind to the enzymatic complex has not been fully resolved.

3.4 Pharmacokinetics

Currently there are no echinocandins available for the oral use. Anidulafungin, caspofungin and micafungin are for intravenous administration only. There is a certain level of the drug available in plasma after oral administration, but this amount is insufficient for treatment of fungal infections. For example the bioavailability of caspofungin is even less than 0,2 % (Caspofungin acetate – FDA advisory committee meeting background,2000).

The recommended dose for treatment of candidaemia for caspofungin is 70 mg/day followed by a maintenance dose 50 mg/day (Cancidas PI, 2010). The recommended dosage for micafungin is 50 mg/day, 100 mg/day or 150 mg/day when the dose 100 mg/day is recommended for treatment of candidaemia (Mycamine PI, 2011). Recommended dose for anidulafungin is 200 mg/day as a loading dose, and 100 mg/day thereafter (Eraxis PI, 2010).

The c_{max} in plasma after intravenous administration of 70 – 75 mg of caspofungin and micafungin are relatively similar, which means 12 mg/L for caspofungin and 10.9 mg/L for micafungin (Stone et al., 2002; Chandrasekar and Sobel, 2006). Anidulafungin reaches the c_{max} 3.55 mg/L which is considerably lower (Eraxis PI, 2010). For all echinocandins high protein binding is typical – more that 95 % (Kofla a Ruhnke, 2011).

Anidulafungin and micafungin are mostly eliminated by the liver. Caspofungin is mostly eliminated by kidneys.

From adverse effects of echinocandins headache, fever, liver toxic effects, histamine release, haemolysis or rush can appear (Cancidas PI, 2010; Mycamine PI, 2011; Eraxis PI, 2010).

3.5 Paradoxical growth effect

An attenuation of antifungal activity in higher concentrations of the drug is called „Paradoxical growth effect" or „Eagle effect" [Figure 9]. This phenomenon was observed for the first time in 1948 in a study where penicillin bactericidal and bacteriostatic activity was tested (Eagle and Musselman,1948). It became apparent that this effect can be observed also in higher levels of echinocandins. It was initially observed for the agent cilofungin, which is a derivative of echinocandin B (Hall et al., 1988) and thereafter it was described in many studies for *Candida* species with currently clinically used echinocandins (Stevens et al., 2004; Shields et al., 2011; Melo et al., 2007; Chamilos et al., 2007; Fleischhacker et al., 2008). The paradoxical effect was observed not only *in vitro* but also *in vivo* studies (Petraitis et al., 1998).

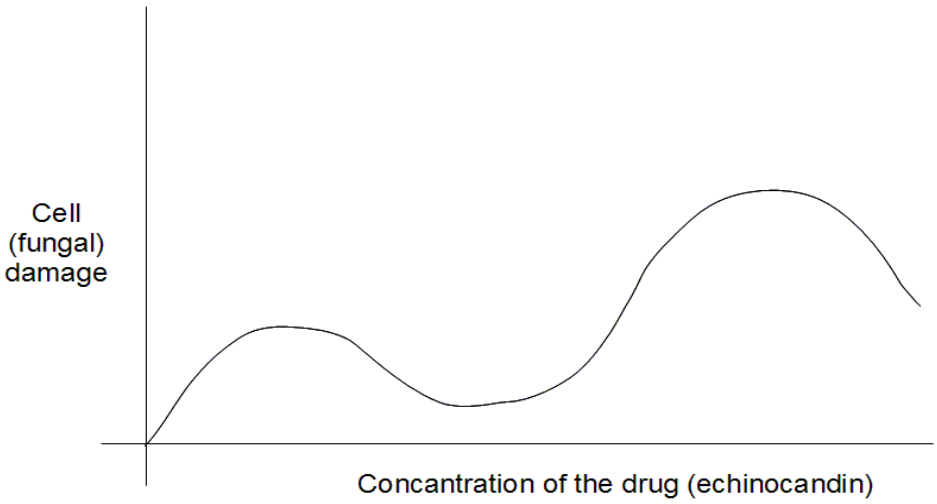

Figure 9: **Paragoxical growth effect** The line is representing fungal damage depending on the concentration of echinocandins (Author: Pešková)

Performed studies revealed that there are profile differences between tested *Candida* species. The concentration at which the paradoxical effect appears is not the same for tested *Candida* species and even for strains of one species differences and diversity in intensity of this effect were observed. In studies the effect also appeared to be media specific. Hall observed that

in RPMI medium the paradoxical effect is more pronounced than in YNB medium.

The cause of attenuation of the antifungal activity in higher concentration of echinocandins remains unknown. One of supposed reasons of development of this effect might be increasing of synthesis of chitin. This contention is based on the before mentioned study of Stevens et al. in which he quantified increase in the amount of chitin after the exposure to caspofungin.

Other potential mechanism can be up-regulation of protein kinase C. A rapid induction of the gene encoding protein kinase C was observed after exposure to caspofungin (Agarwal et al., 2003; Reinoso-Martin et al., 2003).

Other mechanisms responsible for paradoxical effect could be FKS1 mutations and up-regulation of synthesis of β-1,3-D-glucan synthase. Nevertheless it has not been proved that this mechanism occurs specifically after exposure to caspofungin in higher concentrations (Stevens et al., 2005).

Nowadays the research is focused on the clinical implications of findings in this area.

6 Methods and materials

In the practical part we were measuring the susceptibility of *C. lusitaniae, C. guilliermondii* and *C. krusei* biofilm (BF) to antimycotics from a group of echinocandins (anidulafungin, caspofungin and micafungin). We were evaluating their activity against biofilm and planktonic cells (PL). For growth of BF was necessary 48 hours (for *C. lusitaniae* and *C. guilliermondii*) or 72 hours long incubation (for *C. krusei*). Protocol is itemized in appendix.

For evaluation of biofilm susceptibility to echinocandins we used the XTT assay. It is a quantitative colorimetric method which is often used for assessment of eukaryotic cell metabolic activity. When it was proved that it can be also used for testing of fungal cell viability, it becames one of most often used methods for evaluating of susceptibility to antifungal drugs (Hawser et al., 1998; Meshulam et al., 1995).

It is based on conversion of tetrazolinum salt XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]2H-tetrazolium-5-carboxanilide) into water soluble formazan due to mitochondrial dehydrogenase. This enzyme of respiratory chain is produced only by living cells with undamaged mitochondrial membrane.

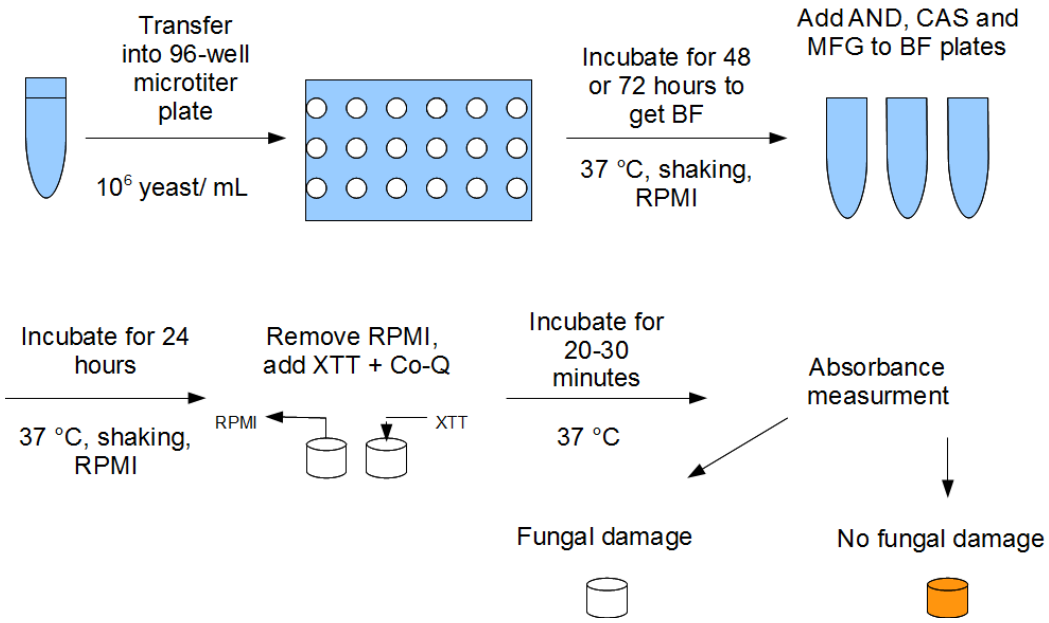

Figure 10: **Schematic plan of the XTT assay for biofilm** BF(biofilm), AND(anidulafungin), CAS(caspofungin), MFG(micafungin), (Author: Pešková)

6.1 Instrumental and material equipment

Laminar box (Gemini, STERIL)
Incubator at 37 °C (GALLENKAMP, MEMMERT)
Light microscope (OLYMPUS)
Benchtop low speed centrifuge (HETTICH UNIVERSAL)
Benchtop high speed refrigerated centrifuge for microtiter plates (HETTICH UNIVERSAL)
96-well microplate absorbance reader (ChroMate, AWARENESS TECHNOLOGY INC)
Electric hot plate with a magnetic stirrer (Cat M 6/1, ZIPPERER GMBH)
Orbital shaker
Vortex (Vortex Genie-2, SCIENTIFIC INDUSTRIES, INC.)
Analytical balance electronic scale (KERN EG)
PC with software ChroMate

Microscope slides
Cover glasses
Neubauer chamber
Centrifuge plastic tubes with a lid (15 mL and 50 mL)
Microcentrifuge safe-lock plastic Eppendorf tube (1.5 mL)
Plastic tissue culture flasks (100 mL)
Petri dishes
Inoculation loop
Bacterial filter (Klari-Flex, WHATMAN)
Automatic pipettes (GILSON)
Automatic multi-channel pipette (Tipor-M, ORANGE SCIENTIFIC)
Automatic pipette tips
96-well polystyrene microtiter plates

6.2 Chemicals

Yeast nitrogen base with amino acids solution (YNB solution)
RPMI 1640 growth medium with 2 % of glucose
Peptone liquid medium used to preserve isolates
Sabouraud agar with antibiotics (SA+)
Phosphate buffered saline solution (PBS)
Coenzyme Q solution
XTT solution

Antimycotic drugs:

Anidulafungin (Ecalta; Pfizer Ltd, US)

Caspofungin (Cancidas; MSD – Merck Sharp and Dohme Ltd, UK)

Micafungin (Mycamine, Atellas Pharma Europe B.V., Holland)

6.2.2 Preparation of solutions and media

Sabouraud agar with antibiotics (SA+)

Contains

30.000 gr of Sabouraud Liquid Medium (with 2 % of glucose)(Scharlau Chemie, S.A)

33.300 gr of agar

750.00 μL of Gentamicin Sulfate (Garamycin; MSD, UK)

300.00 μL of Chloramfenicol Sodium Succinate (Chloranic; Norma Hellas S.A. GR)

Water for injection

Preparation

We mixed Sabouraud liquid medium with agar and refilled with water of injection to 1 L. Whole volume was properly stirred. We sterilized the solution in autoclave and we added antibiotics after the sterilization. The warm solution was poured into Petri dishes and left to solidify.

Peptone liquid medium used to preserve isolates

Contains

250.00 µL of glycerol

750.00 µL of peptone water solution (4 g of Peptone from Casein (AppliChem GmbH, Germany) in 100 mL water for injection)

Preparation

We had prepared peptone water solution stock. We took 750 µL of peptone solution from the stock and mixed with 250 µL of glycerol in a plastic tube to get sufficient amount of medium to preserve one isolate sample.

Yeast nitrogen base with amino acids solution (YNB)

Contains

6.700 gr of Yeast Nitrogen Base with Amino Acids (Sigma-Aldrich Chemie GmbH, Germany)

5.000 gr of glycerol

Water for injection

Preparation

We weighed out yeast nitrogen base with amino acids and glycerol, transferred it into a bottle and filled up with water to 100 mL. Afterwards we mixed it and sterilized it by membrane filtration.

We were using this solution diluted 1:10 as a medium.

RPMI growth medium (RPMI)

Contains

10.400 gr of RPMI 1640 Medium (Sigma-Aldrich Chemie GmbH, Germany)

100.00 mL of water for injection

HEPES buffer (AppliChem GmbH, Germany)

Preparation

We dissolved 10.4 gr of RPMI in water pro injection and added as much HEPES as we needed to reach pH = 7.2. Then we sterilized it by membrane filtration.

Phosphate buffered saline (PBS)

Contains

Solution 1:

0.745 gr of di-Sodium hydrogen phosphate anhydrous p.Analysis (AppliChem, Germany)

0.306 gr of NaCl

Water for injection to 70.00 mL

Solution 2:

0.726 gr of Natruim dihydrogenphosphat-dihydrat (AppliChem, Germany)

1.314 gr of NaCl

Water for injection to 70.00 mL

Preparation

We prepared both solutions. We were adding Solution 2 into Solution 1 until pH reached 7.2. We used membrane filter for sterilization of the solution.

Coenzym Q solution

Contains

0.500 gr of Coenzym Q0 (Sigma-Aldrich Chemie GmbH, Germany)

10.00 mL of acetone

Preparation

We dissolved Coenzym Q in acetone.

XTT solution

Contains

0.025 gr of XTT sodium salt (BioChemica;AppliChem, Germany)

PBS solution to 100 mL

Preparation

We dissolved XTT in 100 mL of PBS solution. We used membrane filter for sterilization.

6.3 Organisms

Biofilms of 3 *Candida species* were observed in this experiment: *C. krusei, C. lusitaniae* and *C. guilliermondii*. Code names of *Candida* BSI isolates from patients are illustrated in the table [Table1].

