Year : 2016

SYSTEMIC AND LOCAL ANTIMICROBIAL ACTIVITY AGAINST PLANKTONIC AND BIOFILM STATE OF MICROORGANISMS IN VITRO AND IN VIVO

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Maryka Maiolo Elena, 2016, SYSTEMIC AND LOCAL ANTIMICROBIAL ACTIVITY AGAINST PLANKTONIC AND BIOFILM STATE OF MICROORGANISMS IN VITRO AND IN VIVO

Originally published at : Thesis, University of Lausanne

Posted at the University of Lausanne Open Archive http://serval.unil.ch
Document URN : urn:nbn:ch:serval-BIB_96AE111894552

UNIL | Université de Lausanne
Faculté de biologie et de médecine

Département de Médecine Interne, Service de chirurgie séptique

SYSTEMIC AND LOCAL ANTIMICROBIAL ACTIVITY AGAINST PLANKTONIC AND BIOFILM STATE OF MICROORGANISMS IN VITRO AND IN VIVO

Thèse de doctorat ès sciences de la vie (PhD)

présentée à la

Faculté de biologie et de médecine
de l'Université de Lausanne

par

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Lausanne 2014

UNIL | Université de Lausanne
Faculté de biologie
et de médecine

Ecole Doctorale
Doctorat ès sciences de la vie

Imprimatur

Vu le rapport présenté par le jury d'examen, composé de

Président	Monsieur Prof. Yvan **Arsenijevic**
Directeur de thèse	Monsieur Dr Olivier **Borens**
Co-directeur de thèse	Monsieur Prof. Andrej **Trampuz**
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	Monsieur Prof. Maurizio **Sanguinetti**

le Conseil de Faculté autorise l'impression de la thèse de

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intitulée

SYSTEMIC AND LOCAL ANTIMICROBIAL ACTIVITY AGAINST PLANKTONIC AND BIOFILM STATE OF MICROORGANISMS IN VITRO AND IN VIVO

Lausanne, le 3 juillet 2014

pour La Doyenne
de la Faculté de Biologie et de Médecine

Prof. Yvan Arsenijevic

Acknowledgements

First, I would like to express my gratitude to all the members of the jury, Professor Yvan Arsenijevic, president of the jury, Doctor Andrej Trampuz, thesis co-director, Doctor Olivier Borens, thesis director, Professor Jacques Meis and Professor Maurizio Sanguinetti, both external experts.

I am grateful to Doctor Andrej Trampuz for giving me the opportunity to do my PhD under his supervision and work on very interesting projects related to foreign-body infections and the development of new methods to testing antifungal susceptibility *in vitro* and *in vivo*. Therefore, it gave me the opportunity to develop different, rich and important collaborations in this field and start an excellent experience in Berlin. Andrej is a very supportive mentor always motivating me in good and bad situations and with great knowledge in his field.

I would like to thank Doctor Olivier Borens for accepting me as researcher in this field and allowing me to follow Doctor Trampuz for the final part of the thesis in Berlin.

I thank all collaborators at the clinical pharmacology and toxicology, Dr Laurent Decosterd for his motivation and enthusiasm and his team for the good and efficacy pharmacokinetic work. A special thanks to Nancy Perrottet Ries for her great help with the pharmacokinetic graphs.

A special thanks to Riccardo Torelli for supervising me during my *in vitro* biofilm experiments, working with him and his colleagues was a great human experience.

Mostly, I thank Ulrika Furustrand Tafin for her precious advices in both science and administrative tasks along the all PhD. I thank Bertrand Betrisey and again Ulrika for teaching me to work with the animal model and Alessandra Oliva for her support and for helping me with the animal experiments.

A special thanks to Laura Rio who started her PhD at the same time as me, for her advices and to Iñes Ferreira who was temporarly by my side in Berlin, for her precious advices regarding the last part of my thesis and for sharing with me the very cold winter of Berlin.

Thanks to all the observers that came to our lab for a short period of time, especially Dr. Christen Ravn, Laura Sessa and Dr Alessandra Oliva for keeping up a stimulating and enriching environment for work and for life.

Finally, I would like to thank my mother for her support, patience and love. A special thanks to my precious friends for their energy, their constant presence and for tolerating my humors.

Abbreviations

AMB	Amphotericin B
AFG	Anidulafungin
ATCC	American type culture collection
AUC	Area under the curve
BA	Blood agar
BAG	Bioactive glass
BHI	Brain heart infusion
CAS	Caspofungin
CFU	Colony forming unit
CLSI	Clinical laboratory standards institute
C_{max}	Maximum (peak) concentration
C_{min24}	Minimum (through) concentration at 24h
C_{min48}	Minimum (through) concentration at 48h
EUCAST	European committee on antimicrobial suceptibility testing
FZL	Fluconazole
GC	Growth control
i.p.	Intraperitoneal
McF	McFarland
MH	Müller hinton
MHIC	Minimal heat inhibitory concentration
MIC	Minimal inhbitory concentration
NaCl	0.9% sodium chloride
PBS	Phosphate buffered saline
PK	Pharmacokinetic
RPMI	Roswell Park Memorial Institute
SD	Standard deviation
SDB	Sabouraud dextrose broth
SEM	Standard error mean
T_{max}	Time at which Cmax is achieved
TSB	Tryptic soy broth
XTT	Tetrazolium salt

Summary

Implant-associated infections caused by *Candida* spp. are difficult to treat due to reduced antimicrobial susceptibility in biofilm. Antifungal susceptibility testing is important due to the increasing number of prosthetic infections caused by *Candida spp.* The aim of this thesis was to investigate innovative methods using calorimetry for microbial detection and antimicrobial susceptibility testing, as well as treatment activity in validated *C. albicans* foreign-body infection animal model (part 1). In addition, new materials with intrinsic antimicrobial activity (bioactive glass) were tested (part 2).

The **first part** of the thesis describes a novel real-time method for evaluation of antifungals against yeast, based on measurements of the growth-related heat production by isothermal microcalorimetry. Current methods for evaluation of antifungal agents against yeast have several limitations, especially when combinations of antifungals are investigated. We therefore evaluated the activity of fluconazole, amphotericin B and two echinocandins (caspofungin and anidulafungin) against *Candida* spp. by microcalorimetry. The minimal heat inhibition concentration (MHIC) was defined as the lowest concentration inhibiting ≥50% (≥90% for amphotericin B) of the heat produced at 24 and 48h for planktonic and biofilm yeast, respectively. Agreement within two-fold dilutions between MHIC and MIC was 50% for fluconazole and 100% for caspofungin, anidulafungin and amphotericin B. As determined by microcalorimetry, echinocandins (especially anidulafungin) were the most active agents against planktonic *Candida*. Subsequently, antimicrobial treatment strategies for infections caused by *Candida albicans in-vivo* were investigated. Since *C. albicans* had not previously been tested in this animal model, we first established an infection profile and investigated the pharmacokinetics of antifungals. In untreated animals, planktonic *Candida* progressively decreased in cage fluid and was cleared in 8% to 24% of cage fluids, however, *Candida* biofilm persisted on all cages, i.e. no spontaneous cure of cage-associated infections was observed. In accordance with *in vitro* experiments, echinocandins showed the highest activity against planktonic *C. albicans*. Against *C. albicans* biofilm, caspofungin showed the highest cure rate (25%), whereas cure rates of other antifungals ranged between 8% - 17%, demonstrating the difficulty of eradicating *Candida* biofilms on implants.

The **second part** of the thesis investigates the activity of bioactive glass (BAG) S53P4 (volumes 1 g and 2 g and sizes 0.5-0.8 mm and <45 µm) against *Staphylococcus aureus, S. epidermidis, Escherichia coli, Enterococcus faecalis* et *Candida albicans* by microcalorimetry. BAG is a surface-reactive glass-ceramic biomaterial which is used as implant material to repair and replace diseased or damaged bones. Besides binding chemically to the bone and being osteoconductive, this material has the property to inhibit the biofilm formation. BAG showed good activity against tested mircoorganisms, except for *E. faecalis* with granula 0.5-0.8 mm.

Résumé

Les infections associées aux implants causées par *Candida* spp. sont difficiles à traiter à cause de la faible susceptibilité du biofilm aux antifongiques. Le test de susceptibilité aux antifongiques est important vu l'augmentation des infections de prothèses dûes à *Candida* spp. Le but de cette thèse est d'étudier des nouvelles méthodes de détection des microorganismes par calorimétrie et de tester la susceptibilité ainsi que l'activité des antifongiques dans un modèle animal (partie 1). De plus, des nouveaux matériaux avec une activité antimicrobienne intrinsèque (bioactive glass) ont été testés (partie 2).

La **première partie** de la thèse décrit une nouvelle méthode d'évaluation de l'effet des antifongiques en temps réel en se basant sur la chaleur produite lors de la croissance mesurée par microcalorimètre isothermique. Les méthodes actuelles visant à évaluer l'effet des antifongiques sont limitées, surtout lorsqu'il s'agit d'évaluer l'effet des combinaisons d'antifongiques. Nous avons évalué l'activité du fluconazole, de l'amphotericin B et des echinocandines (caspofungine et anidulafungine), sur différentes souches de *Candida* spp. La concentration minimale d'inhibition de chaleur (CMIC) a été définie comme étant la plus petite concentration inhibant ≥50% (≥90% pour l'amphotericine B) de la chaleur produite à 24h et 48h pour la croissance planctonique et du biofilm. La concordance entre CMIC et la concentration minimale d'inhibition (CMI), avec 2 dilutions de marge, était de 50% pour fluconazole et 100% pour les echinocandines et l'amphotericin B. Comme déterminé par microcalorimétrie, les echinocandines (surtout l'anidulafungine) ont montré une meilleure activité contre la croissance planctonique de *Candida*. Nous avons ensuite étudié les traitements antimicrobiens contre *C. albicans in vivo*. Étant donné l'absence d'études avec ce modèle animal avec *C. albicans*, nous avons d'abord établi un profil d'infection et étudié la pharmacocinétique des antifongiques. Chez les animaux non traités, *Candida* planctonique a montré une décroissance progressive dans le fluide des cages, tout en restant présent sous forme de biofilm. Pas de cure spontanée des cages infectées a été observée. En accord avec les expériences *in vitro,* les echinocandines ont montré une meilleure activité contre *C. albicans* planctonique. Contre le biofilm la caspofongine montre le plus haut taux de guérison (25%), contrairement aux autres antifongiques où le taux de guérison allait de 8% à 17%, démontrant ainsi la difficulté rencontrée dans l'éradication du biofilm à *C. albicans*.

La **deuxième partie** étudie l'activité du bioactive glass (BAG) S53P4 (volumes 1 and 2 g et diamètres de 0.5-0.8 mm et <45 µm) contre *Staphylococcus aureus, S. epidermidis*, *Escherichia coli*, *Enterococcus faecalis* et *C. albicans*. BAG est un biomatériel en vitre-céramique à surface réactive utilisé en tant qu'implant pour réparer et remplacer les os endommagés ou fracturés. En plus d'avoir la capacité de se lier chimiquement à l'os et être ostéoconductif, ce matériel a la caractéristique d'inhiber la formation du biofilm. BAG a montré une bonne activité contre les microorganismes testés à l'exception de *E. faecalis* avec les granules de 0.5-0.8 mm de diamètre.

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PART I.

Antifungal susceptibility of yeast by isothermal microcalorimetry

Chapter1. General introduction

Fungal infection

Fungal infections are on the rise as advances in modern medicine prolong the lives of severely ill patients. *Candida* spp., *Aspergillus* spp. and *Cryptococcus neoformans* are the most common agents of fungal infections and the mortality rate often remains higher than 50% (1). *Candida* is considered as being the most important cause of opportunistic mycoses, and is the fourth most common cause of nosocomial infections (2, 3).

Candida is a commensal type of yeast well tolerated by healthy humans. It is found on the oral mucosa, on the skin, in the gastrointestinal tract and in the vaginal flora. However, it can become pathogenic and induce mycosis in diabetic people or whenever the immunosystem is deficient (e.g. in HIV infected patients).

Candida is a polymorphic species, meaning that it can grow either as budding yeast or as filamentous cells (pseudohyphal or hyphal form), and can switch from one form to the other depending on environmental conditions such as temperature and pH. The primary mode of reproduction in *C. albicans* is by the budding of yeast cells. Mother cells give rise to daughter cells, which are released to repeat the budding cycle.

In the past decades various risk factors for fungal infections have emerged, including the use of indwelling devices, transplantation procedures, immunosuppression, prolonged intensive care unit stays and use of broad-range antibiotics suppressing normal bacterial flora, increasing the prevalence of fungal disease (4, 5). More than 90% of invasive infections due to *Candida* spp. are attributed to five species, *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei*, and the list of species isolated from clinical specimens continues to grow each year (2, 6).

The evolving epidemiology of non-*C. albicans* species may reflects the increased use of antifungals for prophylaxis and the introduction of new antifungal agents in clinical use. *C. glabrata*, for example, may be less susceptible to fluconazole, the most inexpensive and readily available antifungal agent used to treat candidemia. Similarly to bacteria, fungi can attach to the implant surface and form biofilm, causing persistent and relapsing infections (7, 8).

Fungal biofilm formation on medical devices

In contrast to the vast literature describing bacterial biofilms, less attention has been given to medically important fungal biofilms. Among implant-associated biofilm infections, *Candida* is implicated in about 1-5%. These infections are difficult to treat, and usually requires removal of the implant and prolonged antifungal treatment (9-12).

C. albicans biofilm formation proceeds mainly through three developmental phases: the early phase, the intermediate phase and the maturation phase. The early phase take place between 0 and ~11h and it involves the adhesion of fungal cells to the substrate. The intermediate phase, between ~12 and 30h, imply the coaggregation and the proliferation of blastospores creating communities and producing an extracellular matrix (ECM) rich in carbohydrates. The maturation phase, between ~31and 72h, includes the formation of a thick ECM in which the fungal cells are completely embedded (13).

In a study performed by Depprich *et a.l* comparing the prevalence of microorganisms on different materials *C. albicans* was demonstrated to be able to form biofilm on silicone but not on titanium. This biofilm formation was mainly due to the light roughness of the titanium surface (14). This study also revealed the formation of biofilm on the silicone interior, thus demonstrating the importance of the porosity of the biomaterial. Characteristics such as pores, fissures and structural

defects increase the surface area and provide an accommodation for microorganisms, supporting biofilm formation (8, 15).

Antifungals

Fungi are eukaryotic organism and, as being evolutionarily close to humans, there are limited numbers of targets which can be exploited for antifungal drug development. The first agent with antifungal activity, griseofulvin, was isolated in 1939, whereas the first azole and polyene antifungal agents were reported in 1944 and 1949, respectively. Although their discovery in the 1940-50, it was only in the sixties that oral griseofulvin became available for clinical use (16).

Serious fungal infections increased during the 1980s and 1990s with the increased use of immunosuppressive agents and due to the increased number of immunocompromised or severely ill individuals. The most common reason to immunosupression was the spread of human immunodeficiency virus (HIV) infection, followed by organ transplants, chemotherapy, and improved life-saving medical techniques necessitating indwelling devices (17).

The consequent growing need for antifungal agents brought new drugs to the market and led during the1990s to new lipid formulations of amphotericin B with improved safety profiles (18). In addition, new classes of antifungal agents such as the echinocandins were studied (18, 19).

Antifungals can be classified into different classes according to their mode of action. These classes comprise polyenes, acting on membrane function, allylamines and azoles, targeting specifically the biosynthesis of the major component of the fungal cell membrane, ergosterol (equivalent to cholesterol in mammalian cells) (Figure 1). Other classes of antifungals act on the biosynthesis of cell wall, such as the echinocandins or pradimicin, on the biosynthesis of nucleic acids, such as 5-fluorocytosine, or they inhibits the activity of mitochondrial function, such as the histatins (16, 17,

20). Nowadays, the main classes of antifungals therapeutically used are polyenes, azoles and echinocandins.

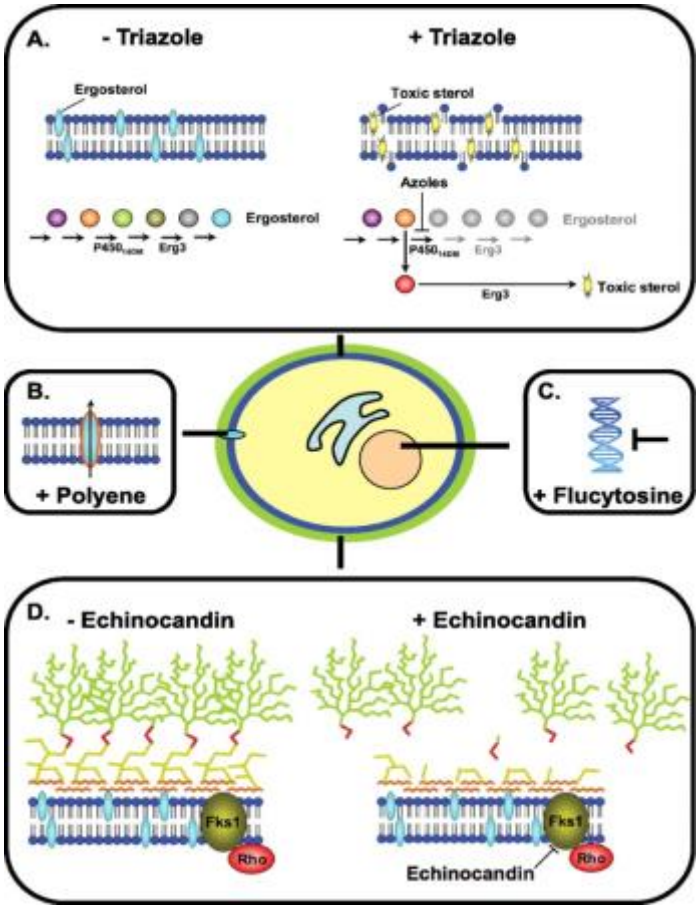

Figure1. Overview of the mechanisms of action of the antifungal classes, azoles (A), polyenes (B), flucytosine (C) and the echinocandins (D), used for treatment of *Candida* infections. From (20)

Polyenes

Since the discovery of the polyenes and their introduction in the 1950-60s, amphotericin B remains the "gold standard" of antifungal therapy. Amphotericin B binds ergosterol, the principal sterol in the fungal membrane, creating pores that compromise membrane integrity causing leakage of cellular components and death (21).

Nevertheless, the efficacy of polyenes is limited by their intrinsic toxicity. Therefore, lipid formulations of amphotericin B have subsequently been developed.

Azoles

Azoles, such as fluconazole, were developed in the 1980s. Azoles are synthetic fungistatic drugs, inhibiting the biosynthesis of ergosterol. They target the cytochrome P 450 14-α-lanosterol demethylase (encoded by ERG11 or CYP51) thus inhibiting its enzymatic activity. This enzyme also plays an important role in the cholesterol synthesis in mammals. Another target of azoles recently described is the enzyme Δ^{22}-desaturase, which is a cytochrome P-450 involved in the last step of the biosynthesis of ergosterol. Azoles cause depletion of ergosterol, leading to accumulation of 14a- methylated sterols into the fungal cell, as well as the disruption of the structure of the membrane and several of its functions, such as nutrient transport and chitin synthesis. These antifungals are the most important agents used therapeutically in clinic due to their moderate toxic effects (16). Although the improved safety profile compared to amphotericin B, the major problem with azoles is the emergence of resistance given by the development of point mutation on the target enzyme as well as the over-expression of genes encoding efflux pumps (22-25).

Resistance to azoles

The appearance of resistance to antifungal drugs led to failure of treatment and persistence of fungal infection despite an appropriate antifungal drug therapy. While resistance to polyenes, such amphotericin B, has been rarely reported in yeast, resistance to azoles has been extensively reported since 1980. The fungistatic effect of azoles and their repeated use led to the increased appearance of resistance to these agents. As a consequence of the increasing number of HIV patients infected by *Candida,* the use of the corresponding agent has also been more frequent. The resistance to

azoles has been explained by three major reported mechanisms: 1. drug efflux transporters, 2. ergosterol biosynthesis pathway modifications and 3.the mediation by Erg11p gene.

1. A large number of *Candida* clinical azole-resistance strains, especially *C. albicans* and *C. glabrata*, showed increased azole efflux which has been correlated to the upregulation of multidrug efflux transporter genes from two distinct families In the ATP binding cassette (ABC) transporters family (*CDR1*, *CDR2* and CSNQ2), ATP is used as driving force for drug efflux, and in the Major Facilitator Superfamily (MFS), a proton gradient is used for drug efflux. Deletion of *CDR1* in *C. albicans* leads to a hypersusceptibility to azoles and an accumulation of fluconazole (26, 27).

2. Some azole-resistant strains showed to have specific alterations in the ergosterol biosynthesis pathway, often resulting in the absence of ergosterol. Some *C. albicans* resistant strains have been found to accumulate 14α-methyl-3,6-diol, which indicates a defect in the enzyme sterol $\Delta^{5,6}$ desaturase encoded by the gene ERG3 (28).

3. The final major mechanism is linked to the overproduction or Erg11p. Upregulation of *ERG11* plays a moderate role in *C. albicans* azole resistance, however, in clinical azole-resistant isolates three specific regions of Erg11p have been reported with amino acid substitutions close to the cytochrome P-450 active site (29).

Echinocandins

Echinocandins are the last new class of antifungal introduced to the market (i.e. caspofungin, micafungin and anidulafungin). This antifungal class has the particularity to be the first that acts against a specific component of the fungal organisms not present in mammalian cells, the cell wall, thus avoiding direct human cell toxicity (30). The echinocandins exhibit fungicidal activity against *Candida* spp., including azole-resistant species, but fungistatic activity against *Aspergillus* spp.

The echinocandins are non-competitive inhibitors of 1,3-β-D-glucan synthase (and, to a lesser extent, 1,6-β-D-glucan synthase), an enzyme complex within the fungal cell wall comprised of at least two subunits: Fks1p (encoded by the genes FKS1, FKS2 and FKS3) and Rho1p. FKS1 transcription is linked to cell wall remodelling in fungi and FKS2 transcription is calcineurin dependent. Rho1p is a key regulatory protein and because of his interaction with multiple proteins, Rho1p is thought to be a key switch driving or arresting the synthesis of 1,3-D-glucan. Specifically, the echinocandins target the FKS1 gene product with Fks1p being the active site of the enzyme, although the precise echinocandin binding site remains unresolved. Fks1p inhibition is concentration dependent. Since 1,3-β-D-glucan is an integral component of the fungal cell wall, changes in its characteristics compromise osmotic stability resulting in cell lysis (31-33).

Clinical relevance of antifungal susceptibility testing

The main objective of all *in vitro* antimicrobial susceptibility testing, antibacterial, antiviral as well as antifungals, is to predict the outcome of the clinical administration of a tested agent on the organism causing the infection. As antibacterial testing, antifungal susceptibility testing aim to provide 1) an estimation of the activity of single or combined antimicrobial agents against the pathogen of interest; 2) a good correlation with the *in vivo* activity; 3) to detect the development of resistance; and 4) to test new antimicrobials and asses their spectrum of activity.

