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- Biofilms are currently a major problem in hospitals, especially for patients with a weakened immune system.
- Here, we introduce bioluminescence imaging (BLI) to monitor and quantify the efficacy of antifungals against mature *Candida albicans* biofilms developed in a mouse model.
- BLI allows monitoring fungal load repeatedly throughout the course of treatment and comparison with baseline in the same animal, thereby providing a better readout of antifungal efficacy against biofilms under in vivo conditions.
- Among several antifungals tested, fluconazole failed to be effective while caspofungin displayed the most efficient activity of the echinocandins tested against mature *C. albicans* biofilms, as documented by BLI and confirmed by CFU counts.
- BLI showed to be a very good method to quantify *C. albicans* biofilm formation and its susceptibility to antifungal drugs under in vivo conditions.

Title

Bioluminescence imaging increases *in vivo* screening efficiency for antifungal activity against device-associated *Candida albicans* biofilms

Short title

BLI of antifungal activity against *C. albicans* biofilms

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Key words: *Candida albicans*, *in vivo*, biofilm, subcutaneous model, bioluminescence imaging, antifungal therapy, *Gaussia princeps* luciferase

Abstract

Fungal infections are a major problem for a growing number of mostly immune-compromised patients. *Candida albicans* is an important human fungal pathogen causing mucosal and deep tissue infections of which the majority is associated with biofilm formation on medical implants. The animal models that are currently in use to test antifungal drugs are limited to *ex vivo* analyses, requiring host sacrifice that excludes longitudinal monitoring of dynamic processes during biofilm formation in the live host. As a solution, we introduce non-invasive, dynamic imaging and quantification of *C. albicans* biofilm formation *in vivo* and subsequent evaluation of treatment efficacy against these biofilms by using bioluminescent *C. albicans* in a catheter-associated mouse model. Bioluminescence imaging (BLI) allowed us to evaluate baseline biofilm load before start of therapy, which is necessary for the correct evaluation and interpretation of anti-biofilm efficacy *in vivo*. Moreover, we demonstrate that our BLI approach monitors the anti-biofilm activity of different antifungal agents efficiently *in vitro* and *in vivo*. In this study, BLI revealed superior anti-biofilm activity for echinocandins compared to amphotericin-B and fluconazole. *In vitro*, anidulafungin showed the highest anti-biofilm activity, followed by micafungin and caspofungin. *In vivo*, caspofungin significantly decreased the biofilm fungal load, as documented by the lower BLI signal and confirmed by CFU counts. In conclusion, our BLI approach increases the power and efficiency of screening and validation of antimycotics under both *in vitro* and *in vivo* conditions, thereby refining preclinical therapy studies.

1. INTRODUCTION

Biofilm formation by fungal pathogens is a major problem in an ever-growing patient population. Especially *C. albicans* biofilms are formed on the surface of many medical implants, leading to the need to physically remove and replace the device to prevent life-threatening device-associated systemic infections [1]. Biofilms are not only offering protection against host defenses, but they are also difficult to treat as they are often resistant to the classical antifungal drugs [2]. For testing antifungals against *C. albicans* biofilm formation in the complex environment of a live host, several different animal models have been developed that are indispensable in the search for novel compounds and strategies to treat fungal biofilms [3]. In these animal studies, fungal load in biofilms is traditionally analyzed *post mortem*, requiring host sacrifice and enumeration of microorganisms from individual explanted biofilms to evaluate the efficacy of antifungal treatment. This means that preclinical screenings are associated with a high effort and ethical burden.