Clinical isolates

C. guilliermondii – 3 strains	*C. lusitaniae* – 14 strains	*C. krusei* – 13 strains
99bl	4241	9908
111bl	5278	10226
112bl	2923	12414
	2588	6865
	4243	9200
	2732	7824
	7892	9346
	8850	7294-2
	5094	8657
	6936	12841
	9949	11154
	0640	11708
	4245	6892
	2885	

Table 1: **Code names of clinical isolates**

Isolates were gathered from the National collection of fungal pathogens stationed at the Microbiology Department, Medical School, National and Kadodistrian University of Athens.

6.4 Methods

6.4.1 Storage of clinical isolates

Tubes with clinical isolates were stored in the freezer (-35 °C).

We had prepared 5 more stock tubes of each sample for our experiment by defrosting original tubes with isolates and inoculating by them SA+ agar plates. Plates were inoculated by spreading the cell suspension over the agar surface. They were incubated for 48 hours at 37 °C. We took appropriate amount of cells after the incubation, transferred them with a loop into 1 mL plastic Eppendorf tubes with peptone liquid medium, vortexed properly and stored in -35 °C .

6.4.2 Biofilm preparation

We had to defrost certain isolate we had chosen for our week experiment. When the sample was defrosted, we vortexed it and used a sufficient amount (approximately 30 µL of suspension) to inoculate a plate with Sabouraud agar with antibiotics. We added suspension to the first quadrant of the plate with a loop and streaked in order to get single colonies. We let the plate incubate for 48 hours at 37 °C .

After 48 hours we removed the plate from the incubator. We took a loop and transferred 2 or 3 colonies from the plate into a sterile tissue plastic flask filled with 20 mL of YNB solution. Then we left the flask in the incubator for 24 hours at 37 °C in dynamic conditions. Dynamic conditions were provided by using a rocker on speed 8. This way we got a cell suspension which could be used to grow biofilm.

After the incubation we had to wash the cells. At first we put all liquid from the flask to a plastic tube and centrifuged at 2000 rpm for 10 min. After 10 minutes we took the tube out of the centrifuge and evacuated the used YNB medium. We resuspended pellets in 10 mL of PBS using the vortex for 20 minutes on maximal speed. When the content of the tube was resuspended we centrifuged again at 2000 rpm for 10 min. After the centrifugation we replaced PBS with 10 mL of RPMI solution and vortexed for 20 minutes at maximum speed.

When the suspension was homogeneous we diluted it with physiological solution 1:1000 and found out the concentration of cells per a millilitre. The Neubauer chamber and a microscope were used for counting cells.

The equation [1] we used for calculation of the concentration of cell per a millilitre was:

$$\mathbf{c = n/4 \times 10^4 \times DF} \qquad [1]$$

n.....amount of cells on 4 squares of the Neubauer chamber
c.....concentration cell/mL
DF = 10^3.....dilution factor of the suspension

Once we knew the concentration of cells per mL (initial concentration) we could continue with the next step of our experiment which was transferring microorganisms into microtiter plates. Prior to the transferring, we had to prepare a suspension of a concentration 10^6 cell/mL in RPMI solution. We calculated the initial volume of suspension and put it into a 50 mL plastic tube. We filled the volume to 28 mL with the RPMI solution.

The volume of the suspension (= initial volume) needed to be transferred into the tube was found out by using this equation [2]:

$$\mathbf{V_i = V_f \times c_f / c_i} \qquad [2]$$

V_i.....initial volume of suspension
V_f.....final volume of suspension
V_f = 28 ml
c_i.....initial concentration of suspension
c_i = c from equation [1]
c_f.....final concentration of suspensio
c_f = 10^6 cell/mL

We had to prepare this suspension in order to have a final concentration 10^5 cell/mL in each well of the microtiter plate (except the last column which was left empty for blankets). We reached this concentration after filling wells with 100 µL of cell suspension and 100 µL of drug solution.

Once the suspension had been prepared, we took three 96-well microtiter plates (one for each drug) and added 100 µL of the suspension into each well of columns from 1 to 11 of every plate with a multi-channel pipette. *C. lusitaniae* and *C. guilliermondii* plates were incubated for 48 hours in order to get BF. *C. krusei* plates were incubated for 72 hours.

6.4.3 Planktonic cell preparation

For growing PL we reused inoculated SA+ plates which we saved in the refrigerator after the biofilm preparation.

At first we put 2 or 3 colonies from the plate into a sterile plastic tissue flask with 20 mL of YNB. This flask was kept in the incubator for 24 hours at 37 °C in dynamic conditions. Dynamic conditions were provided by using the rocker on speed 8.

When the incubation was finished we washed the cells (described in the chapter Biofilm preparation).

From homogeneous suspension we prepared 1:1000 dilution for cells counting and used equation [1] (described in the part Biofilm preparation).

The difference from method of BF preparation was in calculation [2] for the initial volume of suspension which had to be transferred to the plate. The value for final concentration (Cf) was 2×10^6 cell/mL. We used this value in order to get concentration 2×10^5 cell/mL in each well of the microtiter plate filled with 100 µL of the suspension and 100 µL of drug solution.

When the suspension had been ready, we took three 96-well microtiter plates (one for each drug) and added 100 µL of the suspension into each well of columns from 1 to 11 of every plate with the multi-channel pipette .

6.4.4 Drug plates preparation

The concentration of each drug (anidulafungin, caspofungin and micafungin) was tested in pentaplicate.

It was necessary to have 2 plates of each drug for every isolate (one plate for BF and one plate for PL). Firstly we filled every well in columns 1-11 of a sterile 96-well microtiter plate with 150 µL of RPMI solution. Then we added 150 µL of the drug solution (of concentration 1024 mg/L) to wells 1-5 of the row A. We mixed contents properly using the multi-channel pipette and afterwards we transferred 150 µL of the solution from first 5 wells of the row A into first 5 wells of the row B. This way we got half concentration in the row B. We continued as described to reach the lowest required concentration.

We had to consider that the final concentration of drug on the plate with BF and PL will be lower, because we transfered 100 µL of solution from the drug plate to 100 µL of the cell suspension on PL or BF plate. That was the reason why we had to add 150 µL of the drug of the concentration 1024 mg/L into 150 µL of RPMI to get the final concentration of 256 mg/L

Final concentrations of drugs for our experiment were:

Anidulafungin.....256 - 0.007 mg/L
Caspofungin.....256 - 0.015 mg/L
Micafungin.....256 - 0.03 mg/L

The design of the plate had to be adapted to the length of the dilution line of each drug [Figure 11, 12 and 13]. Drugs were put in rows A – H. In the case of anidulafungin we had sixteen dilutions. For caspofungin we had fourteen dilutions so we left free wells in columns 6 - 10 of rows G and H (these wells were later used as extra positive controls for XTT assay). When we were preparing plate with micafungin, we had thirteen dilutions and we got empty wells in columns 6 - 10 of rows F, G and H (hese empty wells were used as positive controls as well).

Usually we prepared these plates earlier in advance and we stored them in the freezer in -35 °C. In the time of need we took them out, defrosted them

and transferred drug solution from them into plates with PL and BF.

ANIDULAFUNGIN

	1	2	3	4	5	6	7	8	9	10	11	12
A	256	256	256	256	256	1	1	1	1	1		
B	128	128	128	128	128	0.5	0.5	0.5	0.5	0.5		
C	64	64	64	64	64	0.25	0.25	0.25	0.25	0.25		
D	32	32	32	32	32	0.125	0.125	0.125	0.125	0.125		
E	16	16	16	16	16	0.06	0.06	0.06	0.06	0.06		
F	8	8	8	8	8	0.03	0.03	0.03	0.03	0.03		
G	4	4	4	4	4	0.015	0.015	0.015	0.015	0.015		
H	2	2	2	2	2	0.007	0.007	0.007	0.007	0.007		

Figure 11: **Final concentrations of anidulafungin** Numbers represent concentration of drug in mg/L

CASPOFUNGIN

	1	2	3	4	5	6	7	8	9	10	11	12
A	256	256	256	256	256	1	1	1	1	1		
B	128	128	128	128	128	0.5	0.5	0.5	0.5	0.5		
C	64	64	64	64	64	0.25	0.25	0.25	0.25	0.25		
D	32	32	32	32	32	0.125	0.125	0.125	0.125	0.125		
E	16	16	16	16	16	0.06	0.06	0.06	0.06	0.06		
F	8	8	8	8	8	0.03	0.03	0.03	0.03	0.03		
G	4	4	4	4	4							
H	2	2	2	2	2							

Figure 12: **Final concentrations of caspofungin** Numbers represent concentration of drug in mg/L

MICAFUNGIN

	1	2	3	4	5	6	7	8	9	10	11	12
A	256	256	256	256	256	1	1	1	1	1		
B	128	128	128	128	128	0.5	0.5	0.5	0.5	0.5		
C	64	64	64	64	64	0.25	0.25	0.25	0.25	0.25		
D	32	32	32	32	32	0.125	0.125	0.125	0.125	0.125		
E	16	16	16	16	16	0.06	0.06	0.06	0.06	0.06		
F	8	8	8	8	8							
G	4	4	4	4	4							
H	2	2	2	2	2							

Figure 13: **Final concentrations of micafungin** Numbers represent concentration of drug in mg/L

6.4.5 Incubation with drugs

We took all plates with BF out of the incubator (after the incubation for 48 or 72 hours) and prepared plates with PL.

We replaced used RPMI medium on BF plates with a new one before we started adding drugs.

When the medium was changed and plates with PL were prepared we defrosted plates with drugs. We transferred 100 µL of the drug solution into each well of columns 1 – 10 of BF and PL plates (to column 11 we added only RPMI). We transferred the drug from a well of a certain number and letter into the well of the same number and letter.

We let BF and PL incubate with drugs for 24 hours at 37 °C using the rocker on speed 8.

6.4.6 XTT assay

We took PL and BF plates with drugs out of the incubator and centrifuged them for 20 minutes at 3000 rpm and at 15 °C. Before the centrifugation we weighted all plates and balanced them very carefully to avoid the damage

of the cell layer during the centrifugation.

We removed the RPMI medium gently from each well with the multi-channel pipette after the centrifugation was finished. At this moment we could see whether the biofilm was created properly.

Then we added mixture of XTT and CoQ solution. We had prepared the mixture of XTT and CoQ solution in advance. These two reagents were in ratio 80 µL of CoQ solution to 1 mL of XTT solution. We transferred 130 µL of this mixture into wells of columns 1 - 11 and in wells G and H of column 12 [Figure 14]. These 2 wells were used as blankets for setting up a microplate absorbance reader.

After we had added XTT and CoQ we put plates into the incubator at 37 °C and kept them inside for approximately 20 minutes. When these plates were ready for reading (the colour of positive controls was orange) we transferred 100 µL of the solution from each well of each plate into wells of clean plates. This way we created a copy of BF and PL plates suitable for reading in absorbance reader.

The wavelength we used for measuring was 450 nm.

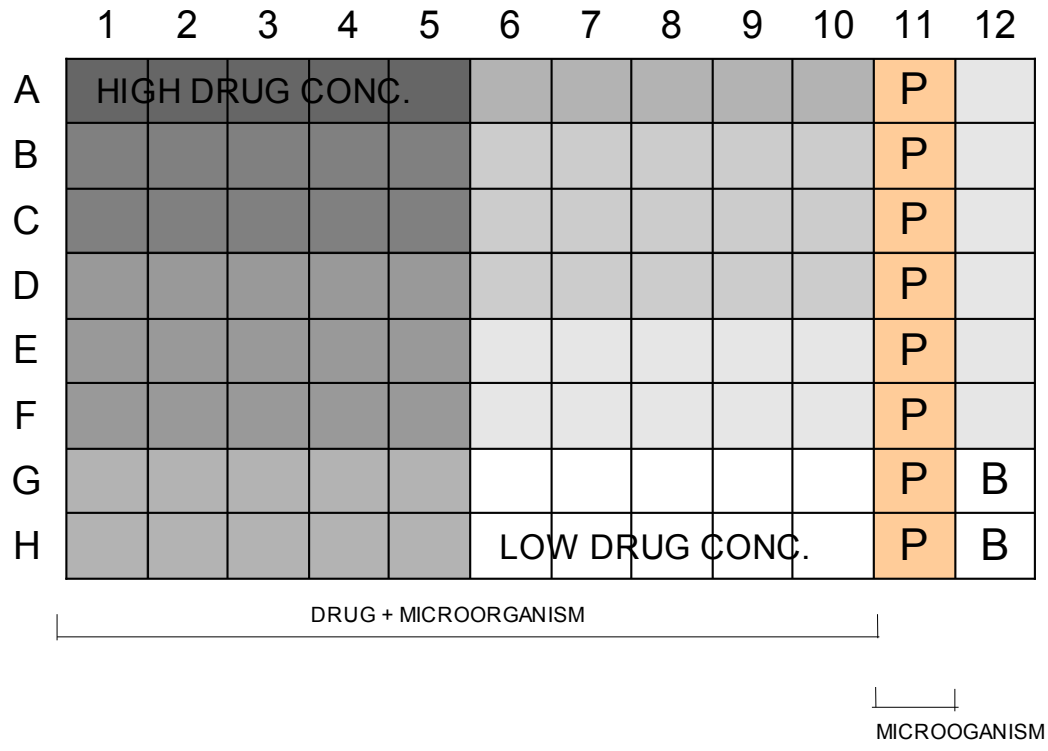

Figure 14: **Final design of the plate for XTT assay** P (positiv control), B (blanket)

We used this equation [3] to calculate the fungal damage from absorbance values:

$$\textbf{\% fungal damage} = 100 - (100 \times A_E/A_C) \quad [3]$$

A_E ..... experimental absorbance value

A_C ..... positive control absorbance value

7 Results

Three *Candida* species were tested – *C. guilliermondii* (3 strains), *C. krusei* (13 strains) and *C. lusitaniae* (14 strains).