The currently available international standard methods for susceptibility determination of yeast to antifungals agents have been developed by the Clinical Laboratory Standards Institute (CLSI) (34) and by the Antifungal Susceptibility Testing Subcommittee of EUCAST (EUCAST-AFST) (35). A microbroth dilution assay is recommended by both guidelines for the determination of the minimal inhibitory concentration (MIC). Furthermore, the two guidelines differ in the inoculum

size (0.5-2.5 x 10^3 vs 0.5-2.5 x 10^5 CFU/ml), the test culture media used (RPMI 1640 with 0.2% vs 2% of glucose), the microtitration plates (U-shaped vs flat-bottom wells) and in the method of reading (visually versus photometrically). Moreover, the MIC is defined as the lowest concentration inhibiting the growth with amphotericin B by 100% vs 90%, with azoles and echinocandins by 50% for both (36).

Several other commercial test assays have been developed to facilitate the antifungal susceptibility testing. E-test and disk diffusion testing have been developed for yeast and moulds producing easy-to-read and sharp zones of inhibition (37, 38). Sensititre Yeast One is a colorimetric antifungal panel including Alamar-blue that converts into pink in the presence of growth and it is easy to interpret. Compared to the reference method (CLSI), Sensititre Yeast One has showed good agreement (39).

Mostly, free-living cells (planktonic) in pure culture are used to assess the MIC. However, different factors have been identified that can affect the results of *in vitro* tests with antifungal agents. For example, the method used for the endpoint determination is complicated for a number of antifungals, especially the azoles, because of the often reported trailing phenomenon. In this case the fungi will initially growth even with high concentrations of the antifungal agent. Furthermore, planktonic results are rarely useful when biofilm is present. Regarding the evaluation of biofilm susceptibility a colorimetric cell proliferation assay (XTT) using reduction of tetrazolium salt has been established for quantitative analysis of biofilm growth (40), however, the validity and reproducibility of this method has been questioned (40, 41). Therefore, other quantitative methods, such as the ATP bioluminescence assay or the incorporation of an isotope have been investigated (36), but none is universally accepted as the reference method for evaluating susceptibility of *Candida* biofilms. Furthermore, most of the models for the formation of microbial biofilm, are cumbersome, time-consuming, labor-intensive and need experienced personnel for interpretation.

These models include the use of catheter disks, plastic slides, cylindrical cellulose filters, modified devices, the Calgary biofilm device, etc. under static or dynamic conditions (42-46).

An early detection of infections caused by antifungal specific resistant pathogens optimises the choice of treatment and the patient outcome. However, it is difficult to correlate *in vitro* susceptibility testing results with the human treatment outcome of an infection. The *in vitro* test does not take into account the dynamics of an *in vivo* infection. Factors such as the host immune response, drug pharmacokinetics and pharmacodynamics, drug interactions as well as the interaction with the host proteins are neglected. All this parameters can also influence the outcome of the treatment of a specific infection (47, 48). The accuracy between the *in vitro* and *in vivo* data has been summarized as the "90-60 rule": "*infections due to isolates that are susceptible to the agent being given respond to therapy approximately 90% of the time, whereas infections due to isolates that are resistant to the agent being given respond approximately 60% of the time*" (47). Great efforts concerning standardisation have been made by both organisations, the CLSI and the EUCAST, establishing clinical breakpoints for *in vitro* antimicrobial susceptibility testing indicating the likely response to a given antimicrobial agent using the approved dosing regimen for that agent. Epidemiological cutoff values have also been established as a sensitive marker for the emergence of decreased susceptibility to the given agent discriminating non-wild type strains (with mutational or acquired resistance mechanisms) from wild-type. Nevertheless, given the differences between the two methods, breakpoints suggested by CLSI cannot be extrapolated to the EUCAST method and vice versa. Antifungal susceptibility testing can aid in the selection and optimization of antifungal treatment and due to the low number of antifungals on the market, having a good and rapid antifungal susceptibility testing is extremely important for surveillance of the emergence of resistance.

Calorimetry in microbiology

Isothermal microcalorimetry is a highly sensitive non-invasive and non-destructive technique, measuring heat produced by microorganisms in the range of microwatt. The release of heat by microorganisms is proportional to their metabolism and growth rate and can be recorder in real time and plotted as heat flow (Watt) versus time. Measurements are performed at constant pressure and temperature as evocated by the name "isothermal". The slope of the heat flow curve at each time point depends on the replication rate of the cells, while the area under the heat flow curve correspond to the total heat (Joule), which is proportional to the final number of cells (Figure 2) (49).

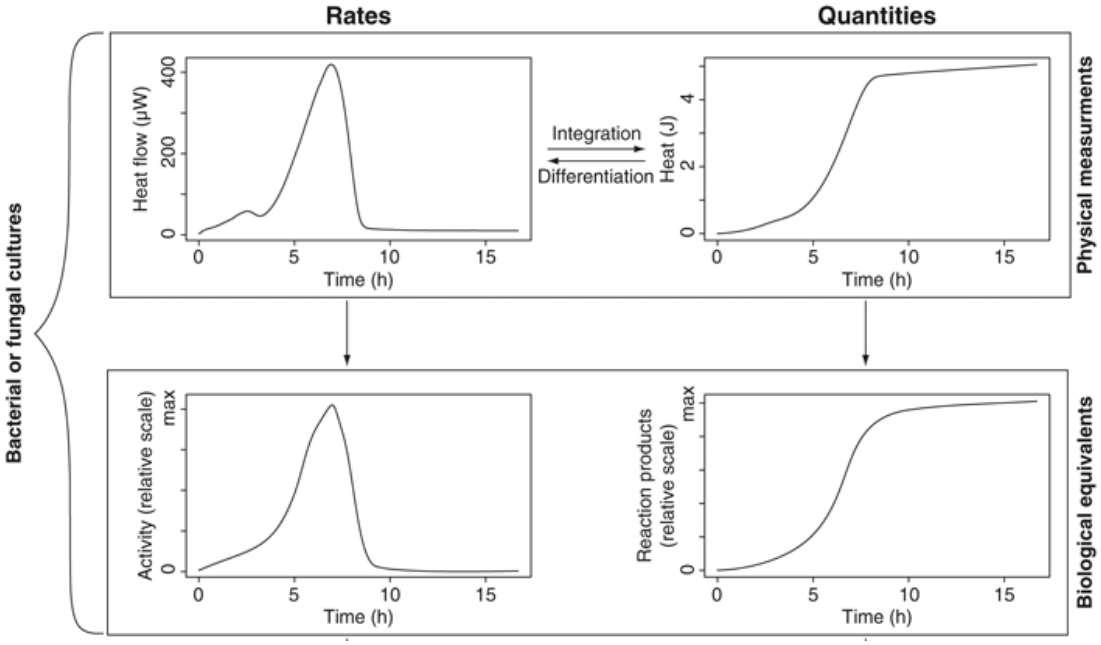

Figure 2. Relation between the microcalorimetric measurements and their biological equivalents. The heat flow represents the activity (growth rate) of a microbial culture. The area under the heat-flow curve gives the total amount of heat produced, representing the final product resulting from microbial activity (total number of cell). Adapted from (49).

Due to its high sensitivity and precision, the multi-channel batch calorimeters of heat-conduction type are the mostly commonly reported for clinical microbiology. The Thermal Activity Monitor

(TAM 48, TA Instruments, New Castle, DE, USA), shown in figure 3 is an example of a batch isothermal thermophile calorimeter allowing parallel measurements of different samples in 48 independent channels with a detection sensitivity on the order of 0.2 µW. Considering that a single bacteria cell produces ~2 pW (1 pW for fungi) when active, only 10^5 bacteria are required to produce a detectable signal. A glass ampoule can contain 1-4 mL of liquid enabling the detectable concentration of active microorganisms between 2.5 x 10^4 and 1.0 x 10^5 bacteria/ml. This cell concentration would not be detectable using a spectrophotometer (i.e. measuring the turbidity at 600nm) (50, 51).

The calorimeter provides a continuous real-time electronic signal proportional to the temperature differential measure between the sample (microorganisms culture) and the heat sink reference, generally made of aluminium, within a well-defined temperature (see figure 3).

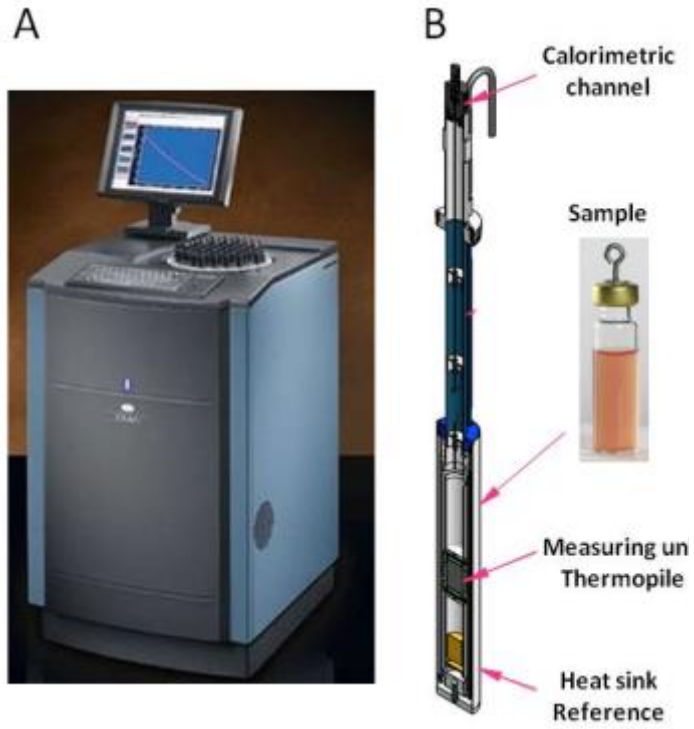

Figure3.

A. The isothermal micrcocalorimeter TAM 48 (TA Instruments) with 48 independent channels.

B. A schematic picture of a calorimetric channel including the heat sink (inert reference) and the sample (a 4 ml glass ampoule hermetically closed). The thermopile measures the difference in heat between the sample and the reference under a constant temperature.

The heat between the sample and the heat sink is transferred through a thermopile consisting of coupled Peltier elements. The Peltiers elements act as thermoelectric generators and convert the temperature difference between the sample and the reference into a voltage signal that is proportional to the heat.

To increase the sensitivity and accuracy, most of the current isothermal microcalorimeters are "twin instruments". The reaction vessel, including the sample and the heat sink, is inserted into a precise liquid thermostat (water or oil), adjustable between 15–150°C, ensuring a temperature stability of 10^{-5} °C. The thermostat temperature in a microbiological setting is normally set at 37°C.

Calorimetry samples are easy to prepare and they do not need specific preparation. Samples are placed in sealed glass ampoules and inserted in one of the measuring channels. Heat production is monitored and recorded as long as there is a heat flow signal (from hours to days). After the heat measurement, samples can also be used for further analysis of interest. Nevertheless, isothermal microcalorimetry has a major drawback which is the non-specificity of the heat-flow signal related to the sum of all chemical and physical processes taking place into the ampoule. Simultaneous exothermic and endothermic processes are unspecifically recorded. Furthermore, since the samples are placed in sealed ampoules, chemical factors such as oxygen depletion and accumulation of metabolic waste products have to be take into account during the interpretation of the results (49). The potential of isothermal microcalorimetry is in the fast detection of microbial infections or contamination, which is of critical importance for the clinical diagnosis and administration of the appropriate treatment. Several studies have shown the potential of the isothermal microcalorimetry to detect growth of different pathogens within a few hours, as, for example, the contamination of donated blood platelets with *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Streptococcus sanguinis*, *Escherichia coli*, *Propionibacterium acnes* and *Candida albicans* (52), the urinary tract

infections (53) or mycobacterial (54). Since the microcalorimeter does not differentiate between growths of different microorganism, the use of selective growth media may allow the recovery and detection of specific microbes when present in the specimens.

It is also possible to determine the minimal inhibitory concentration (MIC) for different antimicrobial agents and microorganisms within hours (55). In the presence of an active antimicrobial agent at concentration above the MIC, susceptible microorganism will be inhibited leading to absence of heat production. Contrarily, resistant microorganism will not be inhibited and heat will be produced. This will allow rapid detection of resistant microorganisms. The use of isothermal microcalorimetry for the differentiation of methicillin-susceptible from methicillin-resistant *Staphylococcus aureus* within 5 h was reported (56), as well as determination of antimicrobial susceptibility of *Escherichia Coli*, *S. aureus*, *Aspergillus* spp. and non-*Aspergillus* molds (55, 57-59). In addition, isothermal miccrocalorimetry has proven to be an effective tool in detecting slow-growing bacteria, such as *Mycobacterium tuberculosis* which could be detected within hours using microcalorimetry (54). This is much faster than traditional methods that may need up to 60 days to detect growth of slow-growing mycobacteria. Anti-parasitic drugs have also being studied with calorimetry against *Trypanosoma brucei rhodesiense* and *Plasmodium falciparum* (60) as well as *Schistosoma mansoni* (61).

One of the main advantages of microcalorimetry is that it can accommodate any type of sample. For biomedical material or biofilm studies this means that a solid sample, with or without previously formed biofilm, can be introduced into the microcalorimeter ampoule. This allows, on one side, to monitor the effect of additives used to improve strength or other properties of bone cements. On the other side, in presence of microorganisms, it allows to study the biofilm adherence on a determined substrate (15).

Although several studies have been performed to demonstrate the potential and benefits for clinical applications, calorimetry methods are not yet fully integrated into the microbiologic routine procedures. The current cost of multichannel calorimeters is, for instance, too high for cost-efficient clinical laboratory tests. However, the non-invasive and non-destructive character of calorimetry, as well as the simplicity in sample preparation, qualifies the isothermal multichannel calorimetry as a valuable tool for the evaluation of antimicrobial inhibitory profiles, including the evaluation of biofilm growth on surfaces (50).

Prosthetic joint infections

The increasing use of implanted devices consequently leads to an increase of implant-associated infections (62). The risk of infectious complications after joint replacement is estimated to be 1-2%. In patients with primary joint replacement, the infection rate during the first 2 years is less than 1% in hip and shoulder prosthesis, less than 2% in knee prosthesis and less than 9% in elbow prosthesis. Prosthetic joint infections (PJIs) are difficult to treat and are associated with morbidity and elevated health care cost (2, 3). Implant-associated infections are typically caused by microorganisms growing in biofilms structures. Eradication of the embedded biofilm requires intensive antimicrobial therapy. Staphylococci are the most frequent infective agents, followed by streptococci, enterococci, gram-negative bacilli and *Propionibacterium acnes*. However, less prevalent organisms, such as fungi, are currently considered a difficult to treat organism, and the optimal eradication treatment is not yet defined. Prosthetic infections caused by fungal agents are considered to be rare, representing 1% of orthopaedic infections, and the optimal antifungal treatment against *C. albicans* biofilm has not been defined. In the biofilm form, fungi are up to 1000-fold more resistant to antimicrobials than in their planktonic form (63). The most frequent

fungal agent is *C. albicans*, followed by *C. parapsilosis* and *C. glabrata*. Although fungal prosthetic joint infections are rare, they represent a diagnostic and therapeutic challenge.

In contrast to bacterial infections, which can be locally treated by insertion of an antibiotic-loaded spacer, data on efficacy of local antifungal therapy is missing. No clear guidelines exist for the surgical management of fungal periprosthetic infections (64). Most patients with chronic infection following joint replacement require removal of the device and the standard treatment is a 2 stage-revision with the use of a temporary spacer impregnated with antibiotics and antifungals allowing elution of the drugs directly into the infected surrounding tissue and maintaining the patient's mobility between stages (65). Amphotericin B and fluconazole are the drug of choice for systemic treatments, nevertheless, in vitro studies of amphotericin B and fluconazole loaded cement have shown only poor elution characteristics for both agents (66, 67). Only a single in vivo study showed the efficacy of fluconazole impregnated cement beads in 2 cases in the treatment of periprosthetic hip infections (68).

The exact mechanism of *Candida* prosthetic joint infection is not elucidated. As for bacterial prosthetic infections, 3 possible modes of infections have been described (69):

1) The haematogenous route is the most frequent mode of infection by *Candida*. In this case the infection originates from microbes coming from a distant infection focus, such as from cutaneous infections, soft tissues infections, oral cavity, or infections of the urogenital or the respiratory tract, or even from infected catheters. Since the manifestations of the symptoms of such infections occur 24 months after surgery, they are classified as "late infection". Late infections are mainly caused by highly virulent microorganisms and present all symptoms of acute infections (70, 71).

2) Direct inoculation after prosthesis implantation into a joint previously infected, or inoculation from the skin microflora at the time of implantation (perioperative).

3) Extension into the synovial space from adjacent infected tissues.

The two last modes of infection constitute the most common cause of implant infection. The delay of the first symptoms occurs, in those cases, either within 3 months or between 3 and 24 months after surgery and such infections have been classified as "early" and "delayed" prosthetic infection respectively. In early prosthesis infections the microorganisms involved are generally highly virulent pathogens such as *S. aureus* and gram-negative bacilli. In the delayed infection, low virulent pathogens such as coagulase-negative staphylococci, including *S. epidermidis* or *Propionibacterium acnes* are implied. In this case the infection usually becomes symptomatic later than 3months after surgery (11, 72-74).

The main problem with implants is that foreign surfaces represent good substrates for microbial adherence and biofilm formation. Elek and Conen showed that in the vicinity of foreign material 100 colony forming unit (CFU) of *S. aureus* was sufficient to induce an abscess, which is more than 100'000-fold lower than in the absence of a foreign device (75). This observations were confirmed by Zimmerli et al. in an animal model of foreign body-associated infection, where 100 CFU were sufficient to infect 95% of the subcutaneous cages (simulating the implants), whereas $>10^7$ CFU of *S. aureus* could not produce any abscess in the absence of a foreign-body device (76, 77).

Animal model of implant-associated infections

The key advantage of studying biofilm infections using animal models is the presence of a physiological environment. Moreover, *in vivo* studies are particularly important for evaluation of the pharmacokinetics and pharmacodynamics of antimicrobial agents since different factors may

influence the interaction between the drug and the microorganism or its efficacy (77). Depending on the problem under study, different models of foreign-body infection have been used to solve the multiples problems associated with the foreign-body device (58, 78-81). Currently, animal models have been described based on catheter-associated urinary tract infections or infections of different materials implanted subcutaneously or intraperitoneally on mice, rats or guinea pigs. Nevertheless, the major disadvantage is linked to the high costs of the experimental animal and the laborious and sophisticated surgical technique required. An animal model for the study of the pathogenesis, the management and prevention of a device-related infection needs to reproduce the same characteristics of the human infection, such as not showing spontaneous healing, if it intends to be clinically relevant. In the case of a human device-associated infection, the model which best reproduces the clinical characteristics is the tissue-cage infection model using guinea pig developed by Zimmerli et al. (76, 82, 83). The guinea-pig tissue-cage infection model has the characteristics of being very similar to human device-associated infection.

Foreign-body infections are convenient to study by inserting an implant which is consequently infected (locally or hematogenously). After a preferred time of infection, the device can be explanted and the presence of biofilm evaluated. Different methods are used to evaluate the presence of biofilm, such as examination by confocal scanning microscopy of microorganism cells and the extracellular matrix stained with specific dyes, or examination by scanning electron microscopy.

The tissue cage infection model

Male albino guinea pigs (Charles River, Sulzfeld, Germany) were kept in the Animal Facility of the University of Lausanne, Switzerland. The experiments were performed according to the regulations of Swiss veterinary law. Guinea pigs were weighted every week to ensure their well-

being. Short-term studies (7-day infection) were performed in a foreign-body cage model with guinea pigs to evaluate the infection profile. Animals were anesthetized with a subcutaneous injection of ketamine and xylazine. Four sterile polytetrafluorethylene (Teflon) cages with 130 regularly spaced perforations of 1 mm diameter (Angst-Pfister AG, Zürich, Switzerland) were subcutaneously implanted in flanks of the guinea pigs (450 – 550 g) under aseptic conditions, allowing accumulation of inflammatory fluid, *Candida* inoculation and pharmacokinetic studies. After complete wound healing (approximately two weeks after implantation), sterility of the cages was confirmed by culturing aspirated cage fluid on blood agar plates and cages were infected by injection of a well-defined *Candida* inoculum. In general, the minimal infective dose needed to achieve a stable infection of the cage in guinea pigs is in the range of 10^2-10^3 CFU for staphylococci and of 10^4-10^6 with *C. albicans*. In rat or mouse models, the minimal infective dose is higher and immunosuppression may be needed to prevent spontaneous healing of the infection (84, 85). Contaminated cages were excluded from further studies. Sterile cages were used for the pharmacokinetic studies by aspirating the tissue-cage fluid after intraperitoneal injection of different doses of the drug. Fungal inoculation was performed only in initially sterile cages.

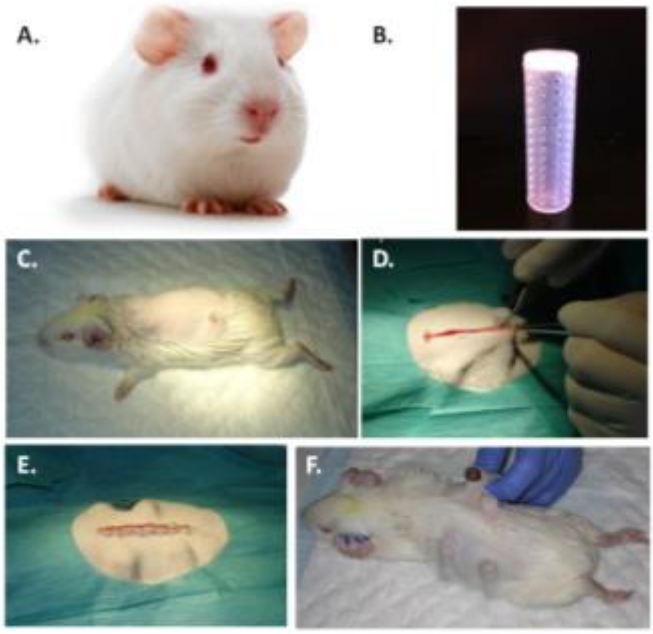

Figure 4. Guinea-pig foreign-body implantation and explantation.

A. Dunkin-Hartley Guinea pig.

B. Tissue-cage (Teflon cylinders).

C. Ventral position of the guinea pig before implantation.

D. Subcutaneous insertion of the cages.

E. Closing wound.

F. Explantation of cages at the end of the experiment

For evaluation of the treatment efficacy the tissue-cages are explanted after the end of therapy (Figure 4, F) and the cure rate is determined by dividing the number of culture-negative cages by the total number of cages in the treatment group. Additionally, the antifungal activity against planktonic *Candida* present in the fluid within the cage can be evaluated by aspirating cage fluid before, during and after treatment.