For studying device-associated infections and their treatment, a subcutaneous catheter rat model system has been developed that has proven useful and relevant for the evaluation of antifungal efficacy against biofilms formed on the inside of implanted catheters [4]. One of the assets of this specific model is that multiple biofilms per animal can be analyzed, thereby improving the throughput for *in vivo* therapy screening studies. In order to further improve the efficiency of this type of therapy studies, the rat biofilm model has been successfully reproduced in mice, which significantly reduces costs and holds potential for biofilm research in different transgenic mouse strains [5]. Moreover, this model has been made compatible for bioluminescence imaging (BLI), thereby tackling the challenge of detecting a quantifiable *in vivo* BLI signal from biofilms formed on the inside of catheters by using a *Gaussia princeps*-luciferase-tagged *C. albicans* strain [5-7]. This strain has been genetically engineered to constitutively express gLuc fused with a cell wall protein, thereby locating the

bioluminescence reaction extracellularly. This strategy has proven to lead to successful BLI of *C. albicans* cells in both the yeast and hyphal stages as well as in biofilms [5, 6, 8].

Based on this recent technological advance and driven by the need for an efficient means to screen the activity of potential antimycotics under *in vivo* conditions, we set out to investigate the potential of BLI to increase the throughput and efficiency of preclinical screening studies for antimycotic activity against device-associated biofilms *in vivo*. Our working hypothesis is that the quantification of a baseline value of fungal load in the mature biofilm before start of treatment followed by longitudinal evaluation of the treatment effect on fungal load, can overcome the obscuring of true efficiency of the antifungal activity of a compound due to inherent variation of biofilm load between different catheters and animals. To this end, we first evaluated different antifungals for their activity against fungal biofilms with BLI *in vitro* and compared this with colony forming unit (cfu) counting, the current gold standard readout for evaluation of fungal load. In a second step, we longitudinally monitored and quantified mature biofilm formation with BLI, before and after start of treatment in an established mouse model for device-associated *C. albicans* biofilm formation, and compared the imaging-based results with *ex vivo* fungal load quantification.

2. MATERIALS AND METHODS

2.1 Ethics

All animal experiments were performed in accordance with the KU Leuven animal care guidelines and were approved by the Ethical Committee of the KU Leuven (project number P125/2011). All animal experiments comply with the ARRIVE guidelines.

2.2 Strains

We used the bioluminescent *C. albicans* strain SKCA23-*ACTgLuc* and the SC5314 as a wild-type control. The bioluminescent strain maintains the same growth characteristics as the wild type strain as described before [5].

2.3 *In vitro* biofilm formation

Cells were grown overnight on YPD plates at 37 °C, washed and resuspended in PBS. The cells were counted to prepare a *C. albicans* cell suspension in RPMI medium (RPMI-1640, R6504, Sigma-Aldrich, Diegem, Belgium; with L-glutamine and without sodium carbonate buffered with MOPS, pH 7.0). To screen antifungal activity against biofilms *in vitro*, sterile 96-well polystyrene cell culture plates were inoculated by adding 100 µl per well of a 1×10^7 cells/ ml *C. albicans* cell suspension in RPMI. The *C. albicans* cells were allowed to adhere to the bottom of the wells by incubation for 90 mins at 37°C, after which non-attached cells were washed away in two steps with PBS. Attached cells were then submerged again in 200 µl of fresh RPMI medium and incubated further at 37 °C for 24h to allow the formation of mature biofilms.

2.4 *In vitro* antifungal screening

In vitro antifungal treatment was initiated after 24h when mature biofilms had been formed (baseline). Twofold serial dilutions of amphotericin-B, fluconazole (Sigma-Life sciences), micafungin (Astellas, The Netherlands), anidulafungin (Pfizer, Belgium), caspofungin (Cancidas, Merck Sharp & Dohme, Belgium) or vehicle alone (control) were added to the wells, covering a concentration range from 64 mg/L to 0,125 mg/L. All conditions were tested in triplicate. Plates were further incubated for 24 h or 48h at 37°C (separate plates), before being quantified by BLI.

2.5 *In vitro* BLI

To obtain *in vitro* bioluminescence measurements, the well plates containing biofilms and antifungal treatment were placed in a BLI-camera (IVIS Spectrum, Perkin Elmer, Waltham, MA, USA) after addition of native coelenterazine (CTZ; Nanolight Technologies, Pinetop, AZ, USA) to all the wells at a final concentration of 6 μ M in PBS [5, 8]. Subsequent frames were acquired with a 5-10 seconds exposure time depending on the signal intensity, at medium binning. The BLI signal intensity was quantified using Living Image software (version 4.2, provided by the manufacturer) and reported as photon flux per second (p/s) for a region of interest (ROI) of fixed size covering each well.