There was observed antifungal activity of anidulafugin, caspofungin and micafungin against biofilm (BF) and planktonic cells (PL).

To create BF cell suspension was incubated on microtiter plates for 48 hours (for species *C. guilliermondii* and *C. lusitaniae*) and for 72 hours (for species *C. krusei*) at 37 °C in dynamic conditions. Both BF and PL were incubated with drugs for 24 hours.

For assessment of the susceptibility to echinocandins was used the XTT assay. The colour conversion was measured at 450 nm with an absorbance reader. Absorbance values of tested concentrations of echinocandins (every drug concentration was repeated in pentaplicate for each strain isolate) were processed and converted into the fungal damage. The drug concentration which cause fungal damage 50 % was defined as the MIC_{50}. In the end an average of MIC_{50} values of tested species strains was made and susceptibility for C*. lusitaniae, C. guilliermondii* and *C. krusei* was evaluated.

7.1 *Candida* species susceptibility profiles

In this part are shown charts illustrating susceptibility profiles of *Candida* species to tested echinocandins.

On charts are plotted values of fungal damage for PL and BF depending on the drug concentration.

Tables with values of fungal damages of single tested strains are itemized in the appendix.

7.1.1 *C. lusitaniae* susceptibility profile

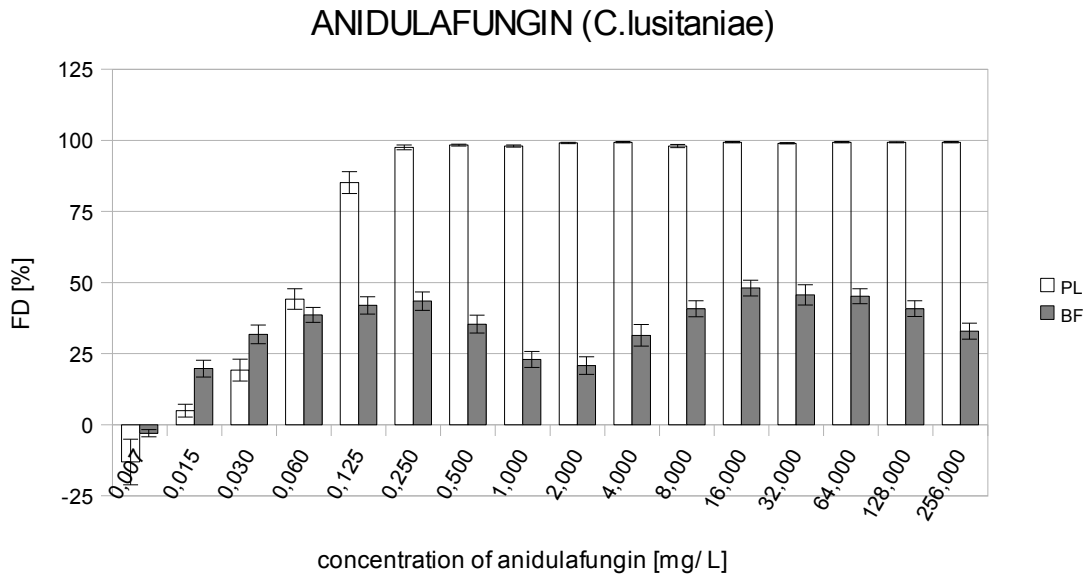

Figure 15: **Susceptibility profile of *C. lusitaniae* to anidulafungin** PL (planktonic cells), BF (biofilm), FD (fungal damage)

	Average FD for C. lusitaniae		**Standard deviation**	
Concentration of anidulafungin [mg/L]:	PL [%]	BF [%]	PL [%]	BF [%]
0,007	-13,09	-3,16	8,01	1,22
0,015	4,92	19,73	2,25	2,99
0,030	19,21	31,80	3,87	3,26
0,060	44,20	38,65	3,59	2,61
0,125	85,15	41,96	3,82	3,07
0,250	97,56	43,48	0,84	3,25
0,500	98,34	35,37	0,35	3,15
1,000	98,01	22,97	0,42	2,81
2,000	99,10	20,80	0,25	3,05
4,000	99,34	31,45	0,21	3,76
8,000	98,01	40,79	0,55	2,83
16,000	99,35	48,05	0,23	2,80
32,000	98,99	45,64	0,27	3,56
64,000	99,35	45,22	0,22	2,64
128,000	99,32	40,81	0,20	2,77
256,000	99,36	32,89	0,20	2,83

Table 2: **Source data for the figure Susceptibility profile of *C. lusitaniae* to anidulafungin**

Anidulafungin did not reach MIC_{50} for biofilm. The closest value to MIC_{50} (48.05 %) it reached was at the concentration 16 mg/L. MIC_{50} for planktonic cells was 32 mg/L.

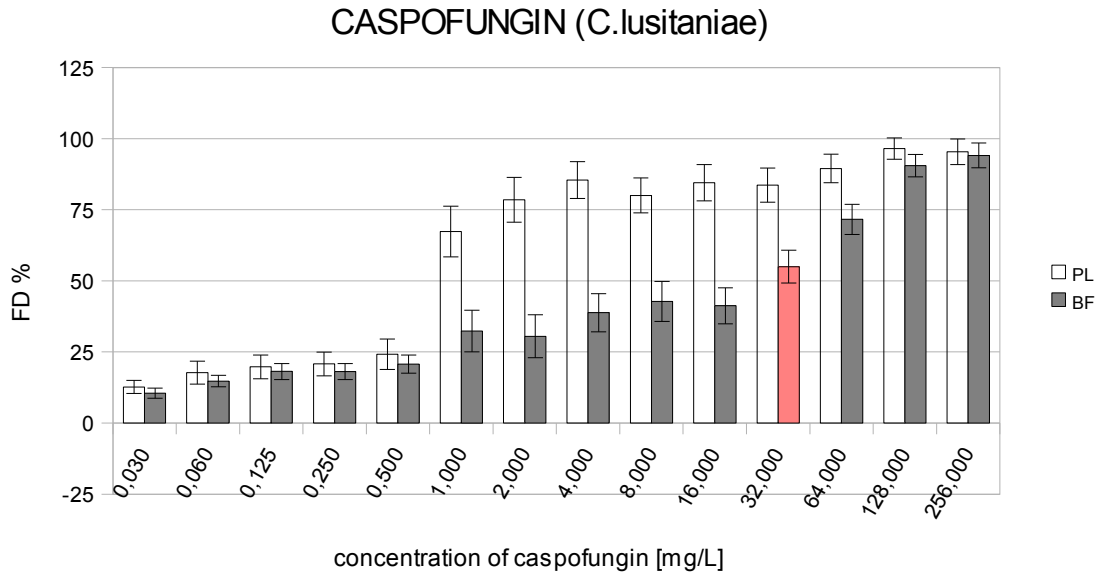

Figure 16: **Susceptibility profile of *C. lusitaniae* to capofungin** PL (planktonic cells), BF (biofilm), FD (fungal damage), red column marks MIC_{50}

Concentration of caspofungin [mg/L]:	Average FD for C. lusitaniae PL [%]	Average FD for C. lusitaniae BF [%]	Standard deviation PL [%]	Standard deviation BF [%]
0.030	12.69	10.52	2.29	1.79
0.060	17.72	14.73	3.99	2.02
0.125	19.74	18.13	4.19	2.81
0.250	20.78	18.08	4.19	2.82
0.500	24.20	20.73	5.35	3.22
1.000	67.34	32.37	8.89	7.32
2.000	78.53	30.51	7.90	7.52
4.000	85.43	38.77	6.44	6.71
8.000	80.04	42.75	6.16	7.01
16.000	84.50	41.22	6.37	6.30
32.000	83.69	54.98	6.01	5.77
64.000	89.50	71.61	5.05	5.28
128.000	96.48	90.51	3.76	3.90
256.000	95.41	94.11	4.49	4.38

Table 3: **Source data for the figure Susceptibility profile of *C. lusitaniae* to capofungin**

Caspofungin reached MIC_{50} for biofilm at 32 mg/L. MIC_{50} for planktonic cells was 1 mg/L.

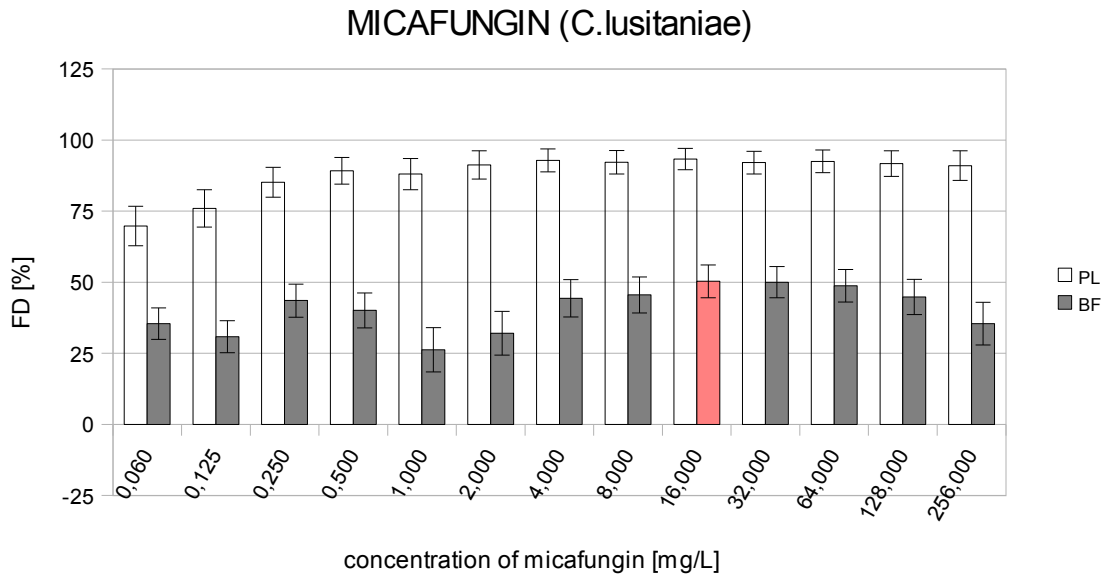

Figure 17: **Susceptibility profile of *C. lusitaniae* to micafungin** PL (planktonic cells), BF (biofilm), FD (fungal damage), red column marks MIC_{50}

	Average FD for C. lusitaniae		Standard deviation	
Concentration of micafungin [mg/L]:	PL [%]	BF [%]	PL [%]	BF [%]
0.060	69.80	35.44	6.96	5.53
0.125	76.00	30.87	6.55	5.63
0.250	85.16	43.55	5.25	5.82
0.500	89.21	40.10	4.70	6.17
1.000	88.04	26.26	5.49	7.77
2.000	91.28	32.10	4.98	7.67
4.000	92.85	44.36	4.01	6.55
8.000	92.19	45.53	4.10	6.33
16.000	93.36	50.34	3.76	5.78
32.000	92.07	50.01	3.99	5.49
64.000	92.49	48.76	3.99	5.72
128.000	91.76	44.81	4.50	6.16
256.000	90.99	35.44	5.21	7.51

Table 4: **Source data for the figure Susceptibility profile of *C. lusitaniae* to micafungin**

Micafungin reached MIC_{50} for biofilm at the concentration 16 mg/L. For planktonic cells the MIC_{50} was less than 0.060 mg/L.

7.1.2 *C. krusei* susceptibility profile

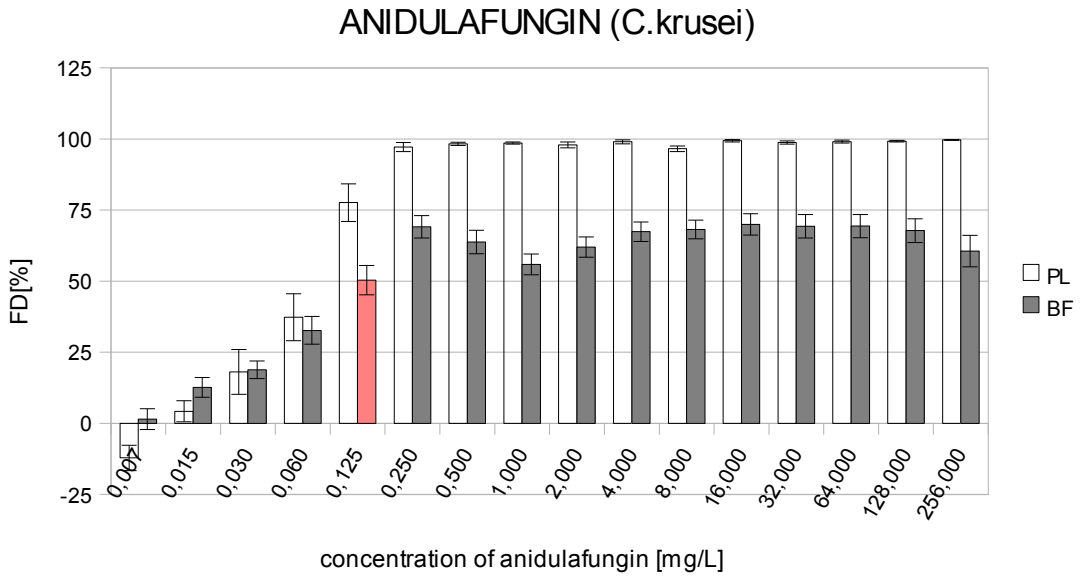

Figure 18: **Susceptibility profile of *C. krusei* to anidulafungin** PL (planktonic cells), BF (biofilm), FD (fungal damage), red column marks MIC_{50}

	Average FD for C. krusei		**Standard deviation**	
Concentration of anidulafungin [mg/L]:	PL [%]	BF [%]	PL [%]	BF [%]
0.007	-12.18	1.47	4.45	3.68
0.015	4.21	12.66	3.69	3.45
0.030	18.12	18.87	7.87	3.09
0.060	37.29	32.67	8.24	4.88
0.125	77.61	50.34	6.60	5.18
0.250	97.13	69.12	1.62	3.90
0.500	98.26	63.78	0.59	4.10
1.000	98.57	55.90	0.41	3.68
2.000	97.89	61.98	1.02	3.52
4.000	99.01	67.39	0.68	3.44
8.000	96.56	68.20	1.01	3.28
16.000	99.37	69.97	0.44	3.79
32.000	98.76	69.30	0.60	4.11
64.000	99.07	69.35	0.53	4.06
128.000	99.24	67.76	0.30	4.19
256.000	99.68	60.58	0.15	5.55

Table 5: **Source data for the figure *C. krusei* susceptibility profile to anidulafungin**

Anidulafungin reached MIC_{50} for biofilm at the concentration 0.125 mg/L. For planktonic cells MIC_{50} was also 0.125 mg/L.