Different treatment regimens for biofilm infections have been evaluated using tissue-cage infection models. When antibiotic are used, guinea pigs are limited by their intolerance to β-lactam and clindamycin, which both cause lethal diarrhea. Furthermore, guinea pigs only support short-term therapy up to 4 days. Rats are more suited for studying chronic infections and long-term therapy, but the infective dose needs to be increased to avoid spontaneous healing (85).

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Chapter2. AIM OF THE STUDY

In this study, we investigated the potential of isothermal microcalorimetry for determination of planktonic and biofilm susceptibility testing of the most medically important *Candida* species. The microcalorimetry results were compared with conventional microbroth dilution testing and the XTT testing. A rapid and accurate reproducible susceptibility testing method may help guiding the choice of an optimal antifungal treatment improving the clinical outcome. Based on *in* vitro results the activity of the same antifungals was tested *in* vivo on planktonic and biofilm *C. albicans* in a foreign-body infection model using guinea pigs.

The aim of the first part (chapter 3) was to investigate the activity of fluconazole, caspofungin, anidulafungin and amphotericin B against planktonic and biofilm *Candida* spp *in vitro*.

The aim of the second part (chapter 4) was to investigate the same antifungals *in vivo*.
First, we established an infection profile for different inocula of *C. albicans*. Second, we studied the pharmacokinetics and the pharmacodynamics of different antifungals and third, we investigated the activity of antifungals against planktonic and adherent *C. albicans in vivo*. To our knowledge, this is the first description of *C. albicans* infection and treatment in this model.

Chapter 3

Activity of fluconazole, caspofungin, anidulafungin and amphotericin B on planktonic and biofilm *Candida* determined by microcalorimetry

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Antimicrobial Agent and Chemotherapy, March 2014

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Editor: Arikan-Akdagli | Section: Susceptibility | Designation: T

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Activities of Fluconazole, Caspofungin, Anidulafungin, and Amphotericin B on Planktonic and Biofilm *Candida* Species Determined by Microcalorimetry

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We investigated the activities of fluconazole, caspofungin, anidulafungin, and amphotericin B against *Candida* species in planktonic form and biofilms using a highly sensitive assay measuring growth-related heat production (microcalorimetry). *C. albicans*, *C. glabrata*, *C. krusei*, and *C. parapsilosis* were tested, and MICs were determined by the broth microdilution method. The antifungal activities were determined by isothermal microcalorimetry at 37°C in RPMI 1640. For planktonic *Candida*, heat flow was measured in the presence of antifungal dilutions for 24 h. *Candida* biofilm was formed on porous glass beads for 24 h and exposed to serial dilutions of antifungals for 24 h, and heat flow was measured for 48 h. The minimum heat inhibitory concentration (MHIC) was defined as the lowest antifungal concentration reducing the heat flow peak by ≥50% (≥90% for amphotericin B) at 24 h for planktonic *Candida* and at 48 h for *Candida* biofilms. Fluconazole (MICs, 0.25 to >512 μg/ml) and amphotericin B (MICs, 0.25 to 1 μg/ml) showed higher MICs than anidulafungin (MICs, 0.03 to 0.5 μg/ml) and caspofungin (MICs, ≤0.125 to 0.5 μg/ml). Against *Candida* species in biofilms, fluconazole's activities were reduced by >1,000-fold compared to its activities against the planktonic counterparts, whereas echinocandins and amphotericin B mainly preserved their activities. Fluconazole induced growth of planktonic *C. krusei* at sub-MICs. At high concentrations of caspofungin (>8 μg/ml), paradoxical growth of planktonic *C. albicans* and *C. glabrata* was observed. Microcalorimetry enabled real-time evaluation of antifungal activities against planktonic and biofilm *Candida* organisms. It can be used in the future to evaluate new antifungals and study resistant strains and antifungal combinations.

Candida species are the fourth most common cause of nosocomial infections (1, 2). Risk factors for invasive *Candida* infections include immunosuppressive treatment, the use of broad-range antibiotics suppressing normal bacterial flora, and the presence of indwelling devices. In recent decades, other risk factors for fungal infections have emerged, including the implantation of foreign bodies like vascular catheters, cardiac pacemakers, neurosurgical shunts, prosthetic heart valves, and orthopedic devices (3). Similar to bacteria, fungi can attach to the implant surface and form a biofilm, causing persistent and relapsing infections (4). *Candida* is implicated in 1 to 5% of implant-associated infections, and these infections are difficult to treat, usually requiring removal of the implant and prolonged antifungal treatment (5–7).

Accurate determination of *Candida* biofilm antifungal susceptibilities and antifungal activities against them is key to successful treatment of retained implants. The conditions for antifungal activity testing against planktonic *Candida* are defined by the Clinical and Laboratory Standards Institute (CLSI) (8) and the Antifungal Susceptibility Testing Subcommittee of EUCAST (9). Both guidelines recommend using the broth microdilution assay for determination of MICs. In contrast to the guidelines for planktonic organisms, only a few assays exist for susceptibility testing of *Candida* biofilms (10), and the relevance of these results and their correlation with therapeutic success are uncertain.

Several studies have found that *Candida* organisms in biofilms demonstrated higher resistance to antifungals than their planktonic counterparts. Amphotericin B and azoles had 30- to 2,000-times-higher MICs for biofilms than for planktonic *Candida* (11). Other researchers have investigated the activities of antifungal drugs against *Candida* biofilms, including ketoconazole, itraconazole, flucytosine, fluconazole (12–14), amphotericin B (15), and echinocandins (16–19). For assessing the antifungal susceptibilities of biofilm *Candida*, microtiter plate-based assays were used (20). A colorimetric cell proliferation assay using reduction of tetrazolium salt [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrozolium-5-carboxanilide (XTT) assay] was used for quantitative analysis of biofilm growth (21); however, the validity and reproducibility of this method was questioned by others (22). Therefore, other quantitative methods, such as the ATP bioluminescence assay or the incorporation of an isotope, have been investigated (23), but none is universally accepted as the reference method for evaluating antifungal susceptibilities of *Candida* biofilms.

Microcalorimetry enables precise measurement of microbial heat production related to growth and metabolism. Isothermal microcalorimetry refers to heat measurement under a constant temperature, which has high sensitivity (in the microwatt range) and allows real-time monitoring of heat production (24). In several studies, microcalorimetry was used to study the metabolic activities and drug susceptibilities of bacteria, mycobacteria, pro-

Received 13 January 2014 Returned for modification 8 February 2014
Accepted 19 February 2014
Published ahead of print 24 February 2014
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doi:10.1128/AAC.00057-14

tozoa, schistosomas, and fungi (yeasts and molds) (22, 25–32). In appropriate growth media, *Candida* organisms produce heat similarly to bacteria, and calorimetry seems to be appropriate to study their metabolic activity. The antifungal activities of two drugs, coptisine and berberine, against *C. albicans* have been studied with a flow microcalorimeter (LKB-2277 bioactivity monitor) (33, 34). However, to our knowledge, the antifungal susceptibilities of other *Candida* species and *Candida* biofilms have not been investigated by microcalorimetry.

In this study, we investigated antifungal activities against planktonic and biofilm *Candida* by isothermal microcalorimetry assay. Furthermore, we compared the susceptibility results determined by microcalorimetry with the results of conventional *in vitro* testing assays and evaluated the potential of microcalorimetry for rapid, accurate, and real-time antifungal susceptibility testing.

MATERIALS AND METHODS

Test strains. *Candida albicans* (ATCC 90028), *Candida glabrata* (DSY 562), *Candida krusei* (ATCC 6258), and *Candida parapsilosis* (ATCC 22019) were used. Stocks of each strain were maintained in cryovials (Roth, Karlsruhe, Germany) at −80°C. *Candida* was cultured on Sabouraud dextrose agar (SAB) for 24 h at 37°C. Inocula were prepared according to a McFarland standard, and the exact inocula were determined by quantitative cultures.

Antifungal agents. Fluconazole was obtained in liquid form (2,000 μg/ml; Teva Pharma AG, Aesch, Switzerland). Amphotericin B (Sigma, St. Louis, MO) and caspofungin (Merck & Co., Inc., Whitehouse station, NJ) were obtained in powder form and dissolved in sterile water. Anidulafungin was kindly provided by Pfizer Pharma AG (Ecalta, Zurich, Switzerland) in powder form and dissolved according to the manufacturer's instructions.

Conventional antifungal susceptibility testing by broth microdilution. The antifungal susceptibilities of planktonic *Candida* organisms were determined by the broth microdilution method according to the EUCAST guidelines (9). In brief, a *Candida* density of 0.5 McFarland, corresponding to 1×10^6 to 5×10^6 CFU/ml, was used to prepare the final concentration of 1×10^5 to 5×10^5 CFU/ml in RPMI 1640. Aliquots of 100 μl of serial 2-fold dilutions of each antifungal were dispensed into microtiter plate wells, followed by the inoculation of 100 μl of fungal suspension. The plate was read by spectrophotometry (at 530 nm) after 24 h of incubation at 37°C. The MIC was defined as the lowest antifungal concentration inhibiting ≥50% of growth, except for amphotericin B, whose MIC was defined as growth inhibition of ≥90%. Experiments were performed in triplicates.

Determination of antifungal susceptibilities of biofilm *Candida* by XTT assay. The antifungal susceptibilities of biofilm *Candida* were determined using the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrozolium-5-carboxanilide (XTT) absorbance assay (10, 35). Three to five colonies of *Candida* organisms were inoculated into 10 ml of yeast extract-peptone-dextrose (YPD) in a sterile 50-ml polypropylene tube and incubated statically at 37°C for 24 h. The cells were then centrifuged at 2,500 × *g* for 5 min at 4°C, the supernatant was discarded, and the cell pellet was resuspended in 10 ml of fresh phosphate-buffered saline (PBS). This step was repeated thrice to remove excess growth medium. The cell pellet was resuspended in 10 ml of PBS, and the cells were counted through a hemacytometer. The final concentration was 10^7cells/ml after 24 h. The cells were diluted in 10 ml of RPMI 1640, and an amount of 100 μl was transferred into each well of a 96-well plate. The plates were incubated at 37°C for 48 h without shaking.

In order to avoid disruption of biofilm, different concentrations of antifungal agents were prepared separately. The plates were carefully washed thrice with PBS, and 100-μl amounts of each antifungal concentration were transferred into the wells. Controls were prepared with medium only (no drug and no biofilm) and biofilm alone (no drug). The plates were incubated at 37°C for 24 h. After 24 h, the plates were carefully washed with PBS. To determine XTT activity, 100 ml of a 1 mg/ml solution of XTT-menadione was added to each well. The plates were covered with aluminum foil and incubated for 1 to 2 h at 37°C statically. Absorbance was measured at a wavelength of 450 nm using a spectrophotometer, and the percent inhibition of metabolic activity for each well containing drug was calculated in comparison with the metabolic activity of biofilms formed in the absence of any drug (growth control). The lowest concentration showing ≥50% reduction in metabolic activity (or ≥90% for amphotericin B) in comparison with the growth control was determined. Experiments were performed in quadruplicates.

Microcalorimetry assay. An isothermal microcalorimeter (TAM III; TA Instruments, Newcastle, DE) equipped with 48 calorimeters and a detection limit of heat production of 0.2 μW was used. The ampoules were sealed for air tightness and introduced into the microcalorimeter, first in the equilibration position and after 15 min in the measuring position. Heat flow was recorded for 48 h. The minimum heat inhibitory concentration (MHIC) was determined at 24 h for planktonic *Candida* and at 48 h for biofilm *Candida*. The MHIC was defined as the lowest concentration reducing the heat flow by ≥50% compared to that of the growth control, except for amphotericin B, where the reduction of heat flow by ≥90% was set as the MHIC (in analogy to the broth microdilution method according to EUCAST guidelines). Experiments were performed in triplicate.

Antifungal susceptibilities of planktonic *Candida* by microcalorimetry. A final cell concentration of 1×10^5 to 5×10^5 CFU/ml was prepared in 3 ml RPMI 1640 supplemented with *l*-glutamine (Sigma) buffered with 0.165 M MOPS (morpholinepropanesulfonic acid) and 2% glucose (pH 7, adjusted with 1 M NaOH). RPMI 1640 contained serial 2-fold dilutions of each antifungal. Growth medium with fungi but without antifungals was used as the positive (growth) control, and growth medium without fungi served as the negative (sterility) control. Experiments were performed in duplicates and quadruplicates for specific strains (*C. parapsilosis* and *C. glabrata*). AQ: A

Antifungal susceptibilities of biofilm *Candida* by microcalorimetry. *Candida* biofilms were formed on porous glass beads having a diameter of 2 to 4 mm, porosity of 0.2 m^2/g, and pore size of 60 μm (Siran carrier, SiKUG 023/02/300/A; Schott Schleiffer AG, Muttenz, Switzerland). The beads were incubated for 24 h at 37°C in RPMI 1640 with 2 or 3 colonies of the *Candida* species. The beads were then washed, incubated for 24 h in serial dilutions of antifungals at concentrations of 0.007 to 1,024 μg/ml, washed again, and placed into the microcalorimeter with fresh RPMI 1640 medium to quantify recovering yeasts. Experiments were performed in triplicates.

Data analysis. Microcalorimetry data analysis was accomplished using the manufacturer's software (TAM Assistant; TA Instruments, New Castle, DE). Figures were plotted using GraphPad Prism 6.01 (GraphPad Software, La Jolla, CA, USA).

RESULTS

Conventional antifungal susceptibility testing by broth microdilution. The MICs obtained by broth microdilution are summarized in Table 1. *C. albicans* showed the lowest MICs (0.03 μg/ml for anidulafungin to 1 μg/ml for amphotericin B), while *C. glabrata* showed the lowest MIC, 0.015 μg/ml for anidulafungin. The other strains showed higher MICs to fluconazole. In general, fluconazole (MICs, 0.25 to >512 μg/ml) and amphotericin B (MICs, 0.25 to 1 μg/ml) showed higher MICs than anidulafungin (MICs, 0.015 to 0.5 μg/ml) and caspofungin (MICs, 0.125 to 0.5 μg/ml). AQ: B T1

Antifungal susceptibility testing of biofilm *Candida* by XTT assay. The MICs obtained by XTT assay at 24 h are summarized in Table 2. Fluconazole showed no activity against *Candida* T2

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TABLE 1 Antifungal susceptibilities of planktonic and biofilm *Candida* determined by broth microdilution MIC and microcalorimetry MHIC values[a]

Antifungal agent	Test strain	24-h MIC (μg/ml)	MHIC (μg/ml) for: Planktonic *Candida* (24 h)	MHIC (μg/ml) for: Biofilm *Candida* (48 h)
Fluconazole	*C. albicans*	0.25	0.25	256
	C. glabrata	**4**	**>512**	>1,024
	C. krusei	32	64	>1,024
	C. parapsilosis	**2**	**>512**	>1,024
Caspofungin	*C. albicans*	0.25	0.125	2
	C. glabrata	0.06	0.125	16
	C. krusei	0.5	0.5	64
	C. parapsilosis	0.125	0.125	64
Anidulafungin	*C. albicans*	0.03	0.03	0.5
	C. glabrata	0.03	0.015	1
	C. krusei	0.06	0.03	32
	C. parapsilosis	1	0.5	2
Amphotericin B	*C. albicans*	1	0.5	64
	C. glabrata	0.25	1	8
	C. krusei	1	1	4
	C. parapsilosis	0.125	0.25	8

[a] MIC (broth microdilution) and MHIC (microcalorimetry) were defined as the lowest antifungal concentration inhibiting ≥50% of growth or reducing the heat flow curve, except for amphotericin B, for which they were defined as ≥90% growth inhibition or reduction of the heat flow curve. Values in boldface indicate discrepancies of >2-fold serial dilutions between MIC and MHIC for planktonic *Candida* spp. Broth microdilution was performed according to EUCAST guidelines (9).

TABLE 2 Antifungal susceptibilities of *Candida* biofilms determined by the XTT assay and by microcalorimetry at 24 h[a]

Antifungal agent	Test strain	MIC (μg/ml)	MHIC (μg/ml)
Fluconazole	*C. albicans*	**>1,024**	**256**
	C. glabrata	>1,024	>1,024
	C. krusei	>1,024	>1,024
	C. parapsilosis	>1,024	>1,024
Caspofungin	*C. albicans*	1	1
	C. glabrata	4	8
	C. krusei	4	8
	C. parapsilosis	64	64
Anidulafungin	*C. albicans*	0.125	0.25
	C. glabrata	0.5	1
	C. krusei	0.25	0.5
	C. parapsilosis	2	2
Amphotericin B	*C. albicans*	16	64
	C. glabrata	8	8
	C. krusei	8	2
	C. parapsilosis	8	8

[a] MIC (XTT assay) and MHIC (microcalorimetry) were defined as the lowest concentration of the antifungal inhibiting ≥50% of growth or reducing the heat flow curve, except for amphotericin B, for which they were defined as ≥90% growth inhibition or reduction of the heat flow curve. Values in boldface indicate discrepancies of more than 2-fold serial dilutions between XTT and microcalorimetry for biofilm *Candida* spp.

biofilms (MICs, >1,024 μg/ml). Caspofungin and amphotericin B were active against *Candida* biofilms (MICs of 4 to 64 μg/ml for caspofungin and 8 to 16 μg/ml for amphotericin B), whereas anidulafungin showed the best activities, with MICs of 0.125 to 2 μg/ml.

Antifungal susceptibility testing of planktonic and biofilm *Candida* organisms by microcalorimetry. The MHICs at 24 h for planktonic *Candida* organisms correlated well with conventional MICs, with 14 of 16 values (88%) in agreement within 2-fold serial dilutions (Table 1). The MHIC for biofilm *Candida* organisms at 24 h correlated well with the XTT assay results as well, with 15 of 16 values (94%) in agreement within 2-fold serial dilutions (Table 2).

Compared to planktonic *Candida* organisms, considerably higher concentrations of fluconazole were required to inhibit the growth of *Candida* biofilms in the microcalorimeter at 48 h. Caspofungin inhibited all planktonic *Candida* spp. at low concentrations (≤0.5 μg/ml). *C. albicans* and *C. glabrata* biofilms were inhibited by caspofungin at 2 and 16 μg/ml, whereas *C. krusei* and *C. parapsilosis* biofilms required higher concentrations (64 μg/ml). Anidulafungin inhibited all *Candida* biofilms at ≤32 μg/ml, whereas amphotericin B required concentrations of ≥4 μg/ml.

Characteristics of microcalorimetry curves of planktonic and biofilm *Candida* organisms. Figures 1 through 4 show representative heat flow curves for the planktonic and biofilm forms of the *Candida* spp. in the presence and absence of antifungals. The variability among triplicate measurements with the same *Candida* species and same antifungal was <10%.

Fluconazole inhibited the growth of planktonic *C. albicans* at a low concentration (MHIC, 0.25 μg/ml), and *C. krusei* was inhibited at 64 μg/ml, whereas *C. glabrata* and *C. parapsilosis* were not inhibited at up to 512 μg/ml (Fig. 1A). Fluconazole at sub-MICs induced growth of *C. krusei*, as indicated by the heat flow peak at 4 to 32 μg/ml being higher than in the growth control (without fluconazole). Fluconazole was active only on *C. albicans* biofilm (MHIC, 256 μg/ml), with no activity observed against *C. glabrata*, *C. krusei*, and *C. parapsilosis* biofilms up to a concentration of 1,024 μg/ml (Fig. 1B).

Caspofungin inhibited planktonic *C. albicans*, *C. glabrata*, and *C. parapsilosis* at 0.125 μg/ml, but paradoxical growth was observed at concentrations above the MICs of caspofungin (>4 μg/ml) (Fig. 2A). This phenomenon was not observed for planktonic *C. krusei* (MHIC, 0.5 μg/ml) and *C. parapsilosis*. Caspofungin exhibited good activity against all *Candida* species biofilms, with MHICs ranging from 2 to 64 μg/ml (Fig. 2B).

Anidulafungin inhibited planktonic *Candida* spp. at low concentrations (MHICs of 0.015 to 0.5 μg/ml), but growth of planktonic *C. albicans* was observed at an anidulafungin concentration of >4 μg/ml (paradoxical effect) (Fig. 3A). Against *Candida* biofilms, anidulafungin showed activities ranging from 0.5 μg/ml (for *C. albicans*) to 32 μg/ml (for *C. krusei*) (Fig. 3B).

Amphotericin B showed activities on planktonic *Candida* at 0.25 μg/ml (for *C. parapsilosis*), 0.5 μg/ml (for *C. albicans*), and 1 μg/ml (for *C. glabrata* and *C. krusei*). On biofilm *Candida* organisms, amphotericin B was active at 4 μg/ml against *C. krusei* and at 8 μg/ml against *C. glabrata* and *C. parapsilosis*. Against biofilm *C. albicans*, amphotericin B showed a delay in heat production (i.e., shift of the heat flow curve to the right) but no reduction of the heat flow peak up to a concentration of 64 μg/ml.

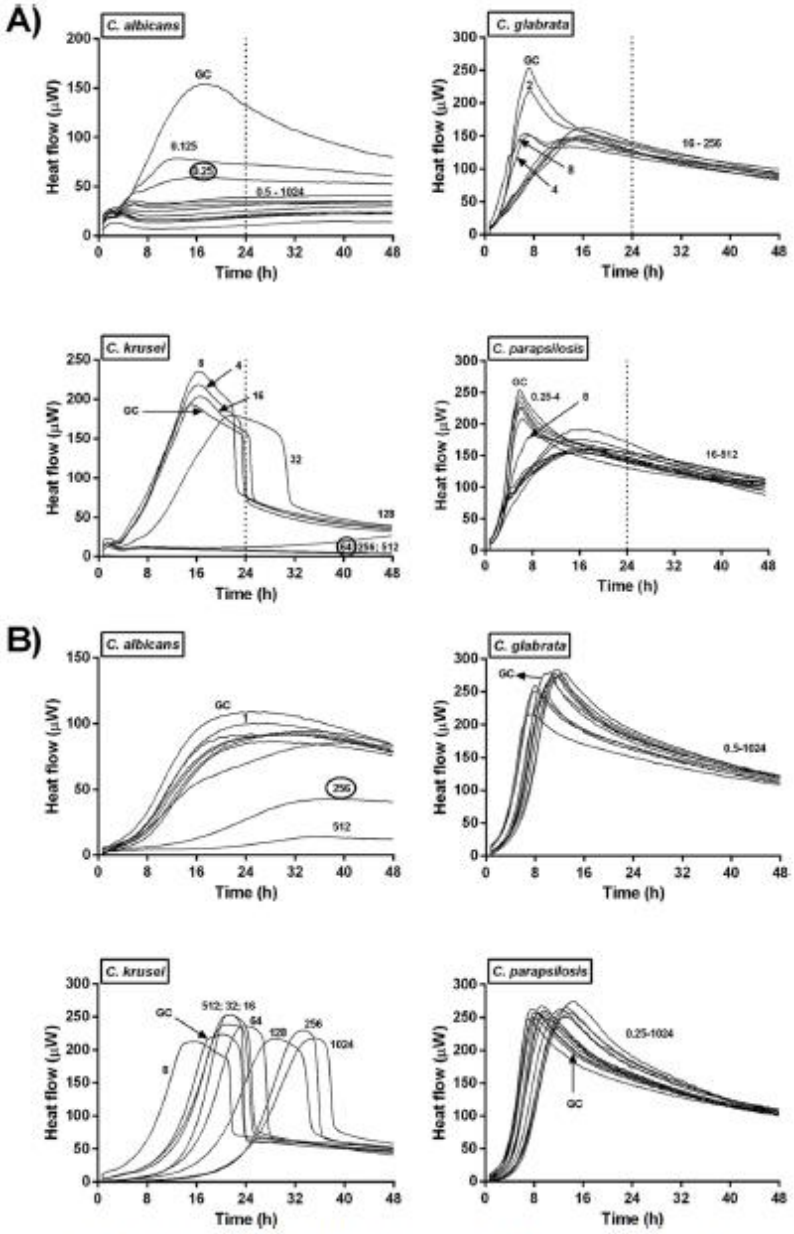

FIG 1 Heat flow curves generated by planktonic (A) and biofilm (B) *Candida* organisms in the presence of fluconazole. Numbers indicate the antifungal concentrations (in μg/ml). GC denotes growth control (without antifungal agent). The vertical dashed line indicates the endpoint of the assay for planktonic *Candida* at 24 h; the endpoint for the biofilm Candida is at 48 h. The circled value denotes the minimum heat inhibitory concentration (MHIC).

DISCUSSION

Measurement of the heat production of representative *Candida* spp. to determine their susceptibilities to antifungals and the activities of antifungals on planktonic and biofilm forms was investigated. The correlation between the conventional broth microdilution MICs and the microcalorimetry MHICs was in agreement within 2-fold serial dilutions for 14 of 16 values (88%). However, this observation needs to be interpreted with caution since it is based on 16 values. Two significant discrepancies were observed for fluconazole, in *C. glabrata* and *C. parapsilosis* (MICs of 2 and 4

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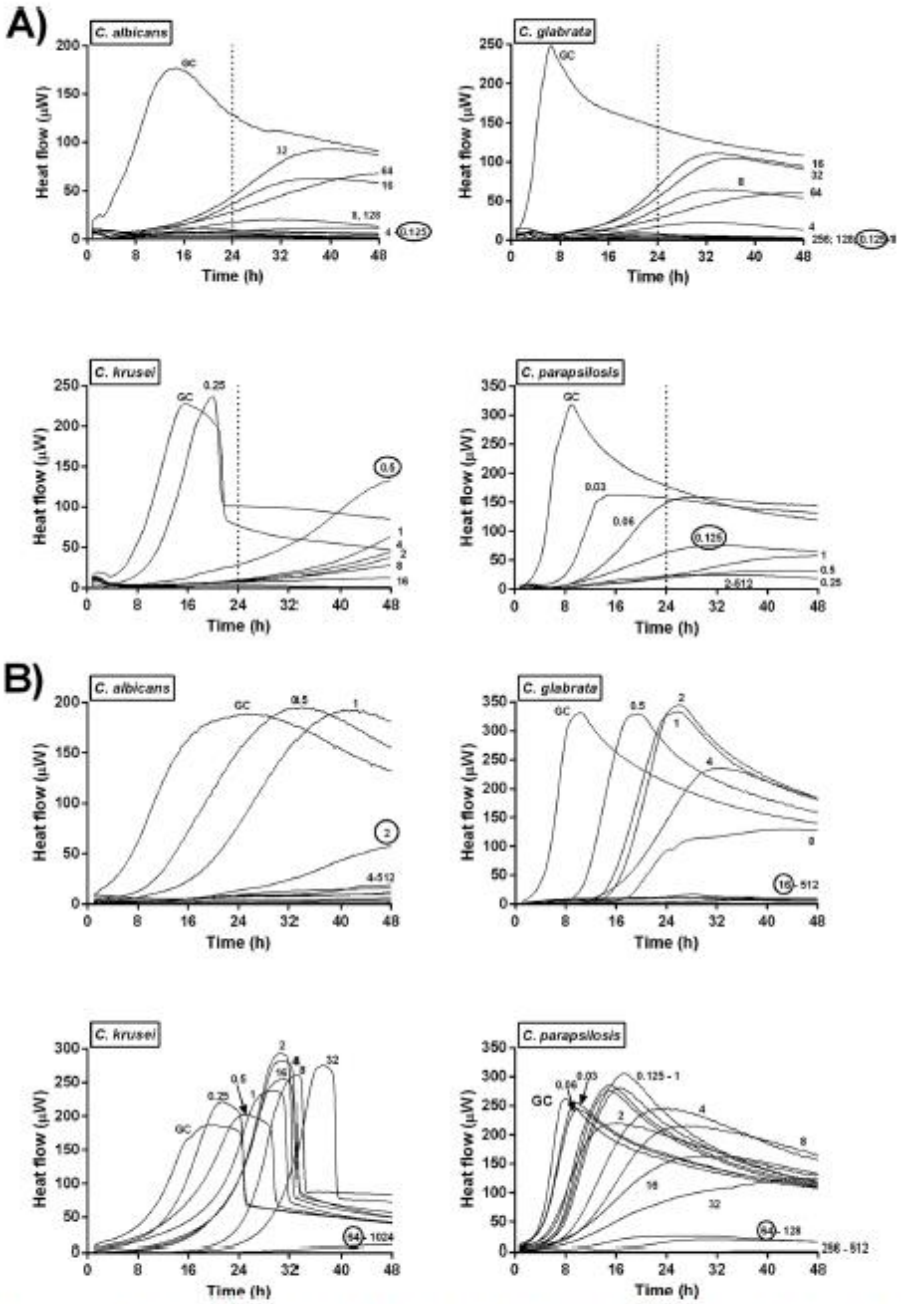

FIG 2 Heat flow curves generated by planktonic (A) and biofilm (B) *Candida* in the presence of caspofungin. For other details, see legend to Fig. 1.

µg/ml, respectively, and MHICs of >512 µg/ml). This difference most likely reflects the fact that the methods are measuring different activities (heat flow versus turbidity). An injectable solution of fluconazole was used in this *in vitro* study because this formulation was used in animal studies. The fluconazole liquid form, including additives, did not produce any additional heat in the microcalorimetric assay (data not shown). We believe that the additives themselves had no antifungal activity since all *Candida*

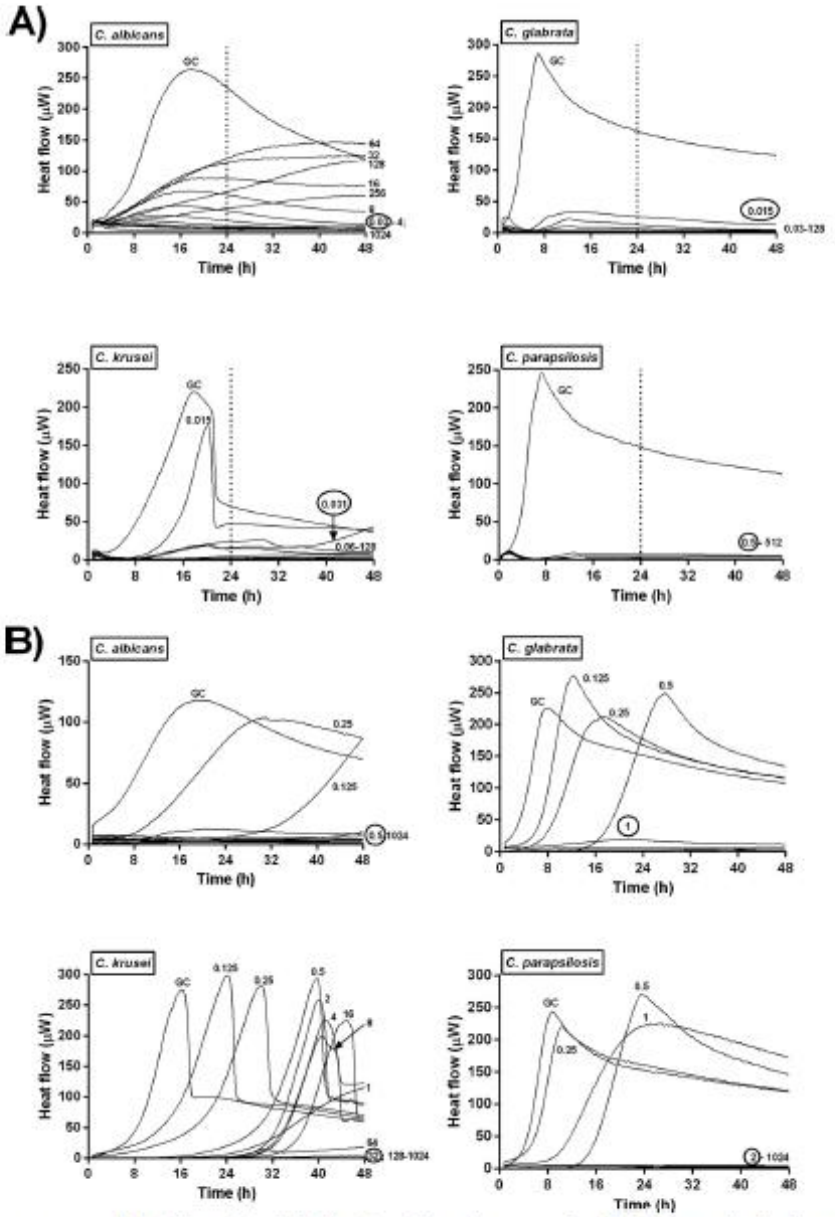

FIG 3 Heat flow curves generated by planktonic (A) and biofilm (B) *Candida* in the presence of anidulafungin. For other details see legend, to Fig. 1.

spp. (except *C. albicans*) were inhibited only at relatively high fluconazole concentrations. However, we cannot exclude a diminished fluconazole potency by using the liquid formulation, which potentially may explain the observed discrepancies.

As mentioned above, additional work on the definition of MHIC and modification or optimization of testing conditions (growth medium, additives, fungal inoculum, temperature, antifungal used, etc.) may further improve the agreement with conventional susceptibility results. Other microcalorimetry parameters (such as area under the heat flow curve, delay of heat production, and slope of the ascending part of the heat flow curve) were investigated, but all showed the same discrepancies, suggesting that the differences are not caused by the definition criteria.

An important aim of this study was to search for antifungals with activity against *Candida* biofilms. *Candida* biofilms on im-

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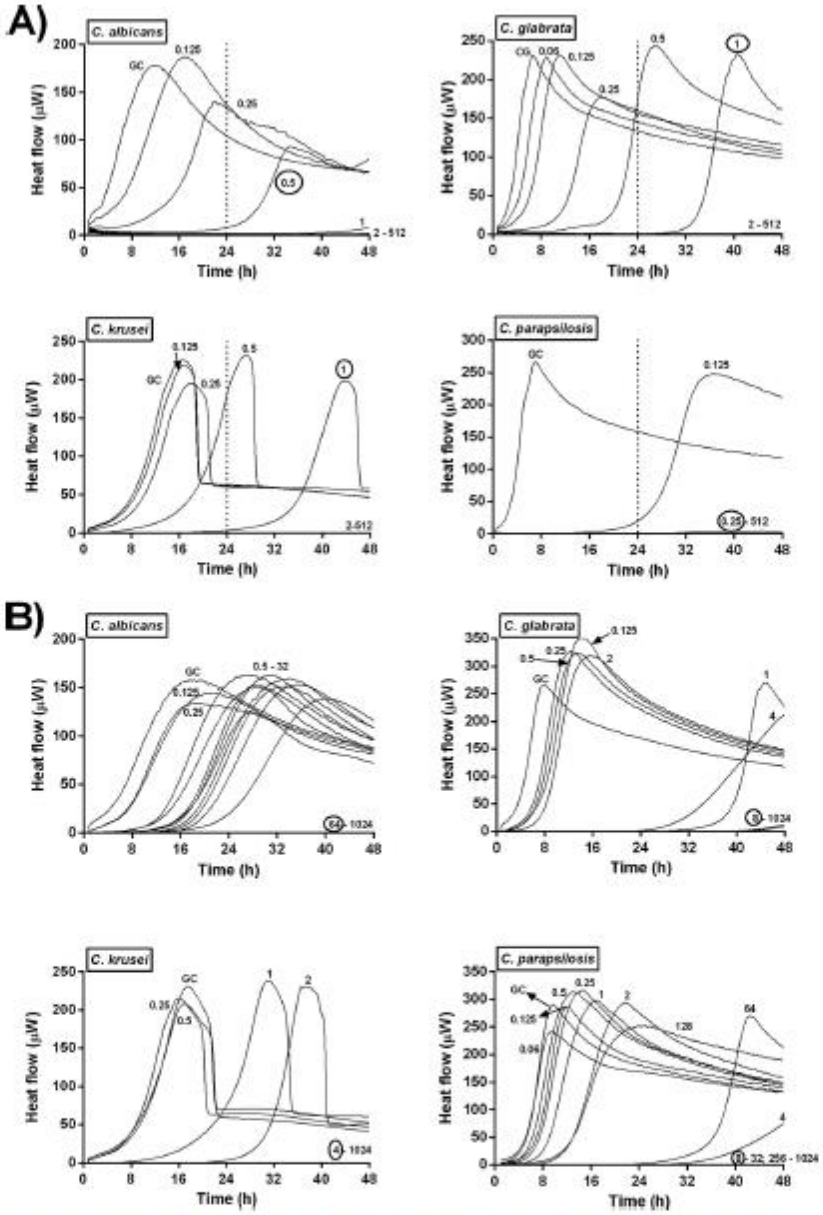

FIG 4 Heat flow curves generated by planktonic (A) and biofilm (B) *Candida* in the presence of amphotericin B. For other details, see legend to Fig. 1.

plants are difficult to eradicate and often cause relapsing infections if the implant remains in place. The correlation between the results of the XTT assay and the microcalorimetry MHICs for *Candida* biofilms was in agreement within 2-fold serial dilutions for 15 of 16 values (94%). With microcalorimetry, antibiotics with antibiofilm activities against bacteria were also found, and these *in vitro* results correlated well with cure rates of implant-associated infections in an experimental foreign-body infection model (36–38) and clinical studies. Therefore, the correlation between the *in vitro* microcalorimetry results and *in vivo* experimental data should be investigated in the next step. In our study, higher concentrations of fluconazole were required to inhibit the growth of *Candida* biofilms than of planktonic *Candida*, whereas caspofungin and amphotericin B preserved their activities against biofilms. Anidulafungin showed the best antibiofilm activities against the *Candida* species.

Microcalorimetry provides additional valuable information on the interaction between the microorganism and the antifungal agent, including the mode of action, real-time determination of the antifungal action, the effects of antifungal combinations (compared to exposure to one drug), and the emergence of resistance and prevention thereof. In our study, additional phenomena were observed by microcalorimetry, such as the induction of growth (exceeding the growth control) at sub-MICs of antifungals or the trailing effect that denotes reduced but still present growth in the presence of echinocandin at concentrations above the MIC (14). Another *in vitro* phenomenon observed was the paradoxical growth observed with echinocandins, especially with caspofungin. Similar reduced activities against *Candida* were observed at high drug concentrations, achievable in human serum, by other authors (39, 40). By broth microdilution, a paradoxical effect with caspofungin is evident for 60% of *C. albicans* bloodstream infections at achievable human serum concentrations (≤8 μg/ml) (41). The paradoxical effect depends on the initial inoculum and the assay method used (41). For example, certain isolates showed the paradoxical effect in the time-kill assay but not the broth microdilution assay. We observed paradoxical growth by microcalorimetry only in planktonic *C. albicans* and *C. glabrata*, most prominently after 24 h of incubation in the microcalorimeter. This phenomenon correlated with observations obtained by standard MIC testing (data not shown), and it was reported previously by other researchers (17, 39–42). With prolonged incubation (>24 h), other, as-yet-unknown phenomena may be observed by microcalorimetry. However, the clinical relevance of these effects remains to be determined.

In summary, this study demonstrated the potential of microcalorimetry for real-time antifungal susceptibility testing and evaluation of antifungal activities against planktonic and biofilm *Candida*. The method could be used for testing new antifungal agents, including their mode of action and antifungal combinations. Another important potential application of microcalorimetry could be the study of factors involved in the antifungal resistance of *Candida* biofilms, such as the architectural properties of the biofilm matrix, reduced fungal growth rates, and the presence of efflux pumps (43–46). In future studies, correlation between microcalorimetry results and *in vivo* activities of antifungals needs to be established.

ACKNOWLEDGMENTS

The strains were kindly provided by Dominique Sanglard from the Institute of Microbiology at Lausanne University Hospital, Switzerland. We kindly thank Riccardo Torelli from the Catholic University of the Sacred Heart, Rome, Italy for help in performing the XTT assay.

The study was supported by an educational grant from Pfizer Pharma GmbH (Nr. WI178378).

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Chapter 4

Antifungal Activity against Planktonic and Biofilm Candida albicans in an Experimental Foreign-Body Infection Model

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Submitted to Antimicrobial Agent and Chemotherapy, March 2014

Manuscript #	AAC00087-14
Current Revision #	0
Submission Date	2014-01-22 17:39:14
Current Stage	Under Review
Title	Antifungal Activity against Planktonic and Biofilm Candida albicans in an Experimental Foreign-Body Infection Model
Running Title	Antifungals against planktonic and biofilm Candida
Manuscript Type	Full-Length Text
Journal Section	Experimental Therapeutics
Corresponding Author	Prof. Andrej Trampuz (Charité - University Medicine, Free and Humboldt-University of Berlin)
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Abstract	The optimal treatment against Candida biofilms is not defined. We investigated the antifungal activity against planktonic and biofilm C. albicans in a foreign-body infection model. Teflon cages were subcutaneously implanted in guinea pigs, infected with C. albicans (ATCC 90028). Animals were treated intraperitoneally 12 h after infection for 4 days once daily with saline, fluconazole (16 mg/kg), amphotericin B (2.5 mg/kg), caspofungin (2.5 mg/kg) or anidulafungin (20 mg/kg). Cage fluid was aspirated and planktonic Candida was quantified before, during and after treatment and the clearance rate from cage fluid was determined. Cages were removed and cultured to determine the biofilm cure rate. In untreated animals, planktonic Candida was cleared from cage fluid in 25% (infected with 4.5 x 103 CFU/cage), 8% (infected with 4.8 x 104 CFU/cage) and 0% (infected with 6.2 x 105 CFU/cage). Candida biofilm persisted on all explanted cages. Compared to untreated controls, fluconazole and amphotericin B did not reduce the number of planktonic C. albicans in cage fluid during and after treatment, whereas caspofungin reduced it to 0.22 and 0.0 CFU/ml, respectively, and anidulafungin to 0.11 and 0.13 CFU/ml, respectively. Fluconazole cured 2/12 cages (17%), amphotericin B and anidulafungin each 1/12 cages (8%) and caspofungin 3/12 cages (25%). In summary, echinocandins showed superior activity against planktonic C. albicans than amphotericin B and fluconazole. Caspofungin showed the highest cure rate of C. albicans biofilm. However, no antifungal exceeded 25% cure rate, demonstrating the difficulty of eradicating Candida biofilms from implants.
Editor	Sevtap Arikan-Akdagli
Suggested Reviewers to Include	Werner Zimmerli (University of Basel), Michael Pfaller (JMI Laboratories), Jacques Meis (Canisius Wilhelmina Hospital, Radboud University Medical Center), Maurizio Sanguinetti (Università Cattolica del S. Cuore), William Kirkpatrick (UTHSCA at San Antonio)
Suggested Reviewers to Exclude	N/A
Keywords	Candida, biofilm, foreign-body, animal model, antifungal agents
Research Areas	Mycology, Pharmacology
Conflict of Interest	No conflict of interest.
Funding Sources	None
Color Charges	No, my article contains no color or my article is exempt from color charges.
Electronic Forms	1 of 1 forms complete - View Electronic Forms Status

Manuscript Items

1. Author Cover Letter (last updated: 01/22/2014 17:34:32) PDF (52KB) Source File (DOCX) 15KB
2. Manuscript Text File (last updated: 01/22/2014 17:33:46) PDF (316KB) Source File (DOCX) 324KB

Antifungal Activity against Planktonic and Biofilm *Candida albicans* in an Experimental Foreign-Body Infection Model

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Keywords: *Candida* spp., biofilm, foreign-body infection, animal model, antifungals

Running title: Antifungal activity against planktonic and biofilm *Candida*

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ABSTRACT

The optimal treatment against *Candida* biofilms is not defined. We investigated the antifungal activity against planktonic and biofilm *C. albicans* in a foreign-body infection model. Teflon cages were subcutaneously implanted in guinea pigs, infected with *C. albicans* (ATCC 90028). Animals were treated intraperitoneally 12 h after infection for 4 days once daily with saline, fluconazole (16 mg/kg), amphotericin B (2.5 mg/kg), caspofungin (2.5 mg/kg) or anidulafungin (20 mg/kg). Cage fluid was aspirated and planktonic *Candida* was quantified before, during and after treatment and the clearance rate from cage fluid was determined. Cages were removed and cultured to determine the biofilm cure rate. In untreated animals, planktonic *Candida* was cleared from cage fluid in 25% (infected with 4.5×10^3 CFU/cage), 8% (infected with 4.8×10^4 CFU/cage) and 0% (infected with 6.2×10^5 CFU/cage). *Candida* biofilm persisted on all explanted cages. Compared to untreated controls, fluconazole and amphotericin B did not reduce the number of planktonic *C. albicans* in cage fluid during and after treatment, whereas caspofungin reduced it to 0.22 and 0.0 CFU/ml, respectively, and anidulafungin to 0.11 and 0.13 CFU/ml, respectively. Fluconazole cured 2/12 cages (17%), amphotericin B and anidulafungin each 1/12 cages (8%) and caspofungin 3/12 cages (25%). In summary, echinocandins showed superior activity against planktonic *C. albicans* than amphotericin B and fluconazole. Caspofungin showed the highest cure rate of *C. albicans* biofilm. However, no antifungal exceeded 25% cure rate, demonstrating the difficulty of eradicating *Candida* biofilms from implants.