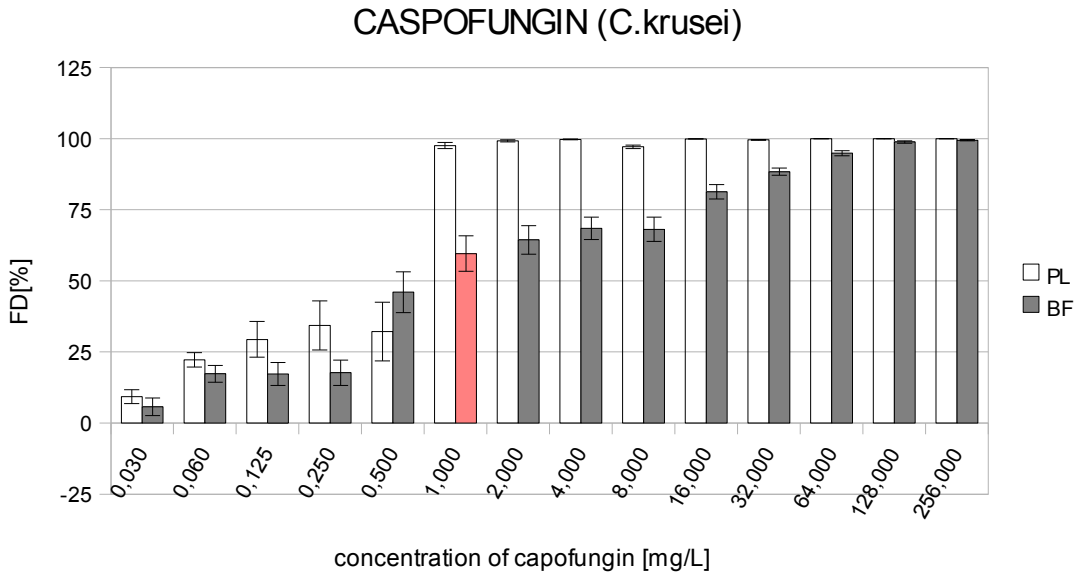

Figure 19: **Susceptibility profile of *C. krusei* to caspofungin** PL (planctonic cells), BF (biofilm), FD (fungal damage), red column marks MIC_{50}

Concentration of caspofungin [mg/L]:	Average FD for C. krusei		Standard deviation	
	PL [%]	BF [%]	PL [%]	BF [%]
0.030	9.25	5.70	2.46	3.07
0.060	22.22	17.31	2.54	2.94
0.125	29.40	17.24	6.28	4.06
0.250	34.33	17.69	8.60	4.46
0.500	32.16	46.00	10.36	7.17
1.000	97.57	59.58	1.10	6.22
2.000	99.25	64.40	0.34	4.99
4.000	99.75	68.48	0.18	3.92
8.000	97.15	68.14	0.59	4.23
16.000	99.92	81.32	0.07	2.56
32.000	99.59	88.37	0.16	1.27
64.000	99.98	94.88	0.01	0.86
128.000	99.99	98.81	0.01	0.38
256.000	99.98	99.47	0.02	0.20

Table 6: **Source data for the figure Susceptibility profile of *C. krusei* to caspofungin**

Caspofungin reached MIC_{50} for biofilm at 1 mg/L. For planktonic cells MIC_{50} was at the concentration 1 mg/L.

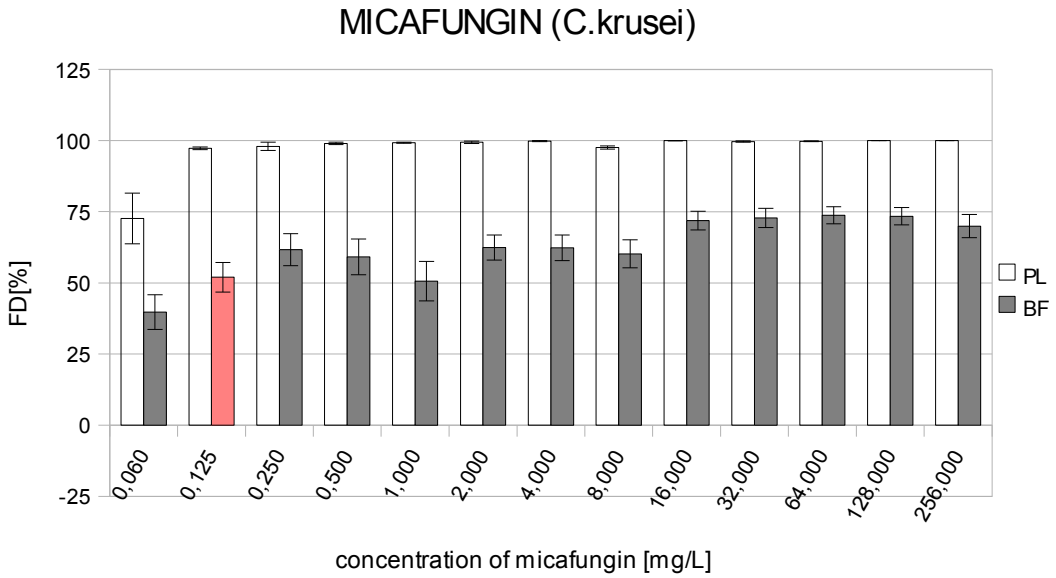

Figure 20: **Susceptibility profile of *C. krusei* to micafungin** PL (planctonic cells), BF (biofilm), FD (fungal damage), red column marks MIC_{50}

	Average FD for C. lusitaniae		**Standard deviation**	
Concentration of micafungin [mg/L]:	PL [%]	BF [%]	PL [%]	BF [%]
0.060	72.60	39.73	8.88	6.09
0.125	97.29	51.99	0.46	5.20
0.250	97.97	61.66	1.47	5.65
0.500	98.97	59.12	0.42	6.26
1.000	99.27	50.60	0.19	6.92
2.000	99.39	62.40	0.39	4.38
4.000	99.78	62.32	0.19	4.53
8.000	97.55	60.19	0.56	4.92
16.000	99.95	71.86	0.04	3.31
32.000	99.65	72.80	0.20	3.37
64.000	99.74	73.75	0.17	3.01
128.000	99.99	73.42	0.01	3.01
256.000	100.00	69.94	0.00	4.08

Table 7: **Source data for the figure Susceptibility profile of *C. krusei* to micafungin**

Micafungin reached MIC_{50} for biofilm at the concentration 0.125 mg/L. For planktonic cells the MIC_{50} was less than 0.060 mg/L.

7.1.3 *C. guilliermondii* susceptibility profile

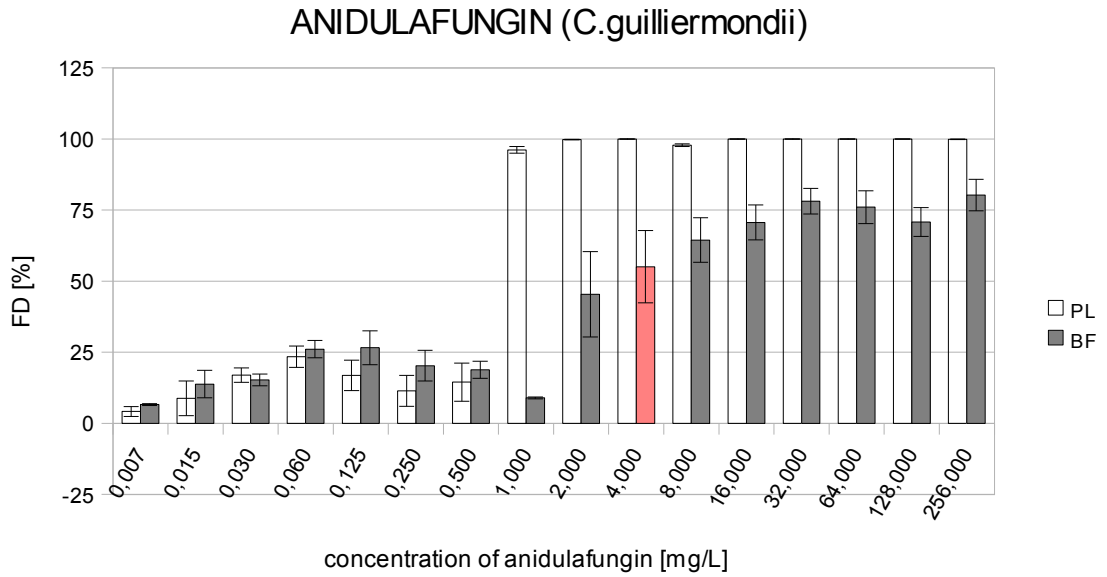

Figure 21: **Susceptibility profile of *C. guilliermondii* to anidulafungin** PL (planktonic cells), BF (biofilm), FD (fungal damage), red column marks MIC_{50}

	Average FD for C. guilliermondii		**Standard deviation**	
Concentration of anidulafungin [mg/L]:	PL [%]	BF [%]	PL [%]	BF [%]
0.007	4.15	6.61	1.77	0.32
0.015	8.76	13.82	6.09	4.81
0.030	16.94	15.28	2.55	2.07
0.060	23.46	26.10	3.76	3.06
0.125	16.86	26.57	5.32	5.93
0.250	11.41	20.27	5.42	5.36
0.500	14.50	18.83	6.69	2.97
1.000	96.13	8.95	1.17	0.29
2.000	99.78	45.38	0.08	14.96
4.000	100.00	55.09	0.00	12.76
8.000	97.84	64.45	0.45	7.79
16.000	100.00	70.64	0.00	6.14
32.000	99.99	78.11	0.01	4.51
64.000	100.00	76.04	0.00	5.77
128.000	99.99	70.81	0.01	5.05
256.000	99.88	80.28	0.07	5.52

Table 8: **Source data for the figure *C. guilliermondii* susceptibility profile to anidulafungin**

Anidulafungin reached MIC_{50} for biofilm at the concentration 4 mg/L. For planktonic cells MIC_{50} was found at the concentration 1 mg/L.

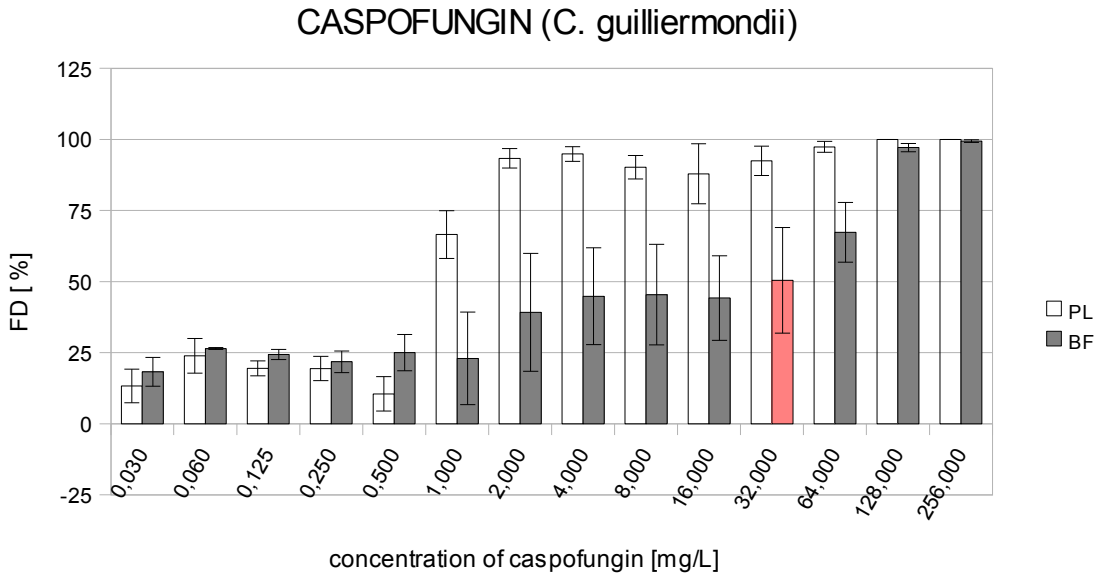

Figure 22: **Susceptibility profile of *C. guilliermondii* to anidulafungin** PL (planktonic cells), BF (biofilm), FD (fungal damage), red column marks MIC_{50}

Average FD for C. guilliermondii			**Standard deviation**	
Concentration of caspofungin [mg/L]:	PL [%]	BF [%]	PL [%]	BF [%]
0.030	13.31	18.29	5.94	5.04
0.060	23.92	26.44	6.10	0.33
0.125	19.48	24.35	2.62	1.78
0.250	19.42	21.83	4.28	3.79
0.500	10.50	25.03	6.05	6.41
1.000	66.56	23.00	8.38	16.26
2.000	93.36	39.18	3.43	20.74
4.000	94.88	44.86	2.59	17.00
8.000	90.22	45.43	4.13	17.68
16.000	87.90	44.26	10.57	14.87
32.000	92.46	50.48	5.14	18.59
64.000	97.37	67.34	1.93	10.48
128.000	100.00	97.13	0.00	1.43
256.000	100.00	99.43	0.00	0.45

Table 9: **Source data for the figure *C. guilliermondii* susceptibility profile to caspofungin**

Caspofungin reached MIC_{50} for biofilm at 32 mg/L. For planktonic cells MIC_{50} was at the concentration 1 mg/L.

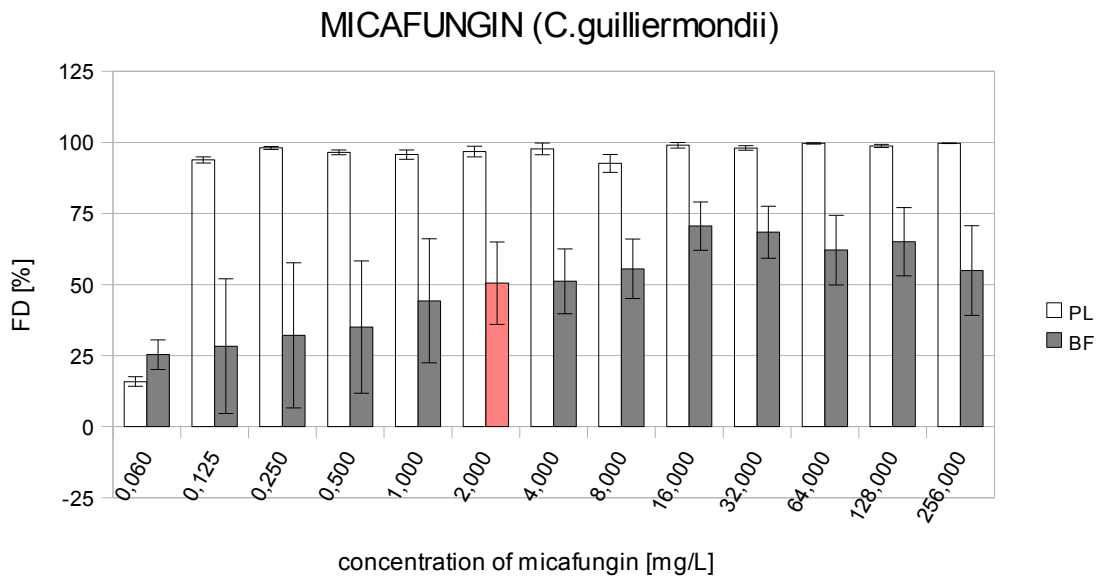

Figure 23: **Susceptibility profile of *C. guilliermondii* to micafungin** PL (planktonic cells), BF (biofilm), FD (fungal damage), red column marks MIC_{50}

	Average FD for C. guilliermondii		**Standard deviation**	
Concentration of micafungin [mg/L]:	PL [%]	BF [%]	PL [%]	BF [%]
0.060	15.86	25.35	1.69	5.21
0.125	93.82	28.33	1.09	23.72
0.250	98.07	32.11	0.51	25.51
0.500	96.50	35.07	0.87	23.26
1.000	95.72	44.27	1.64	21.83
2.000	96.80	50.49	1.89	14.49
4.000	97.68	51.13	2.07	11.36
8.000	92.59	55.53	3.15	10.43
16.000	98.97	70.54	1.03	8.53
32.000	98.01	68.41	0.80	9.16
64.000	99.64	62.12	0.20	12.23
128.000	98.74	65.06	0.60	12.02
256.000	99.66	54.95	0.14	15.75

Table 10: **Source data for the figure *C. guilliermondii* susceptibility profile to micafungin**

Micafungin reached MIC_{50} for biofilm at the concentration 2 mg/L. For planktonic cells MIC_{50} was 0.125 mg/L.

7.2 Comparison of susceptibility profiles of *Candida* species to anidulafungin, caspofungin and micafungin

In charts are compared susceptibility profiles of *Candida* species to tested echinocandins [Figure 24, 25 and 26]. Lines in charts are representing fungal damage depending on the drug concentration and illustrating susceptibility profiles of *C. lusitaniae, C. krusei* and *C. guilliermondii* biofilms.

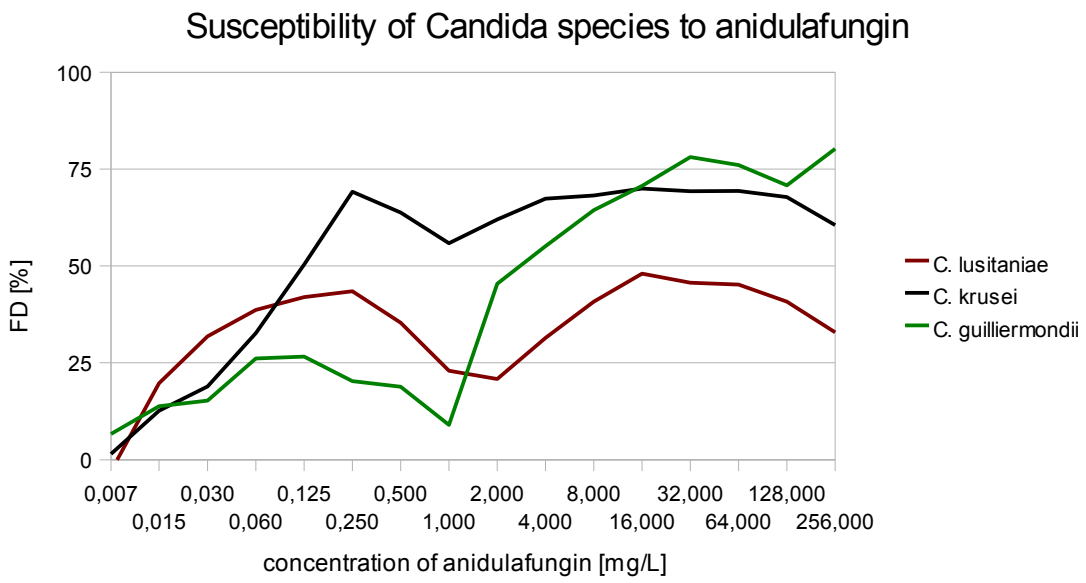

Figure 24: **Anidulafungin - comparison of antifungal activity against C. species BF** FD(fungal damage)

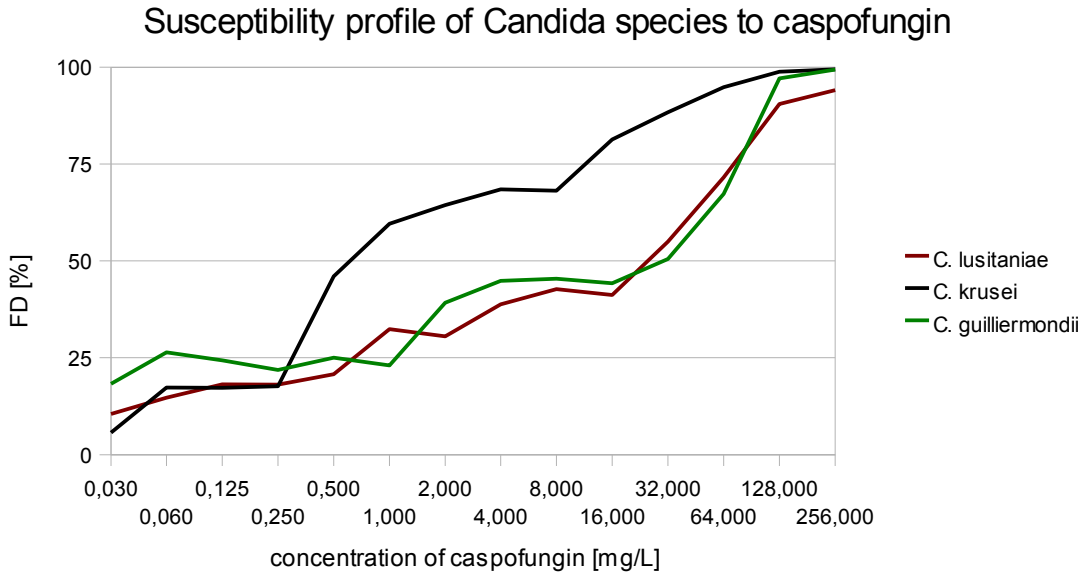

Figure 25: **Caspofungin - comparison of antifungal activity against C. species BF** FD(fungal damage)

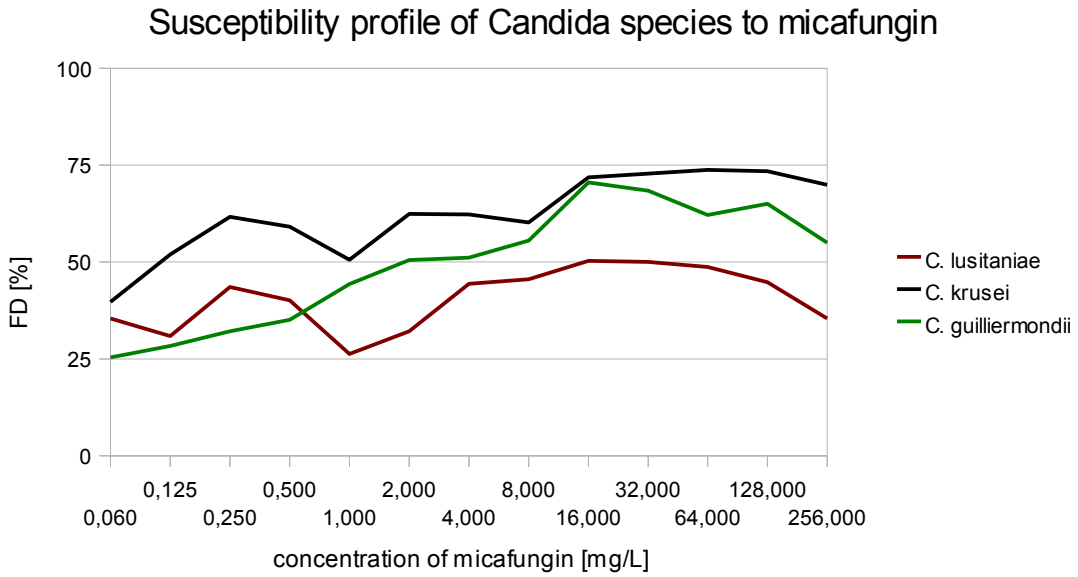

Figure 26: **Micafungin - comparison of antifungal activity against C. species BF** FD(fungal damage)

7.3 Comparison of MIC_{50} of *Candida* species

In the table are compared MIC_{50} values for biofilm (BF) and planktonic cells (PL). For every comparison is calculated the statistical significance with non-parametric Mann-Whitney Test (it was used PC programme InStat 3, GraphPad Software). The result of the comparison of two data rows with the test was considered as significant when the P value was less than 0.05.

***C. krusei* vs *C. lusitaniae* MIC [mg/L]**

AND		CAS		MFG	
PL / PL	BF / BF	PL / PL	BF / BF	PL / PL	BF / BF
0.125 /0.125	0.125 / >256	1/1	1/32	<0.060 /<0.060	0.125 /16
(P=0.0310)	(P=0.0011)	(P=0.2042)	(P=< 0.0001)	(P=0.6431)	(P=< 0.0001)

***C. guilliermondii* vs *C. lusitaniae* MIC [mg/L]**

AND		CAS		MFG	
PL / PL	BF / BF	PL / PL	BF / BF	PL / PL	BF / BF
1 /0.125	4 / >256	1/1	32/32	0.125 /<0.060	2/16
(P=0.0029)	(P=0.3643)	(P=0.4567)	(P=0.8122)	(P=0.3666)	(P=0.0676)

***C. guilliermondii* vs *C.krusei* MIC [mg/L]**

AND		CAS		MFG	
PL / PL	BF / BF	PL / PL	BF / BF	PL / PL	BF / BF
1/0.125	4 /0.125	1/1	32/1	0.125 /<0.060	2/0.125
(P=0.0475)	(P=0.0021)	(P=0.9551)	(P=0.0503)	(P=0.4649)	(P=0.0935)

Table 11: **Comparison of MIC_{50} values** The P value is determinating the statistical significance, PL (planktonic cells), BF (biofilm), AND (anidulafungin), CAS (caspofungin), MFG (micafungin)

8 Conclusion and discussion

We tested the susceptibility of three non-*albicans Candida* species – *C. krusei, C. lusitaniae* and *C. guilliermondii* as biofilm (BF) and as planktonic cells (PL) to anidulafungin (AND), caspofungin (CAS) and micafungin (MFG).

We observed the differences in resistance between species and between strains of single species. There was a large difference between the susceptibility profile of the PL and BF of all strains. BF was more resistant. This reduction of susceptibility in BF is probably caused by specific mechanisms as restricted or decreased penetration of drug into the BF, changes in phenotype as a result of low growth rate and nutrition limitation and surface induced expression of genes increasing resistance and other mechanisms which are not revealed yet.