INTRODUCTION

Candida spp. is rarely causing prosthetic joint infections (PJI), representing about 1-3% of all infections, but are difficult to treat and are associated with high morbidity and healthcare cost (1, 2). Usually a two-step exchange of the prosthesis with a long interval is applied since *Candida* is considered a difficult-to-treat microorganism in implant-associated infection. Little is known about the optimal antifungal treatment of *Candida* PJI. In vitro experiments suggest that microorganisms are considerably more resistant to antifungals than their planktonic counterparts (3). In a recent in vitro study, fluconazole activity against biofilm *Candida* was reduced by >1000-fold compared to planktonic counterparts, whereas echinocandins and amphotericin B mainly preserved their activity (Maiolo EM, Furustrand Tafin U, Borens O, Trampuz A, manuscript in revision AAC01815-13).

By using an established animal model of foreign-body infection, the pharmacokinetic and pharmacodynamic parameters can be studied in a physiological environment, including the interaction between the drug and the microorganism at the site of infection (4). In previous studies, antifungals were evaluated in animal models with different materials implanted subcutaneously or intraperitoneally in mice, rats or guinea pigs (5-11).

In this study we investigated the activity of antifungal agents (fluconazole, amphotericin B, caspofungin and anidulafungin) against planktonic and biofilm *C. albicans* in a guinea pig foreign-body infection model. The tissue cage infection model has been validated for testing the activity of antimicrobial agents against implant-associated infections in preclinical studies (12-15). To our knowledge, this is the first evaluation of antifungal treatment against *C. albicans* foreign-body infection in this model.

(Part of the results of this study was presented at the 23rd European Congress of Clinical Microbiology and Infectious Diseases, Berlin, Germany, 27 to 30 April 2013 [E. Maiolo, U. Furustrand Tafin, A. Trampuz, abstr. P-1097] and at the 32nd meeting of the European Bone and

Joint Infection Society, Prague, Czech Republic, 12 to 14 September 2013 [E. Maiolo, U. Furustrand Tafin, O. Borens, A. Trampuz, oral presentation].

MATERIALS AND METHODS

Study organism. *C. albicans* (ATCC 90028) was used for in vivo antifungal testing. The strain was stored at -80°C by use of a cryovial bead preservation system (Roth, Karlsruhe, Germany) at -80°C. *C. albicans* was cultured on Sabouraud dextrose agar (SDA) for 24 h at 37°C. The inoculum was prepared by McFarland and the exact quantity of organisms was determined by performing quantitative cultures.

Antifungal agents. Fluconazole was obtained in liquid form (2000 µg/ml, Teva Pharma AG, Aesch, Switzerland). Amphotericin B (Sigma, St Louis, MO, USA) and caspofungin (Merck & Co., Inc. Whitehouse station, NJ, USA) were obtained in powder form and dissolved in sterile water. Anidulafungin was kindly provided in powder form by Pfizer Pharma AG (Ecalta, Zurich, Switzerland) and dissolved according to the manufacture instructions.

In vitro antifungal susceptibility. The minimal inhibitory concentration (MIC) was determined by microbroth dilution. Antifungal susceptibility of planktonic *C. albicans* was determined by microbroth dilution method according to the EUCAST guidelines (16). 100 µl of a final concentration of 1-5 x 10^5 CFU/ml in RPMI-1640 (Roswell Park Memorial Institute) were added to 100 µl of a serial two-fold dilutions of each antifungal previously prepared. Plates were subsequently incubated at 37°C for 24h and read by spectrophotometer at 530 nm. The MIC was defined as the lowest antifungal concentration inhibiting 50% of growth. Experiments were performed in triplicates

Animal model. A foreign-body infection model in guinea pig was used, as described previously (17). In brief, male albino guinea pigs (Charles River, Sulzfeld, Germany) were kept in the Animal Facility of the University of Lausanne, Switzerland. The experiments were performed according to the regulations of Swiss veterinary law. Guinea pigs were weighted every week to ensure their well-being. Animals were anesthetized with a subcutaneous injection of ketamine and xylazine. Four sterile polytetrafluorethylene (Teflon) cages (32 x 10 mm) with 130 regularly spaced perforations of 1 mm diameter (Angst-Pfister AG, Zürich, Switzerland) were subcutaneously implanted in flanks of the guinea pigs (450 – 550 g) under aseptic conditions. Two weeks after implantation, cage fluid was aspirated to confirm sterility. Contaminated cages were excluded from further studies.

Study of the infection profile. The tissue cage model has never been tested with *Candida* spp. in guinea pigs. Therefore, in the first experiment an infection profile was evaluated. Cages were infected by percutaneous inoculation of 200 µl of *C. albicans* containing 4.5 x 10^3 CFU/cage (low inoculum), 4.8 x 10^5 CFU/cage (intermediate inoculum) and 6.2 x 10^6 CFU/cage (high inoculum). The infection was confirmed by aspiration of the cage fluid and quantification of the culture on SDA plates. Planktonic *Candida* was quantified in aspirated cage fluid on day 1, 2, 3 and 6 (in CFU/ml), and clearance rate (in %) in cage fluid was determined. On day 6, the animals were sacrificed and the cages were aseptically removed and cultured in 5 ml Sabouraud dextrose broth (SDB) for 48 h to determine the spontaneous cure rate of *Candida* biofilm (in %). Aliquots of 100 µl were spread on a Sabouraud plates and incubated at 37°C for additional 48 h to evaluate the biofilm presence.

Pharmacokinetic studies. Cage fluid was aspirated in uninfected animals during 48 h (1, 2, 4, 8, 24 and 48 h) following intraperitoneal administration of a single dose of fluconazole (8 and 16 mg/kg), amphotericin B (0.62 and 1.25 mg/kg), caspofungin (1 and 2.5 mg/kg) and anidulafungin

(6 and 12 mg/kg). For each antifungal dose three guinea pigs were used (i.e. 12 cages). At each time point, 150 µl aliquots of cage fluid were aspirated from one cage from each animal (three replicates par time point and drug dose). Contaminated cages were excluded from further studies. The collected fluid was centrifuged (4500 rpm for 5min at 4°C) and the supernatant was stored at -20°C until further analysis.

Pharmacokinetic parameters were calculated for each animal: C_{max} was defined as the maximum concentration observed, T_{max} was defined as the time needed to achieve the maximum concentration, C_{min24} was defined as the concentration measured at 24 h, C_{min48} was defined as the concentration measured at 48 h, AUC_{0-24} and AUC_{0-48} (area under the curve) were estimated by trapezoidal method after 24 h and 48 h, respectively. Variability of PK parameters was expressed as mean ± standard deviation. Antifungals concentration profiles were plotted with GraphPad Prism version 6.01, using mean of each sampling time per group, with errors bars representing standard deviation (SD).

Pharmacokinetic analysis. Pharmacokinetic studies were performed using a liquid chromatography tandem mass spectrometry assay (LC tandem MS assay). Calibration curves were established with matrix-matched samples using blank guinea pigs samples spiked with relevant concentration of fluconazole, anidulafungin and caspofungin. This aimed at circumventing the matrix effect of complex biological fluids that potentially adversely affect the performance of the LC tandem MS assay.

Antifungal treatment in animals. For treatment studies animals were infected with the low inoculum (2×10^4 CFU/ml, corresponding to 4×10^3 CFU/cage). Antifungal treatment started 12 h after infection. Cage fluids were aspirated and plated for quantitative analysis, followed by the antifungal treatment. Three animals, each animal holding 4 cages (i.e., 12 cages/treatment regimen), received one of the following treatment regimens: control group (no antifungal treatment); fluconazole (16 mg/kg); amphotericin B and caspofungin (2.5 mg/kg) and

anidulafungin (20 mg/kg). All antifungals were administered intraperitoneally every 24 h for 4 days. The antifungal dose was chosen based on pharmacokinetic studies performed in previously reported studies on rats, mice, guinea pigs and humans.

Activity on planktonic and biofilm *C. albicans* in animals. To determine the activity of antifungals against *C. albicans*, cage fluid was aspirated before (to confirm the presence of infection), during and 10 days after treatment. The fungal counts were expressed as $\log_{10}$ CFU/ml cage fluid. To determine the activity against *C. albicans* biofilm, animals were sacrificed 10 days after treatment and the cages were explanted under aseptic conditions and incubated for 48 h in 5 ml of SDB. After 48 h, 100 µl were spread on a blood agar plate and incubated at 37°C for additional 48 h and assessed for fungal growth.

Statistical analysis. Comparisons were performed by using the Mann-Whitney U test for continuous variables. For all test differences were considered significant when P values were <0.05. Figures were plotted with GraphPad Prism (version 6.01) software (GraphPad Software, La Jolla, CA).

RESULTS

In vitro antifungal susceptibility. The MIC values of *C. albicans* obtained by microbroth dilution were 0.25 µg/ml for fluconazole, 0.25 µg/ml for caspofungin and 0.03 µg/ml for anidulafungin.

Infection profile. Figure 1 represent planktonic *C. albicans* in cage fluid after infection with 10^3 CFU/cage (A), 10^5 CFU/cage (B) and 10^6 CFU/cage (C). A spontaneous progressive reduction of the planktonic counts of *Candida* from tissue cage fluid was observed during 6 days with all inocula. On day 6 (just before explantation), *C. albicans* was cleared from 3/12 cage fluids (25%) with low inoculum of 10^3 CFU/cage and from 1/12 (8%) cage fluids with intermediate inoculum of

10^5 CFU/cage. No clearance was observed with the high inoculum of 10^6 CFU/cage. After explantation of the cages one week after infection, biofilm was detected in all 12 cages, proving the presence of biofilm. No sign of skin inflammation or perforation of the cage was seen during the infection profile.

Pharmacokinetic studies. Figure 2 shows the concentration-time profile in cage fluid after the administration of a single intraperitoneal dose in non-infected animals. Table 1 summarizes the calculated pharmacokinetic parameters. The C_{max} of fluconazole after the administration of a single intraperitoneal dose of 8 and 16 mg/kg were 3.64 µg/ml and 9.07 µg/ml, respectively, which were achieved at ≈6.7 h after dosing. At 8 mg/kg, the fluconazole maximum concentration in the cage fluid reached 14X the MIC of the tested organism (0.25 µg/ml), whereas at 16 mg/kg it reached 36X the MIC. The fluconazole concentrations remained above the MIC for 24 h (C_{min24}, 1.14 and 2.90 µg/ml for doses of 8 and 16 mg/kg, respectively) and decreased below the MIC at 48 h at 8 mg/kg (C_{min48}, 0.20 µg/ml).

The C_{max} of caspofungin after the administration of a single intraperitoneal dose of 1 and 2.5 mg/kg were 0.32 µg/ml and 1.41 µg/ml, respectively, which were achieved at 24 h and 16 h, respectively. The maximum concentration in the cage fluid was above the MIC of *C. albicans* (0.25 µg/ml) at both doses. The maximum concentration reached 1 X and 2.8X the MIC at 24 h (C_{min24}, 0.32 and 1.40 µg/ml for doses of 1 and 2.5 mg/kg, respectively). Concentrations were below the MIC at 48 h at 1 mg/kg (C_{min48}, 0.15 µg/ml).

The *Cmax* of anidulafungin after the administration of a single intraperitoneal dose of 6 and 12 mg/kg were 0.15 µg/ml and 0.22 µg/ml respectively, which were achieved at 6.0 h and ≈13.3 h, respectively. At 6 mg/kg, the maximum concentration in the cage fluid reached 5X the MIC (0.03 µg/ml). The maximum concentration reached 7X the MIC at 12 mg/kg. The anidulafungin

concentrations remained above the MIC for 48 h (C_{min48}, 0.04 and 0.06 µg/ml for doses of 6 and 12 mg/kg, respectively).

Antifungal treatment in animals. Cage fluid sterility was confirmed prior to infection. At 12 h after infection, the median (± SD) concentration of the yeast in the cage fluid was 1 x 10^3 CFU/ml (2.73 ± 0.68 $\log_{10}$ CFU/ml) before treatment. In control animals receiving no drug, fungal counts in cage fluid were 2.22 ± 0.8 $\log_{10}$ and 0.70 ± 1.17 $\log_{10}$ CFU/ml after 4 and 14 days, respectively, which correspond to decrease of 0.51 and 2.03 $\log_{10}$ CFU/ml, respectively. No spontaneous cure of the cage-associated infection occurred in the untreated animals.

Activity on planktonic and biofilm *C. albicans* in animals. Before treatment, cage fluid contained (2.73 ± 0.68 $\log_{10}$ CFU/ml) 1 x 10^3 *C. albicans*/ml. Compared to untreated control, fluconazole and amphotericin B did not reduce planktonic *C. albicans* during and after treatment, whereas caspofungin reduced the numbers to 0.22 ± 0.51 and 0.0 CFU/ml and anidulafungin to 0.11 ± 0.38 and 0.13 ± 0.46 CFU/ml cage fluid (Fig. 3). No spontaneous cure occurred in the untreated controls (Fig. 4), whereas fluconazole cured 2 of 12 cages (17%), amphotericin B and anidulafungin 1 of 12 cages (8%) and caspofungin 3 of 12 cages (25%).

DISCUSSION

The biofilm formation in *Candida* spp. is increasingly recognized as a significant clinical problem, especially in transplant, oncology and intensive care medicine (18). Implant-associated infections caused by yeasts are particularly characterized by high complexity and treatment challenges due to often concomitant immunosuppression of the patient, antifungal resistance and limited therapeutic options against *Candida* biofilms. Data on optimal antimicrobial and surgical management of implant-associated infections caused by *Candida* spp. are limited (19). In most of the cases, explantation of the device is performed, followed by long-term antifungal treatment.

However, the outcome is often characterized with relapses, persistent infection and need of multiple radical surgical interventions.

Azoles, the most studied antifungal agents, especially fluconazole, demonstrated low activity against *Candida* biofilms (20-23). Sessile yeast cells could grow, proliferate and form biofilms after 1 h of adherence despite the presence of high concentrations of fluconazole up to 1024 µg/ml (24). In a time-kill study, fluconazole showed lacking ability to eradicate *Candida* biofilm, whereas caspofungin and amphotericin B deoxycholate showed good activity over 48 h (25). In several studies echinocandins showed superior in vitro activity against *Candida* biofilms than azoles (26, 27), as was also showed in a recent study using the ultra-sensitive microcalorimetry assay (Maiolo E et al., manuscript AAC01815-13, in revision).

In this study, we therefore evaluated the in vivo activity of antifungals in an established foreign-body model using guinea pigs, specifically against planktonic and biofilm *C. albicans*. Several interesting observations were made. During the study of the infection profile of *C. albicans* in untreated animals, a spontaneous decrease of the number of inoculated planktonic *C. albicans* in the cage fluid was observed. Particularly at low inoculum, some cage fluids cleared planktonic *Candida* from the cage fluid completely. However, when infected cages were explanted, all cage cultures grew *Candida* independently on their inoculum size (low, intermediate or high). This observation supports the hypothesis that *Candida* switch from planktonic into biofilm form when a foreign body is present in order to persist on the surface of the cages. Since there was no spontaneous cure of *Candida* biofilms, this model is suitable to test the activity of individual antifungals against biofilms.

The pharmacokinetics of tested antifungals was characterized to determine the appropriate dosing and administration intervals. After 24 h of administration, the concentration of all four tested antifungals in the cage fluid was above the MIC of the test organism, whereas after 48 h with

fluconazole at 8 mg/kg and caspofungin at 1 mg/kg the concentrations in cage fluid were below the MIC. Therefore, the once-daily doing was chosen for further experiments.

In treatment studies, fluconazole and amphotericin B did not reduce planktonic *C. albicans* in the cage fluid during and after treatment and showed limited anti-biofilm activity with cure rates from 8% to 17%. In contrast, caspofungin and anidulafungin had a superior activity against planktonic *Candida* in the cage fluid. Against *C. albicans* biofilm, anidulafungin exhibited similar activity than amphotericin B (cure rate 8%), whereas caspofungin showed superior activity against *C. albicans* biofilm (cure rate 25%).

The observed antifungal activity is in general lower than the one of antibacterial substances against *S. aureus*, *E. coli*, *Enterococcus faecalis* or *P. acnes* (12-15) using the same foreign-body infection model. This fact underlines that *Candida* remains a difficult-to-treat organisms and removal of a device with staged concept of re-implantation seems to be the rational treatment strategy. Other treatment strategies, such as novel antifungals, combination therapies or mechanical (e.g. sonication), biological (e.g. phages) or chemical (e.g. enzymes) biofilm removal strategies may improve the treatment outcome and make the retention and salvage of an infected prosthesis possible.

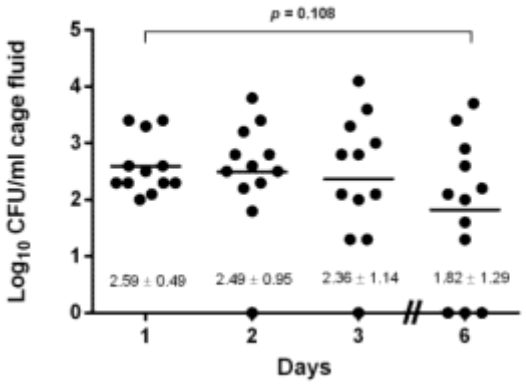
In conclusion, caspofungin and anidulafungin showed superior activity against planktonic *C. albicans* compared to amphotericin B and fluconazole at physiological doses. No antifungal drug administered alone achieved cure rates above 25%, demonstrating the difficulty of eradicating *Candida* biofilms from implants. In further studies, higher doses of the antifungals, their combinations or addition of a non-pharmacological approach may improve the treatment outcome and can help planning rational clinical trials in implant-associated infections.

Acknowledgements

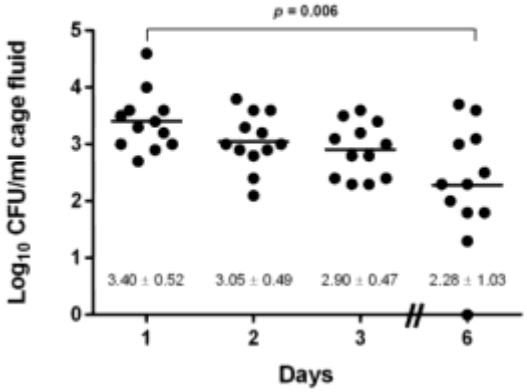
We thank Bertrand Betrisey for helping with the animal experiments and Laurent Decosterd and his team for the pharmacokinetic analysis.

Figure 1. Infection profile, i.e. planktonic *C. albicans* in cage fluid after infection with 10^3 CFU/cage (A), 10^5 CFU/cage (B) and 10^6 CFU/cage (C). Horizontal lines represent means, numbers represent mean ± standard deviation values (in $\log_{10}$ CFU/ml cage fluid).

A. Infection with low inoculum (4.5 x 10^3 CFU/cage)



B. Infection with intermediate inoculum (4.8 x 10^5 CFU/cage)



C. Infection with high inoculum (6.2 x 10^6 CFU/cage)

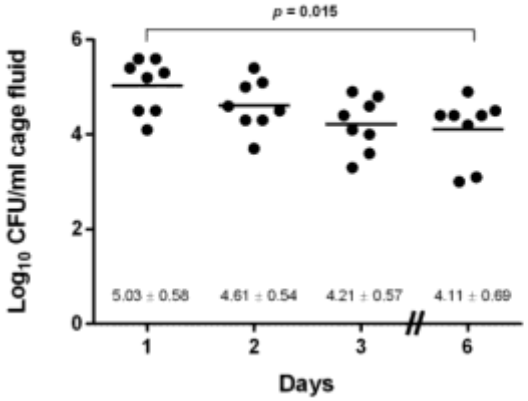


Figure2. Pharmacokinetic profile of fluconazole, caspofungin and anidulafungin in cage fluid after the administration of a single intraperitoneal dose of the drug in non-infected animals. The mean values of three measurements (except for caspofungin and anidulafungin 6 mg/kg, 2 measurements were considered) at each time point are shown. Points represent means; error bars represent SDs.

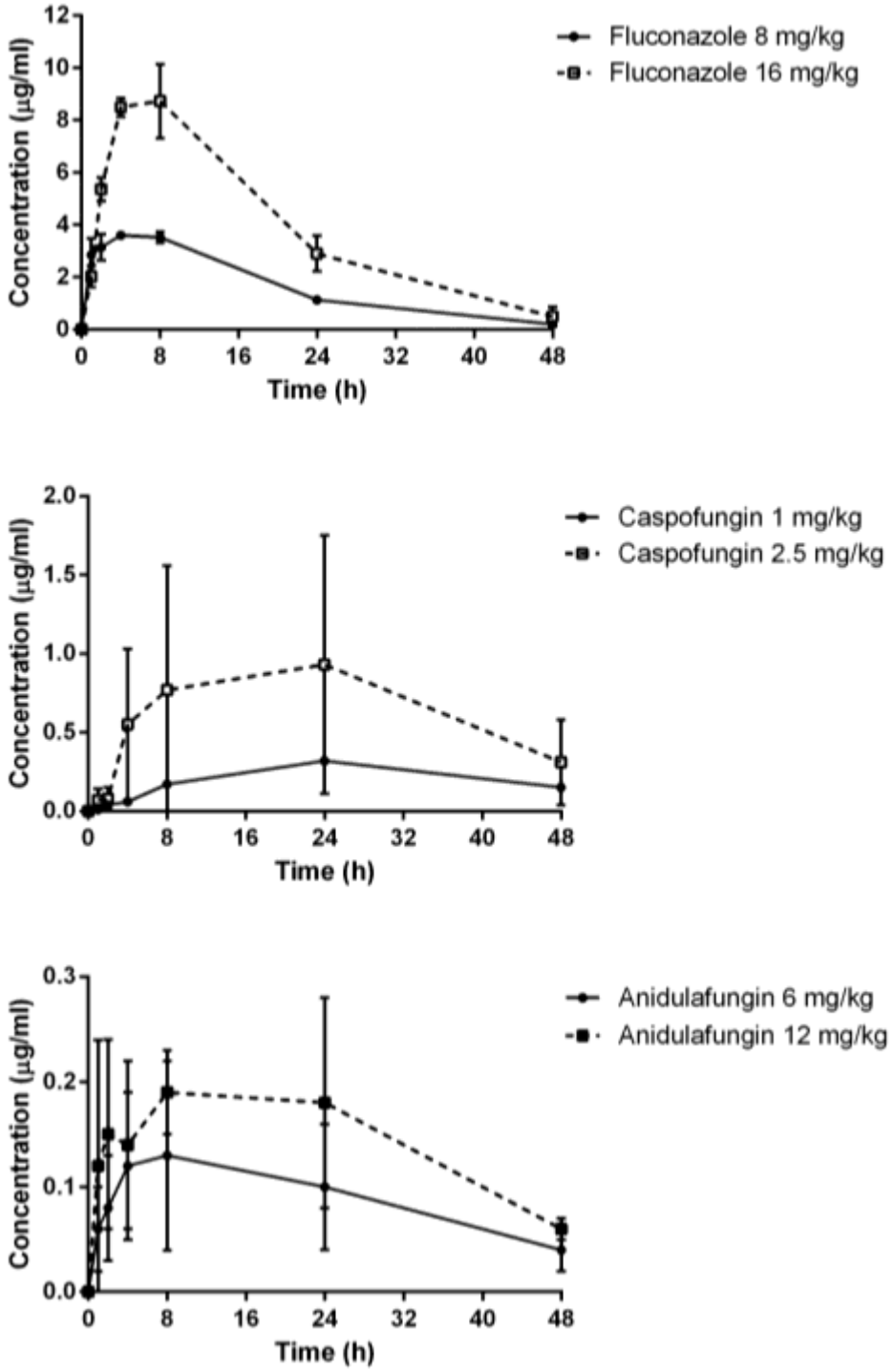


Figure 3. Activity against planktonic *C. albicans* in cage fluid during (day 4) and after treatment (day 14). Animals were infected with 2 x 10^4 CFU/ml, corresponding to 4 x 10^3 CFU/cage. Numbers above error bars represent mean log_{10} CFU/ml ± standard deviations (SD) of antifungal treatments (AMB= amphotericin B; FZL= fluconazole; CAS = caspofungin; AFG= anidulafungin).

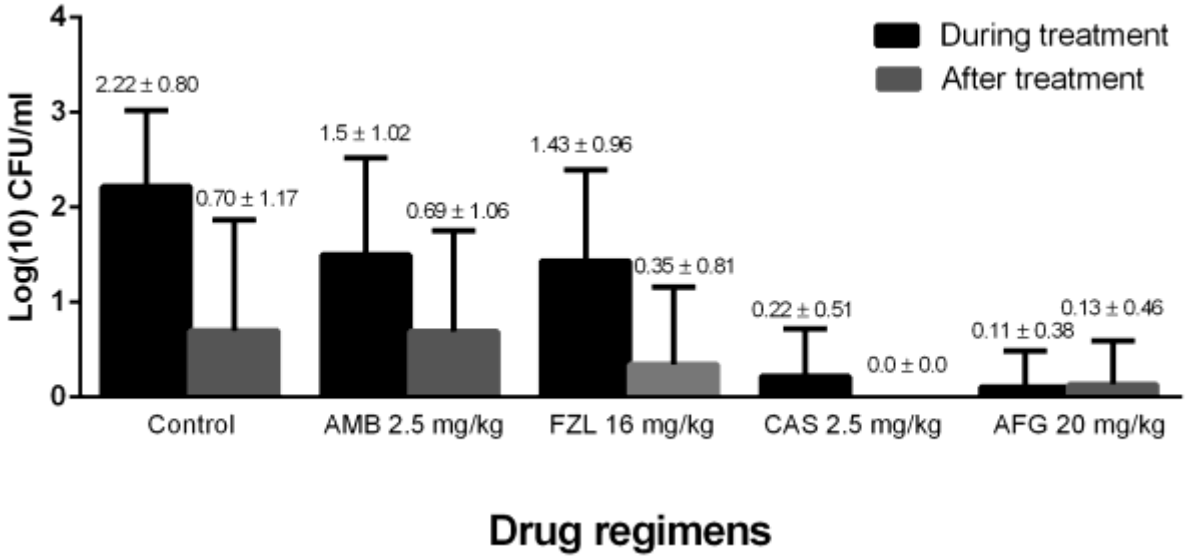


Fig. 4. Treatment efficacy against biofilm *C. albicans*. Numbers in brackets are number of cured / number all cages, followed by the cure rate of biofilm *Candida* (in %).

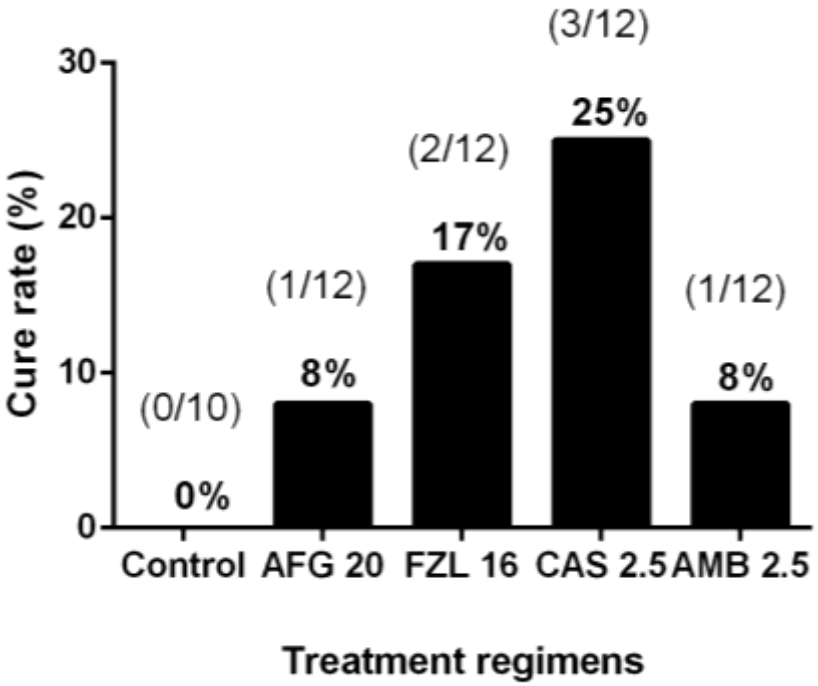


Table 1. Pharmacokinetic parameters for the drugs tested in cage fluid after administration of a single intraperitoneal dose to non infected animals.

Antifungal	MIC (µg/ml)	Dose (mg/kg)	C_{max} (µg/ml)[a]	C_{min} (µg/ml)[a]	T_{max} (h)[a]	C_{min24h} (µg/ml)[a]	C_{min48h} (µg/ml)[a]	AUC_{0-24} (h·µg/ml)[a]	AUC_{0-48} (h·µg/ml)[a]
Fluconazole	0.25	8	3.64 ± 0.10	0.20 ± 0.07	6.67 ± 2.31	1.14 ± 0.02	0.20 ± 0.07	62.80 ± 2.68	78.92 ± 2.58
		16	9.07 ± 1.14	0.49 ± 0.37	6.67 ± 2.31	2.90 ± 0.68	0.49 ± 0.37	146.10 ± 8.55	186.79 ± 3.02
Caspofungin	0.25	1	0.32 ± 0.01	0.15 ± 0.05	24 ± 0.00	0.32 ± 0.01	0.15 ± 0.05	4.54 ± 0.66	10.18 ± 0.21
		2.5	1.41 ± 0.23	0.46 ± 0.01	16 ± 11.31	1.40 ± 0.22	0.46 ± 0.01	25.43 ± 7.54	47.77 ± 10.23
Anidulafungin	0.03	6	0.15 ± 0.05	0.04 ± 0.01	6 ± 2.83	0.10 ± 0.04	0.04 ± 0.01	2.68 ± 1.07	4.35 ± 1.64
		12	0.22 ± 0.07	0.06 ± 0.01	13.33 ± 9.24	0.18 ± 0.10	0.06 ± 0.01	4.25 ± 1.50	7.11 ± 2.69

[a]Values are means ± SDs from three animals.

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Chapter5. General conclusions and outlook

The development of reproducible reference methods for *in vitro* testing of antifungal agents against *Candida* spp. has been a significant step in the improvement of treatment efficacy. The importance of antifungal susceptibility testing is indeed continuously increasing by the emergence and spread of resistant strains. Several sibling genes implied in azoles resistance have been identified leading to a reduced susceptibility to these agents. Thus, studies on new agents and their activity are of great importance. Current biofilm tests are questioned regarding their validity and reproducibility. Most of the tests include a continuous handling and addition of reagents that could destroy the biofilm structure, therefore, a more easy-to perform method needs to be developed.

Isothermal microcalorimetry is commonly used for analysis of chemical and biochemical reactions, either consuming or producing energy. During the last decade, the utility and advantages of isothermal microcalorimetry for highly sensitive detection of microbial growth was demonstrated for a range of microorganisms. In a clinical setting, microcalorimetry was shown to be useful for a rapid detection and discrimination between culture positivity and negativity of fluid samples. In Chapter 3 we demonstrated the potential of isothermal microcalorimetry as a novel method for antifungal susceptibility testing of *Candida* spp. Microcalorimetry showed the potential to be a suitable and precise method for performing antifungal susceptibility testing, as data is obtained continuously in real-time and the test interpretation is not based on subjective visual examination or by spectrophotometry. We demonstrated that fluconazole, echinocandins and amphotericin B affected the growth-related heat production of *Candida* spp. in different ways, depending on their fungistatic or fungicidal properties and phenomena, such as the paradoxical growth, could also be monitored. The MHIC was determined as the lowest concentration inhibiting ≥50% (≥90% for amphotericin B) of the heat produced at 24 h or 48 h for planktonic and biofilm growth,

respectively. For *Candida* spp., agreement within two 2-fold dilutions between MHIC (by microcalorimetry) and MIC (determined by EUCAST guideline) was 50% for fluconazole and 100% for echinocandins and amphotericin B. In order to validate our biofilm assay, XTT testing for biofilm growth evaluation was included. The agreement within two fold dilutions between the MHIC and the XTT test at 24h was 75% for fluconazole and 100% for echinocandins and amphotericin B. As determined by microcalorimetry, echinocandins were the most active agents against planktonic *Candida* spp (MHIC 0.015-0.5 μg/mL). Echinocandins, especially anidulafungin, also showed to have a good activity against biofilm growth (MHIC 0.25-2 μg/mL).

The conventional microbroth dilution method requires experienced personnel and especially in the case of CLSI the interpretation of susceptibility data is fairly subjective. Microcalorimetry, in contrast, offers an objective approach for data interpretation, based on heat production and change in the calorimetric curve, associated with the inhibition of growth. Considering the advantage of real-time growth monitoring, the microcalorimetric assay could be further optimized for a rapid detection of resistant isolates. Indeed, in a future study we will screen a strain collection of different azole-resistant *Candida* isolates, with the goal to establish a rapid and sensitive assay for detection of azole resistance, as was previously described for the differentiation between methicillin-susceptible and methicillin resistant *S. aureus* and for the determination of susceptibility of *Aspergillus* species using the same methodology (Furustrand, 2012). Furthermore, we will evaluate the activity of antifungal combinations against *Candida* spp. by microcalorimetry and compare it with the standard *in vitro* methods used for synergy testing, the time-kill and the checkerboard microdilution methods.

The use of porous glass beads for biofilm formation showed to be a practical and easy method for the evaluation of biofilm growth, without too much handling (which could alter the biofilm). The

addition of dyes or other agents, as in the case of the crystal violet staining (which gives a rough estimation of biofilm mass and differentiate between biotic and abiotic material but it does not give information about viability or activity) could also be avoided. In a future study we will test different materials, with the goal to establish a sensitive assay for detection of biofilm growth and inhibition by antifungals and by the material itself.

Considering a future use of microcalorimetry in a clinical microbiological laboratory, several topics need to be taken into account. First, the currently used isothermal microcalorimeter is still too expensive, mainly due to the low production volume. In order to lower the cost of the instrument it needs to be simplified and adapted to the current microbiological test conditions. Second, the instrument needs to allow a semi- or fully automated processing of multiple samples enabling high-throughput testing and biofilm testing. Promising developments in the calorimetric instrument field need to be performed as the high-throughput measurement calorimeter made by Torres in 2004. He presented a low-cost nano-calorimeter able to detect enthalpies of binding, enzymatic turnover and other chemical reactions in arrays of 96 positions. Adaptations in the development of high-throughput biofilm research also need to be performed in order to meet these criteria.

Based on *in vitro* results, we investigated the different treatment regimens in a guinea-pig model of foreign-body infections. Experimental conditions were assessed and optimized based on previous studies performed on mice, rats and guinea pigs found in the literature. A low inoculum and a short duration of infection (12h) were used for *C. albicans*, since treatment failure occurred when using high inoculum (10^6), 24 h of inoculation and low antifungal doses (data not shown). Infection profile experiments showed that a high inoculum is necessary to induce a persistent infection without spontaneous cure of planktonic yeast in cage fluid, nevertheless, antifungals showed no activity on planktonic cells in the cage fluid nor on the biofilm when using a high

inoculum. Since there was no spontaneous cure of *Candida* biofilms, this model is suitable to test the activity of individual antifungals against biofilms. However, using a low inoculum, an intermediate duration of infection and higher antifungal concentrations, a reduction of planktonic *C. albicans* was observed when echinocandins were used compared to other treatment regimens.

We were able to confirm our *in vitro* findings *in vivo* by performing treatment studies. The best activity was shown with caspofungin and fluconazole with a cure rate of 25%. Pharmacokinetic analysis highlights the importance of giving a good treatment regimen in order to achieve a local antifungal concentration above the MIC, since sub-MIC concentration can cause to resistance.

Considering the growing number of implant-associated infections, as well as the increasing prevalence of antimicrobial resistance, combination therapy could be an alternative, as well as new different treatment strategies for biofilm infections. Another approach could be the prevention of microbial attachment by manipulating the surface of the implant without influencing the host biocompatibility. Several studies are ongoing in this field trying to coat the implant surface with antimicrobials, embed the device and the spacers use in orthopedic surgery with antimicrobial substances, or by rendering the surrounding fluid less acid by ions release from the material leading to an inhibition of biofilm formation. Nevertheless, as already mentioned, sub-MIC concentration of the antimicrobial could lead to microorganism resistance. In order to avoid the emergence of resistance, early inhibition of cellular attachment on the device and biofilm formation, or even enzymatic degradation of biofilm, when present, could represent new strategies to improve the eradication of the infection.

PART II.

Bioactive materials

Chapter6. General introduction

Since bacteria can exist as single cells (planktonic) or in sessile aggregates (biofilm), the primary bacterial adherence to the foreign-body surface starts with planktonic cells and is followed by an irreversible attachment and maturation in a complex three-dimensional structure known as biofilm (Figure 1). The definition of a bacterial biofilm, as for medical microbiology, is a "coherent cluster of bacterial cells imbedded in a matrix, which are more tolerant to most antimicrobials and the host defense, than planktonic bacterial cells" (1). Within the biofilm cells communicate through intercellular signaling described as "quorum sensing" (2), which leads to structural and functional heterogeneity of the biofilm. From the mature biofilm, planktonic bacteria detach and depending on their virulence could cause either a local or a systemic infection (3, 4).

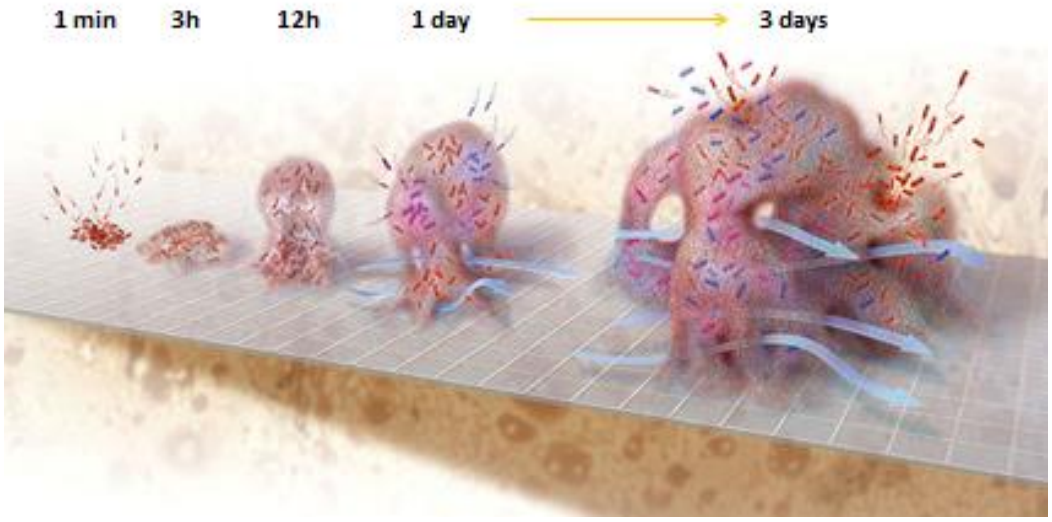

Figure 1. The biofilm life cycle. A biofilm starts to form when bacteria attach to a surface (1 min). An irreversible attachment (3 min) leads to the biofilm maturation through growth of the bacterial cells and production of the complex three-dimensional extracellular matrix (12 h to 3 days). Planktonic bacteria can detach and spread from the biofilm. (Adapted from K. Kasnot, Scientific American, 2001).

The biofilm matrix composed by extracellular polymeric substances (EPS) constitutes an excellent survival mechanism for the pathogen, in which bacteria are protected from the host immune system and the most used antimicrobial agents (2, 5, 6). Moreover, depletion of metabolic substances

and/or waste product accumulation in biofilms causes microbes to enter a slow- or non-growing (stationary) state. The most common infecting microorganisms causing implant-associated infections are staphylococci, followed by coagulase negative staphylococci, etc.(7, 8). Table 1 reports the most common pathogens isolated from prosthetic infections (9).

Microorganism	**Frequency (%)**
Staphylococcus aureus	30-43
Coagulase-negative staphylococci	17-21
Gram-negative bacilli	5-13
Polymicrobial	5-14
Anaerobes	2-5
Enterococci	3-7
Streptococci	11-12
Other/Unknown	5-6

Table 1. Frequency of most common identified microorganisms causing prosthetic joint associated infections. Adapted from (9)

Rare are the infections associated with orthopedic devices like calcium phosphate (CaP) bone grafts, but when it occurs it represents a devastating complication with high morbidity and substantial costs since implant-associated infections are typically caused by microorganisms growing in biofilms (10). The eradication of infection is often only possible by removal of the implant and long-term antimicrobial treatment (11). Determining the risk of infection as well as

the evaluation of efficient treatment options and outcome and the infections characteristics is of considerable clinical relevance.

BONE GRAFT

Bone grafting is a surgical procedure that replaces missing bone in order to repair bone fractures that are extremely complex, cause a significant health risk to the patient, or fail to heal properly. More than one million patients per year need a bone grafting surgery in order to repair a bone defect resulting from a bone disease or a trauma (12-14). Bone transplantation is performed about 10-times more often than any other solid organ transplantation and is the most commonly performed. The use of bone graft is increasingly used in orthopedic surgery to fill bone defects or as antimicrobial delivery system (15), therefore, there is a high probability that infections associated with such devices could also increase. Bone has the ability to regenerate completely but to do so it requires a very small fracture space or some sort of scaffold. Bone grafts should have the characteristic of being completely reabsorbed and replaced as the natural bone heals over a few months.

The main type of bone grafts are (16):

1. **Autologous** (or autogenous) is a bone obtained from the patient's own body, often from iliac crest or the proximal tibia. It is also the most preferred due to the less risk of graft rejection since the graft originated from the same individual receiving the graft. A negative aspect is that an additional surgical site is required, which could leads to additional post-operative pain and complications.

2. **Allograft** is a bone obtained from cadavers, usually sourced from a bone bank, implying that the allograft is harvested from another individual than the one receiving the graft.

3. **Synthetic** variants are artificial bones created with similar mechanical properties of the bone. They are often created from ceramics such as calcium phosphate, Bioglass and calcium phosphate (e.g. hydroxyapatite (HA), tricalcium phosphate or other biocompatible substances). A positive aspect is that the mechanical properties are comparable to bone. Furthermore, growth factors can be added to the materials or mixed with bone marrow in order to increase the biological activity. Infection and rejection of the graft is also less of a risk and no additional surgical site is required.

Biological mechanisms

The main biological mechanisms of bone grafts are the following:

Osteoconduction. Osteoconduction occurs when the bone graft material serves as a scaffold for new bone growth. The bone graft material is used as a framework from osteoblast located in the margin of the defect bone.

Osteoinduction. Osteoinduction occurs when osteoprogenitor cells are stimulated to differentiate into osteoblast which enables the new bone formation.

Osteogenesis. In this case osteoblasts are originating from the bone graft material. Osteogenesis occurs when these osteoblast contribute to the growth of new bone in parallel with bone growth generated by the other mechanisms.

Osteopromotion. Osteorpomotion occurs when osteoinduction is enhanced without having an osteoinductive property.

Nowadays, the use of synthetic bone graft substitutes is replacing the traditional golden standard of autogenous bone grafting (12, 17, 18). In surgery procedure like filling fracture defects, reunion of long-bone, total joint revision or spine fusion, synthetic bone grafts substitutes are expected to be a safer and effective option (19).

BIOMATERIALS

A biomaterial is a synthetic material to be used in intimate contact with living tissue. A more precise definition of a biomaterial was given in 1986, at the Consensus Conference of the European Society for Biomaterials, when a biomaterial was firstly defined as "a nonviable material used in a medical device, intended to interact with biological systems". A definition that evolved within years until the last given in 2011 as “material intended to interface with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body” (European society for biomaterials (ESB), 1976).

The use of synthetic materials with the purpose to replace parts of the human body is old of ages. Gold dental prosthesis has been found in some Egyptian mummies. In the pre-Christian era the gold standard ‘biomaterials’ were copper and bronze. Those materials were subsequently causing problems because of their toxicity. The introduction of aseptic conditions in surgery in 1860 was a step forward in the improvement regarding the application of biomaterials. Since then, different materials were manufactured and used (as ivory or metallic prostheses, gold capsules, etc.). Polymethilmethacrilate (PMMA) began to be used in 1930 to anchor metallic prosthesis to bone and generate a permanent implant. One of the problems most encountered in orthopedic devices

is the biochemical compatibility at the interface between synthetic material and living tissue. Researchers are since then working to find stables and inert materials for devices.

In the past, ceramics and glasses were also commonly used in a wide range of medical-related applications, as for example eye glasses, chemical and chirurgical glassware or even in dentistry. The use of ceramic components inside the body is relatively new and nowadays, bioceramics are widely used for different applications inside the human body (Figure 2) (20).