From tested echinocandins the most efficient was proved to be micafungin. It had 50 % fungal damage for all three species at lowest concentration. Most susceptible to MFG was *C. krusei* (MIC_{50} = 0.125 mg/L), then *C. guilliermondii* (MIC_{50} = 2 mg/L) and *C. lusitaniae* BF (MIC_{50} = 16 mg/L). Another tested echinocandin anidulafungin had the same MIC_{50} for *C. krusei* BF like MFG. Nevertheless the MIC_{50} for *C. guilliermondii* and *C. lusitaniae* BF were higher than those for MFG (4 mg/L and >256 mg/L). Caspofungin had the lowest antimycotic activity against *C. krusei* and *C. guilliermondii* BF from all echinocandins (MIC_{50} were 1 mg/L and 32 mg/L) and medium activity against *C. lusitaniae* BF (MIC_{50} = 32 mg/L). From this comparison follows that the most resistant was *C. lusitaniae* and the most susceptible was *C. krusei.*

According to recent sources as susceptible to echinocandins are concidered *C. krusei* isolates with MIC_{50} lower than 0.25 mg/L and as moderate susceptible with MIC_{50} lower than 0.5 mg/L (Pfaler et al., 2011). This value seems to be more accurate than CLSI clinical breakpoint (CBS) 2 mg/L from 2008 for all three echinocandins (CLSI, 2008). Based on the new statement BFs of *C. krusei* isolates from our experiment were resistant to CAS. Our experiment has detected low occurrence of resistant *C. krusei* BF which factually equates to the results of a study published for PL where only singular resistance to CAS was observed (Pfaller et al., 2011). In most of the studies

it was proved that the connection between the presence of Fks1, Fks2 and Fks3 genes encoded mutations and resistance to echinocandins (Pfaler et al., 2011b; Imtiaz et al., 2012). When MIC_{50} ≥ 1 mg/L the FKS mutation occurs and isolates are almost always clinically resistant (Pfaller et al., 2011). *C. guilliermondii* CBS are 2 mg/L for susceptible strains and 4 mg/L for moderate susceptible strains for echinocandins (Pfaller et al., 2011c; CLSI, 2008). *C. guilliermondii* BF of tested strains seems to be quite resistant taking into consideration this CBS. Especially CAS had a low antimycotic effect against this species.

C. lusitaniae were significantly the most resistant species ($P<0.05$). Taking into account the CBS 2 mg/L which seems to be more of less accurate *C. lusitaniae* BF was resistant to all three echinocandins (Pfaller, 2011c; CLSI, 2008).

Increased resistance of *C. guilliermondii* and *C. lusitaniae* compared to other *Candida* species was described in previous studies (Pfaller, 2011c). BF of all three tested species were also more resistant to echinocandins than *C. albicans* BF except *C. krusei* which was less resistant to anidulafungin. Our results were compared to data from a previous experiment of the laboratory when MIC_{50} for AND was 0.08 mg/L, for CAS 1.08 mg/L and for MFG 1.79 mg/L (Simitsopoulou, 2011). There is a possibility that higher resistance of non-*albicans* species can be the reason for their increasing incidence the last years.

Discussed can be also the probability of achievement of sufficient levels of drugs in the blood of patients when administered in usual doses for treatment of candidaemia. The maximal concentration in blood can be proximetly 8 mg/L for CAS and 8.6 mg/L for AND after the loading and maintenance dose (Eraxis PI, 2010; Cancidas PI, 2001). This concentration is lower than the MIC_{50} we found for *C. lusitaniae* BF and some *C. guilliermondii* BF, which can cause problems in the treatment of patients suffering for infections caused by BF of these two species. Since the concentration of MFG can reach 10.9 mg/L in blood after the usual dose, it seems to be a good option for treatment of the non-*albicans* species BF associated candidaemia (Mycamine PI, 2011).

To summarize conclusions of our experiment we can say that *Candida* BF were more resistant than PL. PL had more similar MIC_{50} than BF which had differences across tested species. Most resistant to echinocandins seems to be

C. lusitaniae BF. *C. krusei* appeared more susceptible to echinocandins than both *C. lusitaniae* and *C. guilliermondii*. Nevertheless echinocandins and especially micafungin seem to be active against non-*albicans Candida* BF in vitro.

9 List of abbreviations

FD	fungal damage
BF	biofilm
PL	planktonic cells
BSI	blood stream infection
MFG	micafungin
CAS	caspofungin
AND	anidulafungin
EPS	extracellular polymeric substance
FDA	U.S. Food and Drug Administration
EMEA	European Medicine Agency
XTT	2,3 - bis[2 - methoxy - 4 - nitro - 5 - sulfophenyl] 2H - tetrazolium - 5- carboxanilide
YNB	Yeast nitrogen base with amino acids solution
SA+	Sabouraud agar with antibiotics
PBS	Phosphate buffered saline solution
CBS	clinical breakpoint

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Appendix

Protocol

We had one week schedule for our project. From 4 to 6 samples were made each week.

Protocol for *C. lusitaniae* and *C. guilliermondii*

DAY 0

- Preparation of SA+ with microorganisms from the stock
  - take one clinical isolate from the freezer (-35 °C)
  - put on a SA+ plate and streak cells using the loop to get single colonies
  - incubate for 48 hours at 37 °C

DAY 1

- Biofilm preparation I
  - add 20 mL YNB + 2 % glucose in 100 mL sterile tissue flask and a loopful of *Candida* from SA+ plate
  - let it grow for 24 hours, 37 °C, using a rocker (at speed 8)

DAY 2

- Biofilm preparation II
  - take out the sterile tissue flask and transfer into a tube
  - separate harvest cells by centrifugation at 2000 rpm, 10 min
  - do washing step – resuspend cells in 10 mL of PBS and wash once
  - resuspend washed cells in 10 mL of RPMI solution
  - vortex 20 min on maximal speed, make 1:1000 dilution, count cells and adjust concentration 10^6 cell/mL using RPMI
  - add to the 96-well microtiter plate 100 µL of 10^6 cell/mL to have a final concentration 10^5 cell/mL in rows 1-11
  - seals plates with parafilm and incubate for 48 hours, 37 °C, using a rocker (at speed 8)

DAY 3

- Planktonic cell preparation
  - add 20 mL YNB + 2 % glucose in 100 mL sterile flask and a loopful

of *Candida* (from saved SA+ plate from first day)

- grow for 24 hours, 37 °C, using a rocker (at speed 8)

DAY 4

- incubation with drugs
  - BF
    - plates with BF centrifuge for 20 min at 3000 rpm (800xg), 15°C
    - then aspirate supernatants and add 100 µL of RPMI and 100 µL of drug (in pentaplicate)
    - 1-10 cells+drug, 11 cells only (positive controls)
  - PL
    - separate harvest PL grown in YNB by centrifugation at 2000 rpm for 10 min
    - resuspend and wash cells in 10 mL of RPMI, pH 7.2
    - vortex 20 minutes on maximal speed, make 1:1000 dilution, count cells and adjust concentration 2x 10^6 cell/mL using RPMI
    - add to the 96-well microtitre plate 100 µL of 2x10^6 cell/mL to have a final concentration 2x10^5 cell/mL in rows 1-11
    - 1-10 cells+drug, 11 cells only (positive controls)
  - incubate PL+BF plates with drugs at 37 °C for 24 hours, using the rocker (at speed 8)

DAY 5

- Evaluation of antifungal susceptibility
  - centrifuge all plates for 20 minutes, 3000 rpm (800xg)
  - aspirate medium and add 130 µL of XTT with CoQ
  - incubate BF and PL cells for 20-30 minutes at 37 °C
  - tranfer into new clean plates and read at 450 nm by spectrophotometer

Protocol for *C. krusei:*

DAY 0

- Preparation of SA+ plates with microorganisms from the stock
  - Viz. Protocol for *C. lusitaniae* and *C. guilliermondii*

DAY 1

- Biofilm preparation I
    - Viz. Protocol for *C. lusitaniae* and *C. guilliermondii*

DAY 2

- Biofilm preparation II
    - Viz. Protocol for *C. lusitaniae* and *C. guilliermondii*

DAY 3

- Biofilm plates incubation
    - incubate BF plates at 37 °C, using a rocker (at speed 8)

DAY 4

- Planktonic cell preparation
    - Viz. Protocol for *C. lusitaniae* and *C. guilliermondii*
- Change of RPMI on BF plates
    - replace used RPMI with 100 µL of a fresh RPMI
    - seals plates with parafilm and incubate at 37 °C, using a rocker (at speed 8)

DAY 5

- Incubation with drugs
    - Viz. Protocol for *C. lusitaniae* and C. *guilliermondii*

DAY 6

- Evaluation of antifungal susceptibility
    - Viz. Protocol for *C. lusitaniae* and *C. guilliermondii*

Values of fungal damages of tested strains

Species itemized in this appendix are: *C. lusitaniae, C. krusei* and *C. guilliermondii*. There are presented six tables with fungal damage values (FD) for each species. Every first three tables are presenting values of FD after 24 hours incubation with anidulafungin, capofungin and micafungin for biofilm plates. Every next three tables are presenting data for planktonic cells.

Fungal damage is in percents.

Concentration of anidulafungin [mg/L]	Fungal damage of C. lusitaniae strains incubated with anidulafungin – biofilm													
	4245	0640	5094	9949	2588	8850	7892	2885	2732	5278	4241	2923	6936	4243
0.007	0.60	-12.85	-3.61	5.19	-7.47	-1.97	-5.41	-3.83	-15.41	3.83	1.60	-1.58	-1.94	-3.14
0.015	30.44	22.41	17.54	17.38	32.56	17.30	17.45	11.56	9.30	16.51	14.61	30.06	-9.70	39.66
0.030	48.72	30.01	32.87	25.49	32.56	17.69	28.38	16.70	15.39	16.76	38.59	33.37	13.39	61.03
0.060	48.09	42.17	34.02	27.63	46.00	27.38	26.36	24.68	25.86	40.70	44.40	36.69	26.14	62.67
0.125	58.41	41.72	36.18	29.52	57.26	42.33	37.51	30.80	24.57	25.80	46.97	24.90	38.50	67.11
0.250	56.11	41.19	39.51	26.55	56.60	46.09	26.95	17.74	24.21	23.80	54.61	43.77	39.93	68.43
0.500	57.02	28.48	34.94	20.04	47.88	28.17	24.43	10.24	20.81	15.63	44.02	35.04	32.96	55.12
1.000	41.74	27.66	31.64	7.66	28.54	14.56	19.16	-2.61	8.53	8.71	24.07	20.72	16.39	41.02
2.000	38.50	16.48	21.84	7.95	25.59	2.82	14.48	5.95	6.81	12.70	40.05	9.86	13.67	40.34
4.000	49.83	32.08	30.45	22.62	52.67	10.03	35.04	14.28	23.81	21.39	40.35	31.20	0.43	55.48
8.000	55.67	42.62	41.81	28.26	52.67	17.45	52.32	22.70	30.39	20.59	42.50	39.18	35.37	59.49
16.000	63.61	53.62	41.81	36.16	59.86	29.70	43.47	25.74	37.86	39.88	53.74	39.85	43.86	68.61
32.000	67.08	55.05	46.13	38.12	59.75	25.08	47.98	36.91	31.18	28.28	43.86	22.54	40.02	71.15
64.000	60.25	46.73	42.76	31.73	55.87	24.51	44.37	34.72	30.32	30.36	51.17	40.87	41.16	64.71
128.000	60.13	44.56	40.09	23.88	51.39	19.39	40.36	31.00	29.53	25.51	43.01	38.40	32.48	58.91
256.000	48.37	33.96	44.37	12.28	41.39	11.26	33.33	17.23	9.35	22.02	40.09	37.44	27.47	45.99

Table : **Fungal damage values of *C. lusitaniae* biofilm incubated with anidulafungin** The fungal damage values are in percents

Concentration of caspofungin [mg/L]	Fungal damage of C. lusitaniae strains incubated with caspofungin – biofilm													
	4245	0640	5094	9949	2588	8850	7892	2885	2732	5278	4241	2923	6936	4243
0.030	13.52	27.24	-7.90	18.33	17.33	6.76	3.80	13.47	1.06	7.21	6.61	9.65	12.07	22.74
0.060	20.39	23.88	-8.43	22.28	11.78	8.18	9.24	18.48	23.14	8.91	11.70	8.62	12.70	27.44
0.125	30.22	51.57	-3.53	24.13	7.17	13.74	13.65	23.40	33.61	10.74	8.71	6.52	18.88	22.21
0.250	30.22	51.57	-3.53	24.13	7.17	13.74	13.65	23.40	33.61	10.74	8.71	6.52	18.88	22.21
0.500	32.75	64.40	24.63	28.48	12.76	7.99	9.69	20.15	29.55	15.10	10.54	5.40	14.74	18.21
1.000	27.47	99.64	99.84	18.33	32.40	-0.81	17.08	2.28	14.99	27.54	49.83	10.68	20.11	34.70
2.000	26.90	100.00	99.82	25.51	27.61	9.23	18.29	4.69	12.33	17.92	42.16	12.20	4.94	37.88
4.000	45.99	100.00	100.00	32.85	46.37	9.27	18.34	15.96	16.13	26.23	36.79	13.86	26.94	43.25
8.000	33.87	98.51	100.00	34.12	42.84	3.99	14.95	12.34	22.38	20.75	29.37	12.62	73.83	44.83
16.000	28.40	100.00	100.00	40.03	39.16	16.03	22.91	22.57	32.45	24.57	32.58	12.62	30.78	52.07
32.000	35.54	99.94	100.00	43.08	49.64	35.68	42.94	34.06	40.18	39.15	34.76	38.12	85.12	53.14
64.000	42.89	100.00	100.00	84.17	74.99	30.83	56.13	51.87	52.66	90.90	60.56	95.61	90.97	67.06
128.000	81.96	100.00	100.00	97.61	97.08	98.94	67.69	85.81	77.38	97.74	99.23	100.00	99.69	99.80
256.000	98.07	100.00	100.00	99.93	97.94	98.98	84.00	99.02	94.60	99.98	99.72	100.00	100.00	100.00

Table : **Fungal damage values of *C. lusitaniae* biofilm incubated with caspofungin** The fungal damage values are in percents