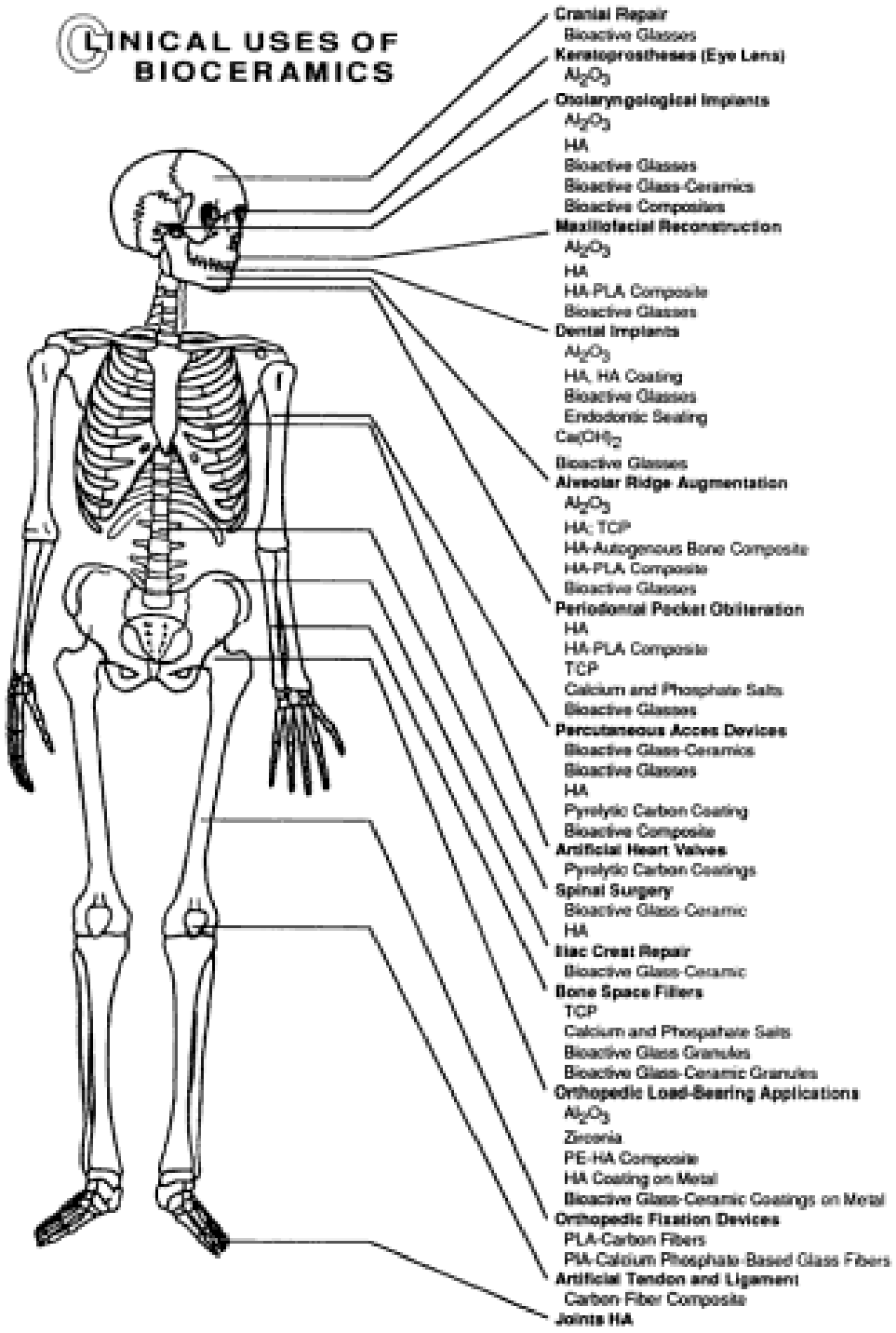

Figure2. Clincal uses of bioceramics. From (20).

BIOACTIVE GLASS

"*The human body rejects metallic and synthetic polymeric materials by forming scar tissue because living tissues are not composed of such materials. Bone contains a hydrated calcium phosphate component, hydroxyapatite and therefore if a material is able to form a HA layer in vivo it may not be rejected by the body*" was the hypothesis at the basics of the bioactive glass discovery and production made by Professor Hench in 1968.

Biocompatible tissue-bonding bioactive glasses (BAGs) are a group of synthetic silica-based bioactive materials with the unique bone bonding characteristic. This property was first discovered by Hench in the 1970s (21). The first bioactive glasses were composed of four-component system: SiO_2,Na_2O, CaO and P_2O_5, as found for example in bioglass 45S5 and S53P4 and the weight percentages of these oxides vary in different glasses (20). These glasses show a tendency to crystallization at high temperatures, therefore, to overcome this disadvantage, new bioactive glasses have been developed based on the Na_2O-K_2O-MgO-CaO-B_2O_3-P_2O_5-SiO_2 system (22). By this way it is possible to manufacture the glasses into different shapes as microspheres, fibers and porous implants leading to an additional advantage for the clinical use. In this study the bioglass S53P4 was tested since it is commercially available for treatment of bone defects.

Molecular mechanism

The chemical surface reaction is initiated as soon as the bioactive glass is in contact with body fluids. Within the first hour ions are released (Na, Ca, P, Si) from the bioactive glass surface leading to an increasing of the pH and the osmotic pressure (Figure 3). After only one day the silica gel layer forms on granule surface through polycondensation of the hydrated silica groups. The Ca^{2+} and PO_4^{3+} precipitate from the extracellular fluids onto the Silica rich layer (19, 23). Within one

week CaP crystallizes to natural hydroxyapatite. The bioactive glass bonds to the bone and promotes the oesteointegration.

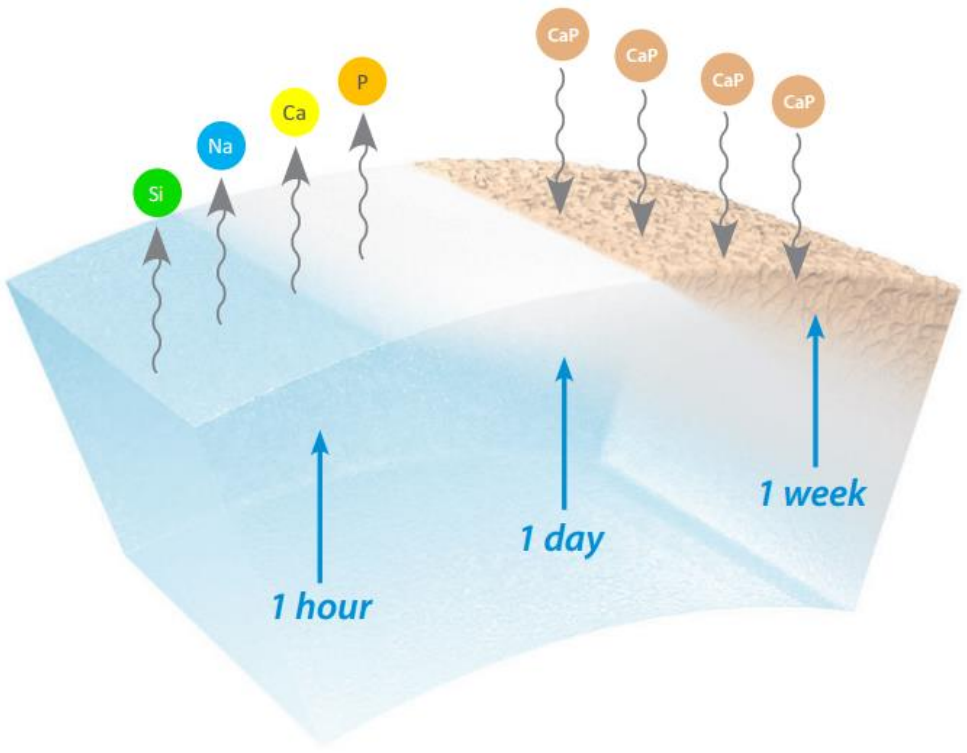

Figure3. Surface reaction cascades. The release of ions (Na, Ca, P, Si) increases the pH and the osmotic pressure, takes place within 1 hour and leads to an inhibition of bacterial growth on granule surface. Silica gel layer forms on granule surface and CaP precipitate on the surface (1 day). Within one week CaP crytallizes to HA enabling bone bonding. (Adapted from the brochure of BonAlive)

Compared with synthetic bioresorbable bioactive ceramics, such as HA, calcium phosphate and tricalciumphosphate, which are the most commonly used osteoconductive bone grafts substitutes (24, 25), silica-based bioactive glasses form themselves a group of bioceramics with unique

properties such as the osteopromotive ability, dissolution or more the inhibition of bacterial growth previously shown *in vitro* (Table 2).

	HA and HA/TCP	Bioactive glasses
Chemical composition	One or two chemical components (hydroxyapatite, tricalcium phosphate, or both)	Several components (at least four components)· • Original: four-component system of SiO_2, Na_2O, CaO and P_2O_5 (Hench et al. 1971) (4)· • Modified systems: Na_2O-K_2O-MgO-CaO-B_2O_3-P_2O_5-SiO_2 (Brink et al. 1997) (6)
Physical forms	Porous blocks or granules	Granules or sintered porous blocks, fibers and woven structures
Basic mechanism	Serves as osteoconductive surface	Forms chemical bonding with ongrowing new bone. Osteopromotive
Molecular mechanism of action *in vivo*	Not defined	Induce high local bone turnover
Regulation of bioactivity	Based only on HA/TCP ratio	Can be regulated by modifying the chemical composition
Resorption rate	TCP is resorbed fast (months) HA is slowly/very slowly resorbed (years, decades)	Resorption can be highly regulated (from weeks to years) by modifying the chemical composition
Mechanism of resorption	Involves chemical dissolution and osteoclastic resorption	Chemical dissolution
Antimicrobial properties	Not reported	Inhibition of bacterial growth *in vitro*, dependent on the chemical composition

Table 2. Comparison of synthetic HA/TCP and bioactive glasses as bone graft substitutes. Adapted from (19).

Nowadays BAGs are gaining use in both dental and orthopedic applications. Since the 1980s they have been applied in several surgeries such as the reconstruction of orbital floor fractures and defects of facial bones, in filling of frontal sinuses, in treatment of depressed tibial plateau fractures and in treatment of osteomyelitis (26-31). BAG S53P4 has been shown to have an angiogenesis-promoting properties and an antibacterial effect on some oral microorganisms as well as on a variety of clinically important aerobic and anaerobic pathogens (32-37). Several factors contribute to the antibacterial action of BAGs as the osmotic effects caused by the concentrations of ions dissolved from the glass and the increasing pH (20, 35).

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Chapter 7.

Activity of bioactive glass S53P4 on different planktonic microorganisms performed by microcalorimetry

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Submitted to Journal of Orthopedic Research, March 2014

Activity of bioactive glass S53P4 on different planktonic microorganisms performed by microcalorimetry

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Keywords: Bioactive glass S53P4, microcalorimetry, microorganisms

Running title: In vitro activity of bioactive glass on microorganisms

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ABSTRACT

We tested the activity of bioactive glass (BAG) S53P4 on planktonic microorganisms using a highly sensitive assay measuring growth-related heat production (microcalorimetry). BAG was tested in different granule sizes (0.5-0.8 mm and <45 µm) and volumes (1 and 2 g).

S. aureus (ATCC 29213), *S. epidermidis* (ATCC 35984), *E. faecalis* (ATCC 19433), *E. coli* (ATCC 25922) and *C. albicans* (ATCC 90028) were tested. Test strains were added in sealed glass ampoules containing 2, 25 ml Müller Hinton broth with or without BAG and placed into the microcalorimetry at 37°C. The heat flow produced was recorded by microcalorimetry and the total heat analysed. The heat flow pick decreased with the addition of different volumes BAG 0.5-0.8 mm, except for *E. coli* where a higher production of heat was detected. The heat flow pick decreased for all the microorganisms tested when <45 µm BAG was used.

BAG <45 µm showed a better activity against all the microorganisms than BAG 0.5-0.8 mm, this phenomena was better shown when analysing the area under the curve and by taking into account the heat produced by the controls. Microcalorimetry allowed real-time evaluation of antibacterial activity of bioactive glass.

INTRODUCTION

A new era in development of materials for use in medicine began in the 1970s, when Professor Larry Hench discovered glasses capable of forming interfacial bonding with bone. The compositions showing this special property were called bioactive glasses (1). Bioactive glasses (BAGs) are a group of surface reactive glass-ceramic biomaterial, which, thanks to their biocompatibility, are used as implant materials in the human body to repair and replace diseased or damaged bones. The composition of the first bioactive glass, named 45S5, was approved from the Food and Drug Administration (FDA), and after the invention of BAG 45S5, numerous glasses and glass ceramic with different composition have been developed and studied.

Bioactive materials are synthetic osteoconductive bone substitutes able to bind chemically to the bone, with documented antibacterial and angiogenesis-promoting properties (2-6). The BAG used in this study is named S53P4 and it is composed of SiO_2 (53%), Na_2O (23%), CaO (20%), P_2O_5 (4%), which are all naturally found in the human body. BAG S53P4 surface is activated by blood or physiological solutions. The first reaction that we can observe, when the glass is in contact with a solution, is the exchange of alkalis from the glass surface with H^+ and H_3O^+ in the same solution. The siloxane bonds in the glass interface subsequently starts breaking, leading to loss of soluble silica in the form of $Si(OH)_4$ in the solution. In the following step, the thickness of the silica rich layer is increased, thanks to the condensation and repolymerization of a SiO_2 rich layer on the surface. The layer grows by incorporation of Ca^{2+} and PO^2_4 ions from the solution: the CaP-layer mineralizes to natural hydroxyapatite, which will bond to the surrounding bone. The hydroxyapatite starts forming in a few hours, within 3-6 weeks osteogenic cells are organizing osteoid tissue between the granules and, in about 6-8 weeks, the lamellar bone is formed. On the other side, the presence of those ions can inhibit bacteria growth. Two principal mechanisms give rise to the bacterial growth inhibiting property and prevent the bacteria to attach on the surface: 1) the release of sodium (Na) and the subsequent formation of NaOH, increases slightly the pH (9-11) in the close vicinity of the surface of the glass. 2) the increasing of the osmotic pressure, which occures

when other ions, Ca, Si and P, are released (4). Several studies have been performed *in vitro* and *in vivo* to assess the best structrure fro the activity of the bioactive glass to satisfy the biological and medical need (6-13).

The purpose of this study was to evaluate the activity of BAG S53P4 and different parameters (amount and size) on planktonic *S.aureus*, *S. epidermidis, E. faecalis*, *E. coli* and *C. albicans* and by microcalorimetry.

MATERIALS AND METHODS

Test strains. *S. aureus* (ATCC 29213), *S. epidermidis* (ATCC 35984), *E. faecalis* (ATCC 19433), *E. coli* (ATCC 25922) and *C. albicans* (ATCC 90028) were used. Stocks of each strain were maintained on cryovials (Roth, Karlsruhe, Germany) at -80°C. Bacteria were cultured on MH agar plates, *Candida* was cultured on Sabouraud dextrose agar (SAB) for 24 h at 37°C. Inoculum was prepared by McFarland and the exact inoculum was determined by quantitative cultures. The final concentration in the ampules, filled with 2,25 ml MH, was ~10^5 CFU/ml.

Materials. The BAG S53P4 powders (BonAlive Biomaterials Ltd, Finland) were produced by Process Chemistry Center, Åbo Akademi University, Turku. Tested granules of BAG S53P4 used have two different sizes, 0.5-0.8 mm and <45 µm. The composition of this synthetic material is, by weight, SiO_2 53%, Na_2O 23%, CaO 20%, P_2O_5 4%. For each experiment we used 1 or 2 g of bioactive glass in each ampule.

Evaluation of thermal growth characteristics. An isothermal microcalorimeter (TAM III, TA Instruments, Newcastle, DE), equipped with 48 calorimeters and a detection limit of heat production of 0.2 µW was used. The ampoules were air-tightly sealed and introduced into the microcalorimeter, first in the equilibration position and after 15 minutes in the measuring position. Temperature was set at 37°C and heat flow was recorded for 48 h. Calorimetric ampoules (4 ml total volume) containing 2,25 ml of MH media and 1 or 2 g BAG were inoculated with 10^6 CFU/ml.

For the negative control the ampoules were filled with 2,5 ml BHI and 1 or 2 g of BAG. The growth-related heat production of tested bacterial strains was investigated. Heat flow in µW Joule was recorded and the total heat in J analysed.

BAG were also analysed alone without microorganisms addition in order to analyse the heat produced by the BAGs. The activity of BAG after 24h were also analysed by leaving the different BAGs in MH for 24h before addition of the tested strain (*S. aureus* in this case).

Data analysis. Microcalorimetry data analysis was accomplished using the manufacturer's software (TAM Assistant, TA Instruments, New Castle, DE). Figures were plotted using GraphPad Prism 6.01 (GraphPad Software, La Jolla, CA, USA).

RESULTS

Evaluation of thermal growth characteristics.

All the experiments were performed in duplicate by microcalorimetry, showing clear results in terms of heat production from microorganisms. In order to assess a correlation between the amount and sizes of BAG and the subsequent effect on the growth of the microorganisms, different sizes of granules (0.5-0.8 mm and <45 µm) and different amounts (1 and 2 g) have been tested. The effect of BAG on the growth of the different microorganisms analysed by microcalorimetry is shown in Figure 1 and 2. All the volumes and sizes of BAG used inhibited the growth of the tested microorganisms, except for *E.* coli with BAG 0.5-0.8 mm. Figure 1 and 2 also shown the total heat (considered as the area under the curve) obtained by microcalorimetry. The analysis of the total heat shown, for some experiments (Fig1. A, B, C, D and Fig2. C), that a higher heat is produced with 1 or 2 g of BAG compared to the growth control (without BAG). It also shown that, surprisingly, the BAG controls alone, in contact with the media, produced some heat (especially shown in Figure 4). Therefore, this phenomena was taken into account by subtracting for each experiment the heat flow produced by the controls (1 and 2 g), in order to consider only the heat produced by the microorganisms itself when added to the different BAG volumes (Fig.3 A and B). BAG 0.5-0.8 mm had a good activity especially against *S. aureus*, *S. epidermidis* and *C. albicans* (Fig.3A), wherease BAG <45 µm shown a very good activity against all the microorganisms (Figure. 3B). In order to try to avoid the heat phenomena produced by the BAGs alone we left the different BAGs (0.5-0.8 mm and <45 µm; 1 and 2g) in contact with the media for 24h and added *S. aureus* afterwards. The results showed that the heat phenomenon is no more present and that the BAGs still have their antimicrobial activity against *S. aureus* (Fig. 5).

DISCUSSION

We examined the antibacterial effect of BAG S53P4 by microcalorimetry on different gram-positive, such as *S. aureus* (coagulase-positive), *S. epidermidis* (coagulase-negative), *E. faecalis*,

on gram-negative bacteria, such as *E. coli* and a resistant strain, *E. coli* Bj HDE and on fungi, as *C. albicans*. We evaluated the activity of the glass on planktonic microorganisms by calorimeter, testing different parameters as the amount of glass and the size of glass. The tested strains are above the most common microorganisms involved in infections against which the glass could be used: osteomyelitis (14), tibial plateau fractures (15) and also benign bone tumors (16). Different aerobic (5) and anaerobic (4) bacteria have already been tested by flow cytometric (FCM) showing a better activity of BAG S53P4 compared to other type of BAGs. The good antibacterial activity of BAG S53P4 against planktonic bacteria, as gram+ and gram- is known. Nevertheless, this is the first study of BAG activity assessed with microcalorimetry and against fungi. The mechanism of action of the glass against microorganisms is due to the high pH and the osmotic pressure created in the media surrounding the BAG. Bacteria normally live with an optimal pH about neutral, nevertheless, the release of NaOH from the BAG lead to an acid pH around 9-11 (17). All the other released ions cause an increasing of the osmotic pressure, leading to the loss of water from the cell and perturbations of the membrane potential of the microorganism.

In these experiments, we found a correspondence between the volumes of glass: the more glass we used the more and faster effect we had, especially when BAG <45 µm were used. We tested two different sizes of granules, 0.5-0.8 mm and <45 µm, and according to our results the smallest one had a better activity than the biggest one, as expected. This can be explained if we consider that from a smaller surface we have a faster and easier release of ions and if we consider that the smaller are the granules the bigger is the total surface area for a given volume. We also shown that BAG alone produced some heat and in order to take this phenomena into account when using microcalorimeters, it is suggested to subtract the heat produced by the controls alone or leave the granules for 24h alone with the media before adding the microorganisms of interest.

In conclusion, this study demonstrates the effect of BAG against different species. These experiments show the good activity of BAG against *S. epidermidis* and *C. albicans* when using the larger diameter granules, and against the Gram – bacteria when using the smaller diameter

granules. The study also demonstrates the potential of microcalorimetry for real-time analysis. Several studies have previously shown the potential of the isothermal microcalorimetry to detect growth of different pathogens and determine the minimal hinibitory concentration (MIC) for different antimicrobial agents and microorganisms (18-20) In this study, microcalorimetry allowed high precision real-time evaluation of bioactive glass activity against various microbial species. Further studies need to be performed against different species, including resistant and anaerobe species, against biofilm and combined with antimicrobial drugs in order to have a better overview on BAG S53P4 antibacterial activity and his efficacy.

Acknowledgment: This study was supported by BonAlive Biomaterials Ltd, Finland.
We thank Dr. Fredrik Ollila, from BonAlive Biomaterials Ltd, Turku, Finland, for his kind help and useful suggestions. A special thanks to Annette Moter and her research group to giving me the opportunity of using her laboratory to perform part of the experiments.

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Legend to figures

Figure1. Heat flow (µW) and Total heat (J) for *S. aureus* (A), *S. epidermidis* (B), *E. faecalis* (C), *E. coli* (D) and *C. albicans* (E) in presence of BAG 0.5-0.8 mm.

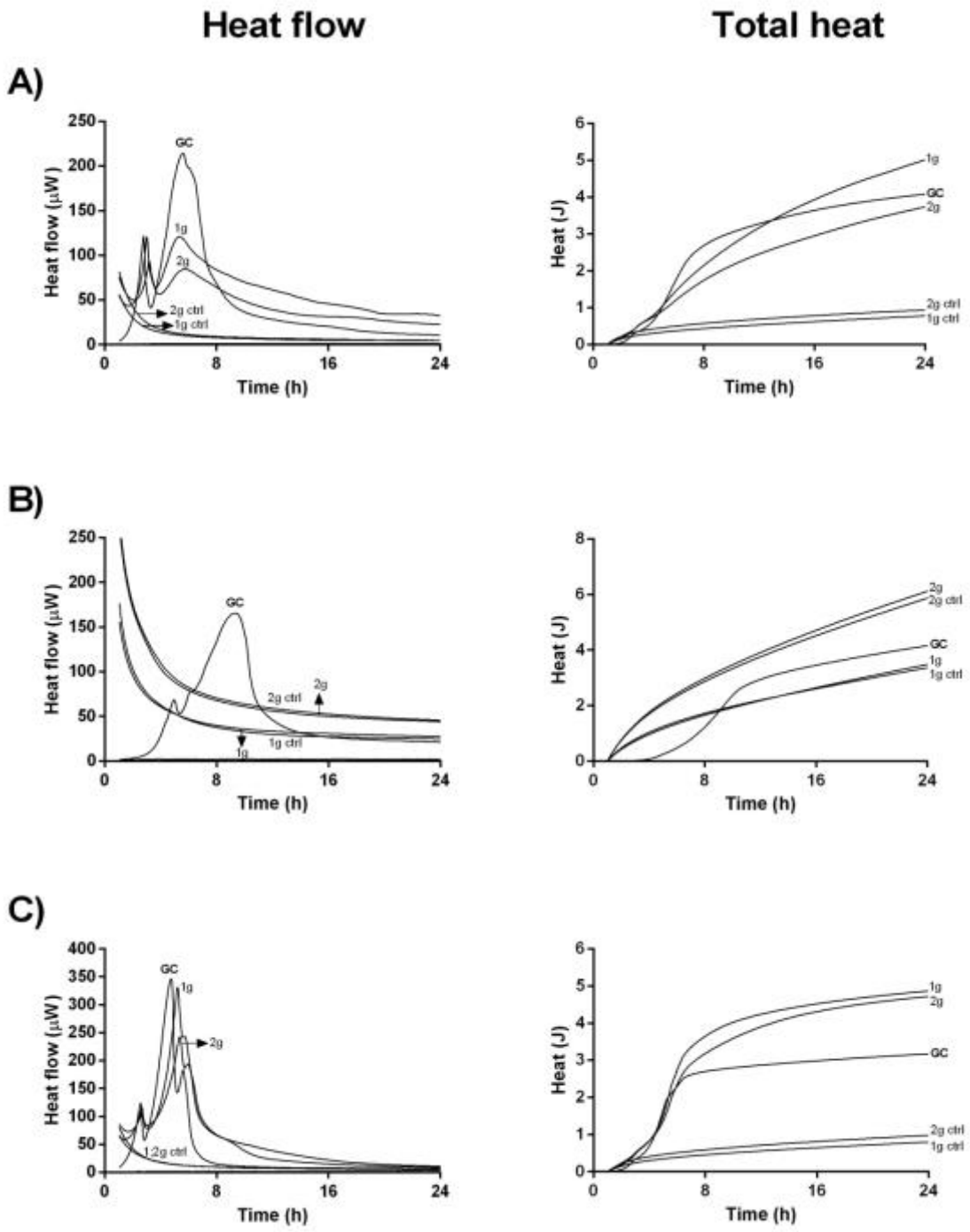
Figure2. Heat flow (µW) and Total heat (J) for *S. aureus* (A), *S. epidermidis* (B), *E. faecalis* (C), *E. coli* (D) and *C. albicans* (E) in presence of BAG <45 µm.