Concentration of micafungin	Fungal damage of C. lusitaniae strains incubated with micafungin – biofilm													
[mg/L]	4245	0640	5094	9949	2588	8850	7892	2885	2732	5278	4241	2923	6936	4243
0.060	25.99	18.51	30.42	14.77	40.53	36.51	12.40	20.18	21.45	23.51	36.61	44.59	9.85	44.53
0.125	39.62	26.93	26.93	13.11	43.27	37.47	19.00	31.96	23.52	23.45	37.36	45.04	14.91	45.01
0.250	26.73	32.89	27.44	16.89	51.32	40.41	20.10	36.42	26.05	24.04	43.95	45.35	51.22	29.10
0.500	21.96	16.50	24.17	16.18	44.80	32.67	26.13	26.46	40.40	15.22	39.73	35.57	50.86	41.35
1.000	2.01	-13.34	14.68	15.89	23.28	21.94	12.22	16.58	11.40	6.10	23.36	21.68	56.73	4.27
2.000	16.61	5.72	18.44	11.95	28.60	15.37	6.65	5.87	6.19	11.09	38.57	17.37	87.70	36.57
4.000	33.76	35.82	29.46	20.88	49.69	38.74	27.93	10.94	10.79	7.98	45.94	37.48	89.00	41.30
8.000	26.38	35.66	35.20	11.79	50.36	41.66	28.08	16.90	25.80	21.46	42.32	37.63	94.76	43.57
16.000	35.74	37.51	36.53	21.90	57.01	47.70	37.71	35.01	25.35	24.69	49.71	41.13	92.22	44.28
32.000	32.40	36.38	35.64	26.54	57.26	46.29	41.92	44.79	30.97	25.09	47.67	38.45	81.32	41.91
64.000	41.60	36.13	32.14	35.04	55.31	39.73	33.76	45.36	25.59	23.61	49.41	36.49	82.66	21.06
128.000	29.36	30.37	31.28	32.49	53.13	27.90	25.58	37.38	26.99	16.32	44.21	26.43	84.31	43.33
256.000	24.12	-0.64	16.52	30.25	43.07	21.78	30.11	30.64	12.37	11.96	31.41	17.01	89.41	5.65

Table : **Fungal damage values of *C.lusitaniea* biofilm incubated with micafungin** The fungal damamges are in percents

Concentration of anidulafungin [mg/L]	Fungal damage of C. lusitaniae strains incubated with anidulafungin – planktonic cells													
	4245	0640	5094	9949	2588	8850	7892	2885	2732	5278	4241	2923	6936	4243
0.007	2.65	-18.84	1.68	3.21	-12.41	-1.21	-5.41	0.61	-0.39	-0.87	2.91	-4.37	2.14	0.97
0.015	9.90	0.71	16.22	6.25	-7.96	13.39	17.45	4.21	7.02	14.20	18.12	-12.31	11.71	-1.90
0.030	23.49	15.46	26.74	15.05	5.79	22.46	28.38	7.91	11.48	20.91	20.95	58.61	14.81	-2.10
0.060	44.64	36.35	83.18	38.42	48.56	37.81	26.36	17.70	24.17	39.42	53.20	54.48	39.89	53.11
0.125	87.35	92.37	90.57	93.51	96.37	61.08	37.51	77.27	96.37	98.16	81.96	91.69	75.48	96.89
0.250	100.00	99.61	89.49	100.00	98.66	99.82	26.95	98.91	98.83	99.84	99.94	90.48	98.09	98.85
0.500	100.00	98.76	100.00	100.00	99.18	99.76	24.43	99.41	98.03	99.43	98.16	94.86	97.82	98.12
1.000	100.00	95.59	99.91	100.00	98.64	99.22	19.16	99.56	98.68	99.02	98.48	94.65	98.17	97.73
2.000	100.00	100.00	100.00	100.00	99.61	99.92	14.48	100.00	98.75	100.00	99.97	97.16	97.79	98.48
4.000	100.00	100.00	100.00	100.00	99.90	100.00	35.04	100.00	99.45	100.00	100.00	97.63	98.02	99.43
8.000	100.00	99.13	100.00	100.00	99.05	99.48	52.32	98.25	98.15	97.90	98.51	92.06	97.51	98.32
16.000	100.00	100.00	100.00	100.00	100.00	100.00	43.47	100.00	99.56	100.00	100.00	97.23	98.32	99.51
32.000	100.00	99.83	100.00	100.00	99.38	100.00	47.98	100.00	98.70	100.00	99.90	97.09	97.56	98.66
64.000	100.00	100.00	100.00	100.00	99.92	100.00	44.37	100.00	99.44	100.00	100.00	97.81	97.81	99.58
128.000	100.00	100.00	100.00	100.00	99.75	100.00	40.36	100.00	99.34	100.00	100.00	98.17	97.91	99.18
256.000	100.00	100.00	100.00	100.00	99.86	100.00	33.33	99.93	99.48	100.00	100.00	97.70	98.57	99.15

Table : **Fungal damage values of *C. lusitaniae* planktonic cells incubated with anidulafungin** The fungal damage values are in percents

Concentration of caspofungin [mg/L]	Fungal damage of C. lusitaniae strains incubated with caspofungin – planktonic cells													
	4245	0640	5094	9949	2588	8850	7892	2885	2732	5278	4241	2923	6936	4243
0.030	45.65	27.24	-7.90	3.60	17.74	14.52	4.29	29.80	5.71	6.04	31.19	6.46	17.59	10.90
0.060	57.90	23.88	-8.43	4.70	21.78	23.58	16.06	75.43	11.41	14.05	27.99	7.23	12.08	13.37
0.125	71.20	51.57	-3.53	0.77	22.92	19.88	15.84	75.70	12.27	8.13	22.16	8.96	21.07	16.39
0.250	71.20	51.57	-3.53	0.77	22.92	19.35	15.84	75.70	12.27	7.10	22.16	8.96	21.07	16.39
0.500	82.47	64.40	24.63	-2.99	21.10	5.70	13.85	98.41	15.29	36.52	26.81	9.88	33.08	20.54
1.000	99.71	99.64	99.84	100.00	97.04	57.53	96.66	98.39	96.82	98.45	27.69	19.76	98.54	32.20
2.000	99.76	100.00	99.82	100.00	99.86	100.00	97.87	99.41	97.83	99.63	100.00	95.81	98.92	100.00
4.000	100.00	100.00	100.00	100.00	99.87	100.00	94.64	99.77	97.69	99.85	100.00	95.98	99.62	100.00
8.000	91.50	98.51	100.00	98.05	99.22	98.81	80.86	97.61	93.83	95.14	99.89	97.73	98.24	98.36
16.000	77.85	100.00	100.00	98.49	97.43	99.44	99.10	99.21	81.54	98.42	100.00	99.97	99.73	98.08
32.000	99.92	99.94	100.00	99.98	98.77	90.25	98.22	98.88	98.33	98.30	99.89	98.97	98.96	98.23
64.000	100.00	100.00	100.00	100.00	99.97	100.00	98.72	99.97	99.17	99.24	100.00	99.85	99.73	100.00
128.000	100.00	100.00	100.00	100.00	99.81	99.90	99.02	99.93	99.10	100.00	100.00	99.64	99.66	100.00
256.000	100.00	100.00	100.00	100.00	100.00	100.00	99.37	99.99	99.70	99.63	100.00	99.83	99.87	100.00

Table: **Fungal damage values of *C. lusitaniae* planktonic cells incubated with caspofungin** The fungal damage values are in percents

Concentration of micafungin	Fungal damage of C. lusitaniae strains incubated with micafungin – planktonic cells													
[mg/L]	4245	0640	5094	9949	2588	8850	7892	2885	2732	5278	4241	2923	6936	4243
0.060	92.15	59.71	99.49	43.90	97.55	82.83	29.28	17.47	92.37	93.12	97.39	48.29	99.91	99.67
0.125	99.75	80.08	97.67	56.02	98.40	74.00	41.98	18.61	96.66	97.98	99.47	85.52	99.51	98.64
0.250	100.00	99.74	99.62	83.93	99.58	82.64	71.88	29.04	98.35	99.02	100.00	99.40	100.00	99.73
0.500	100.00	99.54	99.46	99.81	99.44	82.34	86.02	95.89	98.19	100.00	100.00	99.16	99.97	99.64
1.000	99.84	99.17	97.07	100.00	99.03	82.74	89.21	99.62	97.69	99.75	100.00	98.90	99.90	98.89
2.000	100.00	100.00	100.00	100.00	99.92	86.95	98.85	99.47	99.08	100.00	100.00	97.82	99.95	99.91
4.000	100.00	100.00	100.00	100.00	100.00	87.15	98.59	100.00	99.70	100.00	100.00	99.19	100.00	100.00
8.000	99.67	100.00	100.00	99.81	99.53	81.66	97.76	99.51	98.05	99.90	99.94	97.18	99.60	98.51
16.000	100.00	100.00	100.00	100.00	100.00	85.97	99.23	99.96	99.61	100.00	100.00	99.35	100.00	100.00
32.000	100.00	100.00	99.79	100.00	99.95	79.11	98.60	99.27	98.99	99.85	100.00	98.87	100.00	99.73
64.000	100.00	100.00	100.00	100.00	100.00	83.32	99.01	99.86	99.26	100.00	100.00	99.83	100.00	100.00
128.000	100.00	100.00	100.00	100.00	100.00	83.03	98.75	99.89	98.96	100.00	100.00	99.55	100.00	100.00
256.000	100.00	100.00	100.00	100.00	100.00	84.70	98.81	99.92	98.68	100.00	100.00	99.85	100.00	100.00

Table : **Fungal damage values of *C. lusitaniae* planktonic cells incubated with micafungin** The fungal damage values are in percents

Concentration of anidulafungin [mg/L]	Fungal damage of C.krusei strains incubated with anidulafungin – biofilm												
	6865	12414	9346	9908	7394-2	7824	9200	10226	8657	6892	11154	12841	11708
0.007	-12.08	1.10	-8.99	7.46	1.66	9.72	-3.97	-15.24	-21.07	-14.20	27.93	0.23	0.74
0.015	-1.12	7.99	2.87	8.53	12.16	14.55	18.68	7.10	-2.76	-1.72	44.80	11.69	9.14
0.030	-1.93	25.69	15.69	17.09	14.48	12.86	24.66	0.33	7.05	19.67	42.98	13.72	19.65
0.060	-8.83	26.73	32.82	21.10	20.74	29.75	62.91	19.53	35.33	42.43	80.19	32.22	30.40
0.125	18.29	32.86	52.45	48.56	34.47	52.54	65.32	35.53	87.31	74.83	85.07	63.45	28.13
0.250	49.53	52.60	54.73	57.42	82.36	65.24	95.05	84.53	92.37	82.62	64.40	82.50	45.94
0.500	48.71	40.70	54.79	50.38	82.26	54.67	92.50	79.27	82.50	74.05	69.28	74.90	34.18
1.000	38.63	49.70	48.88	39.39	82.11	50.26	79.31	71.25	75.35	67.65	49.90	53.98	39.90
2.000	34.88	61.01	53.67	57.99	86.50	71.27	69.71	71.82	80.31	72.95	49.47	68.61	42.14
4.000	45.82	63.50	53.78	54.76	89.61	70.74	80.49	70.55	88.17	73.04	77.60	73.50	47.13
8.000	52.31	60.94	61.91	47.91	88.25	73.30	85.29	75.06	83.47	75.98	66.26	75.39	48.03
16.000	57.93	60.77	57.93	40.12	89.69	77.37	87.30	82.04	83.13	89.93	53.20	75.45	57.36
32.000	37.07	66.30	50.53	38.92	89.17	74.27	87.99	80.66	81.47	85.74	64.40	81.63	62.41
64.000	37.12	62.81	55.59	38.85	89.97	72.46	88.68	79.58	80.60	84.76	70.14	78.65	58.87
128.000	43.50	50.52	47.93	38.85	90.51	70.21	85.54	86.84	83.32	75.38	65.55	80.20	51.72
256.000	46.11	62.60	54.31	27.23	78.91	67.35	84.74	77.66	71.60	68.59	-3.08	68.54	55.15

Table : **Fungal damage values of *C. krusei* biofilm incubated with anidulafungin** The fungal damage values are in percents

Concentration of caspofungin [mg/L]	Fungal damage of C. krusei strains incubated with caspofungin – biofilm												
	6865	12414	9346	9908	7394-2	7824	9200	10226	8657	6892	11154	12841	11708
0.030	3.69	6.91	-2.71	4.04	4.74	5.23	13.51	4.76	19.87	-3.12	-30.07	-0.05	18.72
0.060	11.82	21.88	51.31	15.51	10.08	16.65	20.04	13.71	22.15	6.15	-3.71	9.04	26.24
0.125	3.63	14.29	61.48	-4.23	26.94	13.84	17.10	17.93	20.69	-6.96	23.30	1.36	35.77
0.250	46.01	13.04	36.83	0.09	8.33	6.22	17.10	17.93	52.46	8.56	-19.31	9.26	35.77
0.500	46.18	55.89	73.72	1.40	37.48	88.36	11.07	24.38	81.60	56.29	88.73	72.84	33.79
1.000	15.71	61.64	47.85	30.44	85.35	88.43	71.74	92.04	85.46	61.96	85.84	70.32	22.36
2.000	58.97	70.96	62.36	66.13	92.11	84.34	76.22	79.27	70.62	76.90	6.04	76.04	44.98
4.000	59.89	72.37	37.72	60.35	93.78	87.23	77.72	84.53	72.14	80.71	51.32	83.64	52.80
8.000	49.47	64.22	32.58	44.15	93.03	79.61	78.85	79.60	77.46	78.22	87.58	80.71	58.24
16.000	84.02	80.48	63.63	75.69	94.72	94.00	85.16	88.75	85.22	80.79	100.00	73.07	75.11
32.000	85.15	89.84	87.72	88.42	92.87	94.09	85.28	90.13	90.13	81.65	100.00	79.83	89.31
64.000	94.28	93.99	88.42	94.81	90.37	99.47	96.56	96.58	94.98	89.05	100.00	91.65	97.75
128.000	99.14	100.00	98.51	99.96	97.29	100.00	99.44	99.74	99.77	94.57	100.00	99.74	98.70
256.000	99.89	100.00	99.21	99.88	99.48	100.00	99.84	100.00	100.00	100.00	100.00	99.89	97.26