Figure 3: Total heat (J) for different microorganisms in presence of BAG 0.5-0.8 (A) and <45 µm (B) when subtracting the heat produced by the control for each experiment. CG, control growth; 1g, in presence if 1g of BAG; 2g, in presence of 2g of BAG. All the experiments where analysed at 24h except for *C. albicans* where 40h were considered.

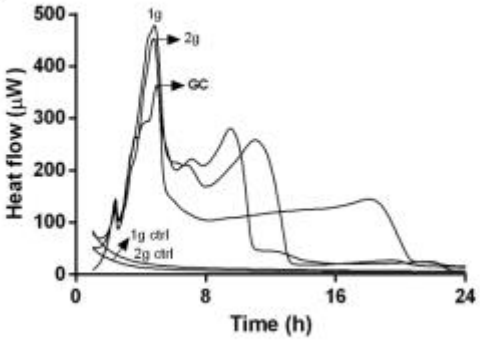
Figure 4: Thermodynamic activity of BAG with different sizes and volumes.
2g 0.5-0.8 mm = adding 2g of BAG 0.5-0.8 mm of size. 1 or 2g <45 µm = adding 1 or 2g of BAG <45 µm of size.

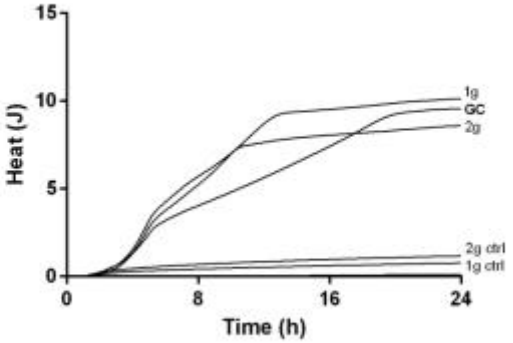
Figure 5. BAGs were put in MH for 24h before adding *S. aureus*. Experiments were performed in duplicate,

Fig. 1

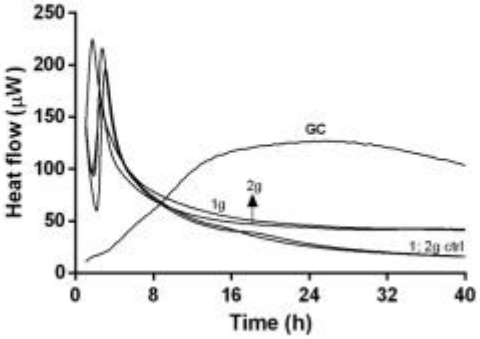

Heat flow
Total heat
A)
B)
C)
Heat flow (µW)
Heat (J)
Time (h)
GC
1g
2g
2g ctrl
1g ctrl
0
8
16
24

D)


Heat flow (µW)
Time (h)
1g
2g
GC
1g ctrl
2g ctrl


Heat (J)
Time (h)
1g
GC
2g
2g ctrl
1g ctrl

E)


Heat flow (µW)
Time (h)
GC
1g
2g
1, 2g ctrl

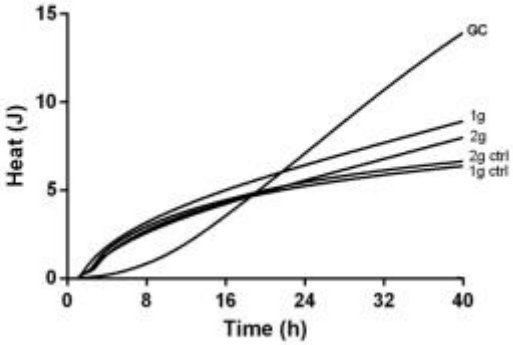
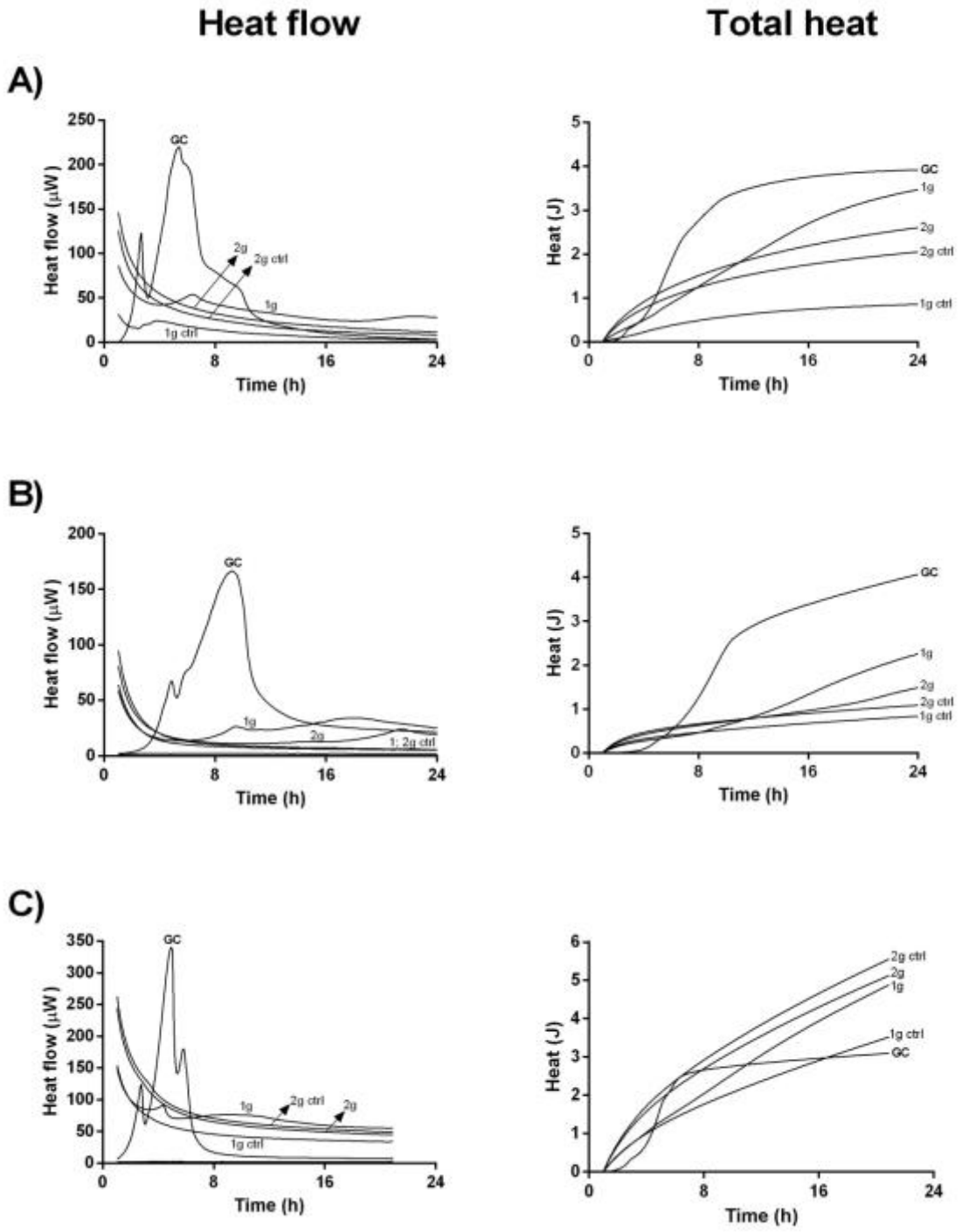
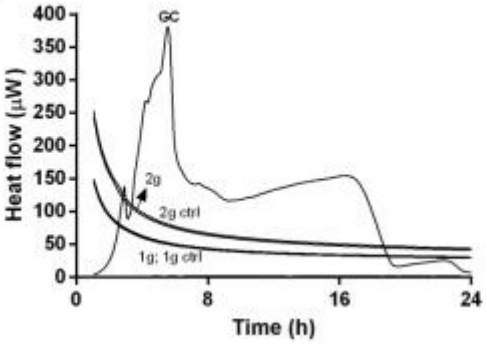
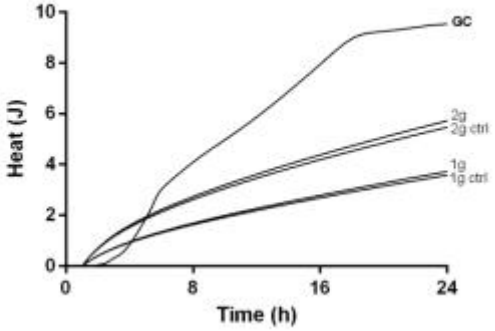

Heat (J)
Time (h)
GC
1g
2g
2g ctrl
1g ctrl

Fig. 2

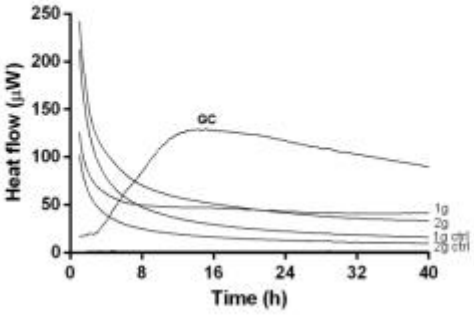

Heat flow
Total heat
A)
B)
C)
Heat flow (µW)
Heat (J)
Time (h)
GC
1g
2g
2g ctrl
1g ctrl
1; 2g ctrl

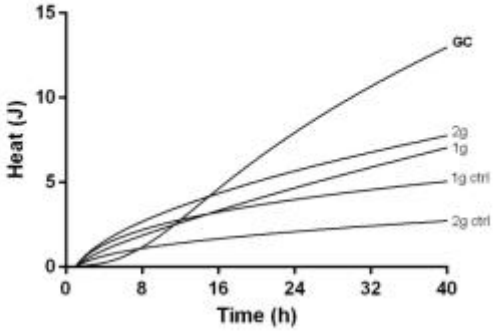
D)


GC
2g
2g ctrl
1g; 1g ctrl
Heat flow (µW)
Time (h)


GC
2g
2g ctrl
1g
1g ctrl
Heat (J)
Time (h)

E)


GC
1g
2g
1g ctrl
2g ctrl
Heat flow (µW)
Time (h)


GC
2g
1g
1g ctrl
2g ctrl
Heat (J)
Time (h)

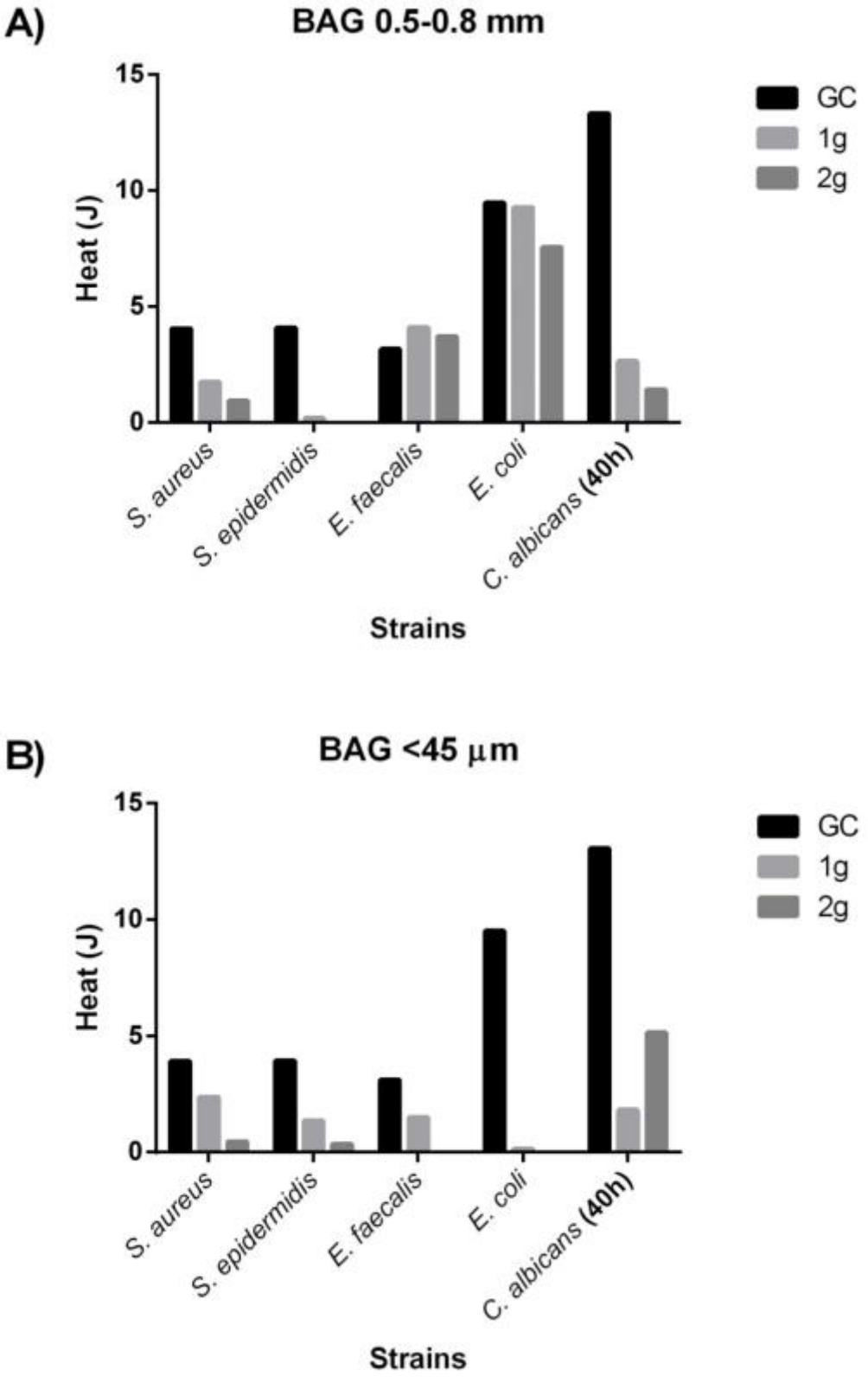

A)
BAG 0.5-0.8 mm
Heat (J)
15
10
5
0
GC
1g
2g
S. aureus
S. epidermidis
E. faecalis
E. coli
C. albicans (40h)
Strains
B)
BAG <45 µm
Heat (J)
15
10
5
0
GC
1g
2g
S. aureus
S. epidermidis
E. faecalis
E. coli
C. albicans (40h)
Strains

Fig. 3

Fig.4

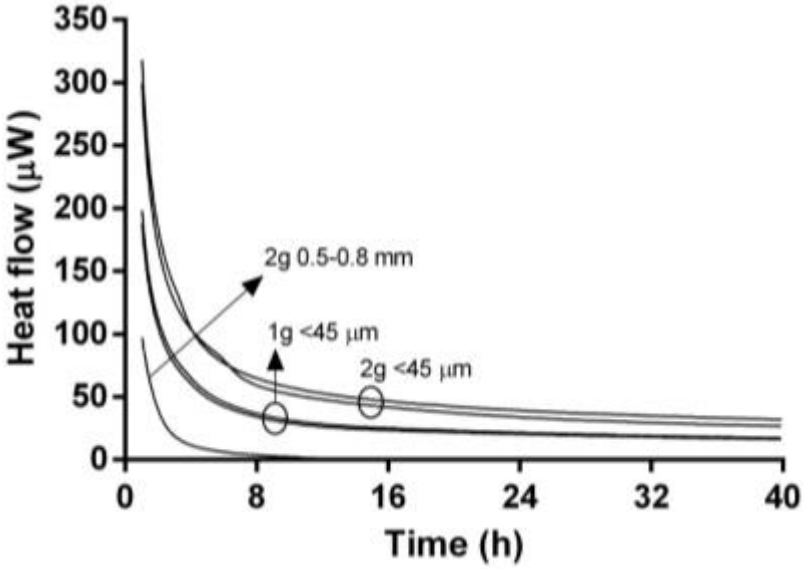
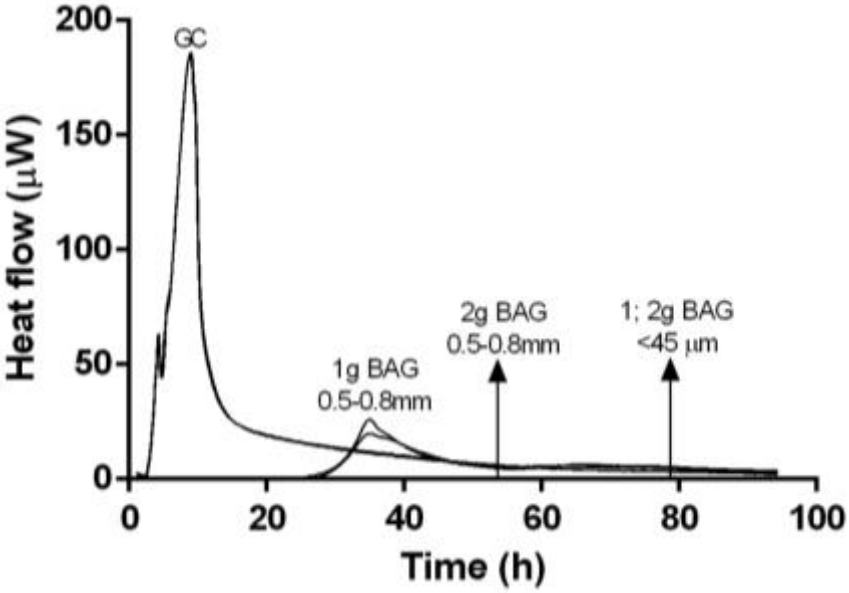

Heat flow (μW)
350
300
250
200
150
100
50
0
0
8
16
24
32
40
Time (h)
2g 0.5-0.8 mm
1g <45 μm
2g <45 μm

Fig.5


Heat flow (μW)
200
150
100
50
0
0
20
40
60
80
100
Time (h)
GC
1g BAG
0.5-0.8mm
2g BAG
0.5-0.8mm
1; 2g BAG
<45 μm

Chapter8. General conclusions and outlook

Isothermal microcalorimetry is commonly used for analysis of chemical and biochemical reactions, either consuming or producing energy. During the last decade, the utility and advantages of isothermal microcalorimetry for highly sensitive detection of microbial growth was demonstrated for a range of microorganisms.

In chapter 3, we demonstrated the potential of isothermal microcalorimetry, as a novel method for antifungal susceptibility testing of *Candida* spp. In chapter 8,we further evaluated our assay for studying the activity of BAG S53P4 against the most important infective microorganisms: gram-positive bacteria, such as *S. aureus* (coagulase-positive), *S. epidermidis* (coagulase-negative) and *E. faecalis*, gram-negative bacteria, such as *E. coli* and a resistant strain of *E. coli* Bj HDE (results not shown) and fungi, as *C. albicans*. Due to the different reproduction rates of the microorganisms, microcalorimetry appears to be a suitable and precise approach for performing antifungal susceptibility testing and inhibition growth testing by other materials, since the test interpretation is not based on subjective visual examination or spectrophotometric reading and the data is obtained continuously in real-time (as heat-flow curve). Besides enabling microbial rapid growth detection, microcalorimetry allowed also the detection of the exothermic reaction of the bioactive material, not detected with common used susceptibility testing.

The mechanism of action of the bioactive glass against microorganisms is due to the high pH and the osmotic pressure created in the media surrounding the material. Bacteria normally live with an optimal pH about neutral, nevertheless, the release of NaOH from the BAG lead to an acid pH around 9-11. All the other released ions cause an increasing of the osmotic pressure, leading to the loss of water from the cell and perturbations of the membrane potential of the microorganism. In these experiments, we tested two different sizes of granules, 0.5-0.8 mm and <45 μm, and according to our results the smallest one had a better activity than the biggest one. When smaller

particles are used, cation released from the surface during the reaction seems to be faster, therefore, higher values of pH are reached. Furthermore, the surface area increases for small particles than for higher diameter ones: the smaller are the granules the bigger is the total surface area for a given volume. With these experiments we also could show that bioactive glass alone produces heat.

In summary, this study also demonstrated the good activity of bioactive glass against microorganisms growth and the potential of microcalorimetry for real-time analysis. Considering the advantage of real-time growth monitoring, the microcalorimetric assay could be further optimized for a rapid detection of resistant isolates or anaerobic bacterial. Indeed, in a future study we will screen the activity of bioactive glass S53P4 against different anaerobic strains.

The work presented here represents only a small step towards the goal of understanding the interactions between bioactive glasses and microorganisms. Many possible directions can be taken as future developments. On one hand, some of the experiments here exposed could be try to be reproduced with other types of bioactive glasses, like, for example, bioactive glasses containing also other ions in their composition. Others bioactive glasses sizes and volumes could also be tested as well as the addition of antibiotics and antifungals, in this case the activity of the antimicrobials in a high pH environment should also be assessed. Characterization of the surface interaction sites with cells or proteins and simulated body fluids in order to have a higher similarity with the real biological situation in clinic, is indeed an interested area of study. Furthermore, the *in vivo* activity of the bioactive glass will also be a very exciting area to explore; indeed, in a future study we will screen the activity of bioactive glass in cage fluids in a guinea-pig model.