Table : **Fungal damage values of *C. krusei* biofilm incubated with caspofungin** The fungal damage values are in percents

Concentration of micafungin	Fungal damage of C. krusei strains incubated with micafungin – biofilm												
[mg/L]	6865	12414	9346	9908	7394-2	7824	9200	10226	8657	6892	11154	12841	11708
0.060	45.85	35.01	35.32	12.16	65.51	38.29	31.29	40.32	31.87	87.72	65.22	81.92	16.74
0.125	58.00	58.18	38.74	31.73	77.81	61.09	51.33	58.44	57.44	84.27	67.24	79.93	16.86
0.250	51.88	59.46	41.49	52.38	89.25	54.36	69.05	83.24	74.95	83.41	98.75	80.42	21.38
0.500	36.67	69.37	52.84	29.43	90.52	33.80	73.75	83.62	80.04	85.30	78.07	91.02	31.16
1.000	-6.73	58.12	45.70	29.52	90.38	25.41	77.26	66.20	73.80	70.41	90.60	57.29	18.35
2.000	46.23	70.34	65.80	60.18	92.86	70.16	76.21	79.96	74.25	76.00	23.83	67.83	40.62
4.000	46.95	54.87	62.10	62.20	96.36	67.19	79.34	82.85	71.98	79.80	21.63	68.14	44.88
8.000	47.91	59.96	56.13	58.73	92.51	53.05	75.17	74.28	76.18	85.88	8.14	64.09	47.03
16.000	59.03	69.47	67.42	58.76	95.81	65.42	76.67	84.33	81.11	88.50	91.74	73.00	54.20
32.000	64.25	63.23	67.01	55.51	94.65	64.48	87.56	85.15	81.89	88.64	95.04	61.66	60.25
64.000	60.25	61.33	64.53	69.25	98.72	63.80	73.31	89.24	82.89	87.50	88.99	67.00	64.48
128.000	62.53	64.14	67.85	68.96	98.03	61.82	62.73	90.01	88.03	77.87	92.60	66.61	67.51
256.000	44.39	66.12	59.37	65.43	94.05	39.33	79.47	87.88	89.86	76.67	92.84	59.98	67.86

Table : **Fungal damage values of *C. krusei* biofilm incubated with micafungin** The fungal damage values are in percents

Concentration of anidulafungin [mg/L]	Fungal damage of C. krusei strains incubated with anidulafungin – planktonic cells												
	6865	12414	9346	9908	7294-2	7824	9200	10226	8657	6892	11154	12841	11708
0.007	-11.59	-1.14	-24.13	3.94	-19.48	-9.07	-8.37	-6.16	-19.58	-55.60	-2.05	-40.06	3.87
0.015	9.83	20.47	-2.27	-11.51	6.87	3.82	1.59	10.19	-2.54	-26.20	22.06	-20.69	18.85
0.030	11.59	30.95	-0.40	82.20	0.05	98.30	-5.91	11.32	12.26	-18.07	9.90	-13.54	-0.52
0.060	44.05	98.55	30.00	99.39	15.59	100.00	35.46	35.85	5.31	5.76	14.24	3.30	31.97
0.125	71.82	98.22	99.07	99.67	73.32	99.14	59.58	74.24	4.04	88.75	50.67	60.51	97.07
0.250	100.00	99.73	100.00	100.00	92.28	99.95	98.24	98.37	93.36	100.00	100.00	99.83	99.44
0.500	100.00	99.52	100.00	100.00	93.29	100.00	94.41	97.69	93.54	99.33	100.00	98.75	98.09
1.000	99.95	99.14	100.00	100.00	95.85	98.29	94.99	97.95	97.98	100.00	100.00	98.41	98.87
2.000	100.00	99.64	100.00	87.69	95.08	100.00	99.20	99.21	88.34	100.00	100.00	99.70	100.00
4.000	100.00	100.00	100.00	100.00	93.51	100.00	100.00	99.95	90.73	100.00	100.00	100.00	100.00
8.000	98.59	98.36	100.00	98.00	88.19	99.24	96.43	96.16	85.83	98.90	100.00	98.02	96.09
16.000	100.00	100.00	100.00	100.00	97.18	100.00	100.00	100.00	93.24	99.51	100.00	100.00	100.00
32.000	100.00	99.70	100.00	100.00	91.54	100.00	98.83	98.26	94.20	99.76	100.00	99.66	100.00
64.000	100.00	99.79	100.00	100.00	92.87	100.00	97.50	99.89	95.10	100.00	100.00	100.00	100.00
128.000	100.00	98.19	100.00	100.00	95.96	100.00	97.92	99.11	97.55	100.00	100.00	99.57	100.00
256.000	100.00	99.85	100.00	100.00	98.51	100.00	98.08	99.53	99.28	100.00	100.00	99.70	100.00

Table : **Fungal damage values of *C. krusei* planktonic cells incubated with anidulafungin** The fungal damage values are in percents

Concentration of caspofungin [mg/L]	Fungal damage of C. krusei strains incubated with caspofungin – planktonic cells												
	6865	12414	9346	9908	7394-2	7824	9200	10226	8657	6892	11154	12841	11708
0.030	21.86	16.30	2.21	6.13	-7.52	14.14	3.92	4.21	10.35	32.41	16.29	5.93	12.41
0.060	39.59	25.02	11.72	35.93	6.95	26.58	23.62	16.86	21.49	30.51	24.44	13.59	29.56
0.125	70.88	98.64	41.23	41.71	5.40	22.97	11.65	7.20	14.91	26.46	20.68	17.39	13.30
0.250	99.44	99.90	76.23	84.84	5.40	18.84	11.65	7.20	11.84	17.46	19.54	17.70	13.30
0.500	99.86	99.48	99.74	99.96	1.91	5.82	2.02	1.53	16.39	-2.02	12.51	13.00	5.85
1.000	100.00	99.74	99.28	100.00	97.66	99.37	94.77	99.54	85.40	100.00	100.00	99.91	88.47
2.000	100.00	99.58	99.74	99.68	95.64	100.00	99.92	100.00	96.21	100.00	100.00	99.88	98.54
4.000	100.00	100.00	100.00	100.00	97.37	100.00	100.00	100.00	98.69	100.00	100.00	100.00	100.00
8.000	98.26	98.97	97.26	100.00	91.42	97.70	95.93	98.47	93.37	99.33	99.65	99.94	96.84
16.000	100.00	100.00	100.00	100.00	99.83	100.00	100.00	100.00	98.87	100.00	100.00	100.00	100.00
32.000	100.00	98.87	100.00	100.00	99.14	100.00	100.00	99.85	97.84	100.00	100.00	99.60	98.66
64.000	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	99.91	100.00	100.00	100.00	99.84
128.000	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	99.92
256.000	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

Table : **Fungal damage values of *C. krusei* planktonic cells incubated with caspofungin** The fungal damage values are in percents

Concentration of micafungin	Fungal damage of C. krusei strains incubated with micafungin – planktonic cells												
[mg/L]	6865	12414	9346	9908	7394-2	7824	9200	10226	8657	6892	11154	12841	11708
0.060	100.00	100.00	100.00	100.00	-2.59	49.25	96.86	99.53	99.40	100.00	100.00	100.00	31.28
0.125	99.27	99.13	97.23	98.66	94.20	96.83	94.69	95.45	99.60	98.30	99.13	99.17	97.18
0.250	100.00	100.00	99.86	100.00	96.79	100.00	99.26	99.42	99.96	100.00	100.00	100.00	76.20
0.500	100.00	100.00	99.29	99.68	98.22	99.93	97.94	98.68	99.95	99.79	93.17	100.00	99.96
1.000	99.92	99.93	99.15	99.90	97.53	100.00	99.43	98.44	100.00	99.62	100.00	99.76	99.29
2.000	100.00	100.00	100.00	100.00	98.86	100.00	93.77	98.52	99.60	99.83	100.00	100.00	100.00
4.000	100.00	100.00	100.00	100.00	100.00	100.00	96.92	100.00	100.00	99.79	100.00	100.00	100.00
8.000	100.00	98.32	98.58	99.81	96.00	96.97	92.06	97.39	99.68	98.86	99.59	97.68	99.34
16.000	100.00	100.00	100.00	100.00	100.00	100.00	99.31	100.00	100.00	100.00	100.00	100.00	100.00
32.000	100.00	100.00	100.00	100.00	99.06	100.00	96.97	98.68	100.00	99.86	100.00	100.00	100.00
64.000	100.00	100.00	100.00	100.00	100.00	100.00	98.06	100.00	100.00	100.00	100.00	100.00	98.06
128.000	100.00	100.00	100.00	100.00	99.95	100.00	99.83	100.00	100.00	100.00	100.00	100.00	100.00
256.000	100.00	100.00	100.00	100.00	99.95	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

Table : **Fungal damage values of *C. krusei* planktonic cells incubated with micafungin** The fungal damage values are in percents

Concentration of anidulafungin [mg/L]	Fungal damage of C. guilliermondii strains incubated with anidulafungin – biofilm		
	99	111	112
0.007	5.85	4.91	9.08
0.015	13.67	11.10	16.69
0.030	15.86	19.76	10.21
0.060	23.52	28.62	26.18
0.125	29.11	26.17	24.44
0.250	18.47	19.07	23.27
0.500	10.56	10.16	35.77
1.000	8.66	9.24	27.64
2.000	70.06	48.30	17.76
4.000	84.81	56.45	24.01
8.000	84.65	71.71	37.00
16.000	86.09	79.12	46.72
32.000	88.78	84.99	60.55
64.000	89.32	80.02	58.79
128.000	87.13	83.29	42.02
256.000	83.20	83.71	73.95

Table : **Fungal damage values of *C. guilliermondii* biofilm incubated with anidulafungin** The fungal damage values are in percents

Concentration of caspofungin [mg/L]	Fungal damage of C. guilliermondii strains incubated with caspofungin – biofilm		
	99	111	112
0.030	7.33	24.74	22.78
0.060	24.92	26.01	28.40
0.125	19.68	25.51	27.86
0.250	20.01	11.91	33.57
0.500	22.78	15.23	27.29
1.000	34.14	15.59	30.40
2.000	47.71	21.59	30.65
4.000	57.29	28.44	32.43
8.000	56.30	28.68	34.55
16.000	59.12	39.39	44.26
32.000	65.62	57.62	35.34
64.000	77.55	79.90	57.13
128.000	99.71	95.58	96.11
256.000	100.00	98.66	99.62

Table : **Fungal damage values of *C. guilliermondii* biofilm incubated with caspofungin** The fungal damage values are in percents

Concentration of micafungin [mg/L]	Fungal damage of C. guilliermondii strains incubated with micafungin – biofilm		
	99	111	112
0.060	41.76	24.58	26.12
0.125	40.54	11.31	33.13
0.250	49.88	10.27	36.20
0.500	54.52	14.87	35.83
1.000	54.12	17.02	61.69
2.000	53.74	46.33	51.41
4.000	58.63	60.34	34.43
8.000	50.32	65.51	50.77
16.000	70.51	72.23	68.88
32.000	70.12	67.84	67.28
64.000	64.79	60.69	60.88
128.000	64.50	59.22	71.45
256.000	62.54	46.18	56.14

Table : **Fungal damage values of *C. guilliermondii* biofilm incubated with micafungin** The fungal damage values are in percents

Concentration of anidulafungin [mg/L]	Fungal damage of C. guilliermondii strains incubated with anidulafungin – planktonic cells		
	99	111	112
0.007	5.94	0.61	5.91
0.015	19.19	8.99	-1.89
0.030	21.42	16.80	12.60
0.060	30.67	21.69	18.02
0.125	27.34	13.22	10.02
0.250	21.34	10.19	2.70
0.500	18.47	23.58	1.45
1.000	96.72	97.80	93.88
2.000	99.76	99.66	99.92
4.000	100.00	100.00	100.00
8.000	97.16	97.65	98.70
16.000	100.00	100.00	100.00
32.000	100.00	99.97	100.00
64.000	100.00	100.00	100.00
128.000	99.96	100.00	100.00
256.000	99.76	99.87	100.00

Table : **Fungal damage values of *C. guilliermondii* planktonic cells incubated with anidulafungin** The fungal damage values are in percents

Concentration of caspofungin [mg/L]	Fungal damage of C. guilliermondii strains incubated with caspofungin – planktonic cells		
	99	111	112
0.030	22.78	2.36	14.80
0.060	33.41	12.55	25.80
0.125	23.21	14.42	20.81
0.250	22.40	10.99	24.89
0.500	11.04	70.95	9.96
1.000	50.96	79.66	69.07
2.000	99.19	93.57	87.31
4.000	99.90	93.44	91.29
8.000	96.89	91.13	82.66
16.000	98.47	77.32	70.80
32.000	97.60	87.32	77.32
64.000	100.00	93.60	98.50
128.000	100.00	100.00	100.00
256.000	100.00	100.00	100.00

Table : **Fungal damage values of *C. guilliermondii* planktonic cells incubated with caspofungin** The fungal damage values are in percents

Concentration of micafungin [mg/L]	Fungal damage of C. guilliermondii strains incubated with micafungin – planktonic cells		
	99	111	112
0.060	23.66	17.56	14.17
0.125	93.09	95.95	92.41
0.250	97.05	98.67	98.48
0.500	95.83	98.22	95.45
1.000	96.29	98.22	92.64
2.000	98.47	98.89	93.03
4.000	99.95	99.56	93.54
8.000	95.53	95.95	86.29
16.000	100.00	100.00	96.91
32.000	98.73	98.89	96.40
64.000	99.69	99.96	99.27
128.000	98.88	99.69	97.64
256.000	99.64	99.91	99.44

Table : **Fungal damage values of *C. guilliermondii* planktonic cells incubated with micafungin** The fungal damage values are in percents