2.6 *In vivo* biofilm model

Female, 8 weeks-old Balb/C mice (internal stock, KU Leuven animal facility) were kept in individually ventilated filter top cages with free access to standard food and water *ad libitum*. One group of animals was immunosuppressed starting 24 hours before and during the entire experiment by adding dexamethasone (0.4 mg/ L; Fagron SAS, Paris, France) to their drinking water [9]. A second group of animals was immune suppressed under a more moderate regime with half the dose of dexamethasone. All animals were administered ampicillin (ampicillin sodium powder 0.5 g/ L; Duchefa Biochemie, Haarlem, The Netherlands) via the drinking water to prevent possible bacterial infections [9]. *In vivo* biofilms were grown on the inside of catheter pieces implanted subcutaneously on the mouse's back, as described previously [5, 7]. Three *C. albicans* colonized catheter pieces were inserted on the lower left of the back and three more on the right, so that each mouse carries in total six catheter pieces. After the last imaging time point, mice were euthanized by cervical dislocation and catheters sterilely explanted and washed for further quantification of biomass.

2.7 *In vivo* antifungal screening

Mature biofilms were left to form *in vivo* for two days after catheter implantation. The mice underwent BLI to assess the baseline of mature biofilm fungal burden on day 2, after which treatment was started and continued during 7 consecutive days (until day 9 post implantation) by daily intravenous injection of vehicle alone (negative control) or the following antifungal compounds with the respective dose: caspofungin (10 mg/kg/day), anidulafungin (10 mg/kg/day), micafungin (30 mg/kg/day), fluconazole (125 mg/kg/day), amphotericin-B (5 mg/kg/day).

2.8 *In vivo* BLI

The animals were anesthetized using a gas mixture of 1.5-2 % isoflurane in oxygen and imaged at 2, 6 and 9 days after catheter implantation using the IVIS Spectrum system as described before with minor modifications [8]. Freshly prepared CTZ (100 µL, 1.2 mM in PBS) was topically applied simultaneously to each catheter trio by subcutaneous injection. Immediately thereafter the animal was placed in the BLI camera. Consecutive scans with acquisition time of 20-60 seconds (depending on the signal intensity) were acquired starting immediately (*i.e.* within 10 seconds) after CTZ administration until maximum signal intensity was reached [8].

2.9 Biofilm fungal burden quantification

For fungal burden assessment by cfu quantification, biofilms formed on the bottom of cell culture plates and explanted catheters were washed twice with PBS, sonicated for 10 min at 40000 Hz in a water bath sonicator (Branson 2210) and vortexed for 30 s in PBS. Original samples, 1:10 and 1:100 dilutions were plated on YPD agar in duplicate. Cfus were counted after incubating the plates for 2 days at 37 °C.

2.10 Statistics

For statistics and representation of the results, we used Microsoft Office Excel 2007 (Microsoft Corporation, Seattle, WA, USA) and GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Significance tests were performed using two-way (repeated measures) ANOVA followed by Bonferroni tests for analysis of the longitudinal data, and with one-way ANOVA and Dunnett tests for comparison of data across one single time point. P-values of < 0.05 were considered significant, and represented as follows: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.005$.

3. RESULTS

3.1 BLI allows for rapid and efficient screening of antifungal activity against biofilms formed under *in vitro* conditions.

In order to evaluate BLI for the comparative screening of the fungistatic/ fungicidal activity of different compounds against biofilms under *in vitro* conditions, mature biofilms were left to form on the bottom of 96-well plates. After completion of mature biofilm formation, the different antifungal compounds – three echinocandins, fluconazole and amphotericin-B – were added to the biofilms in a broad concentration range to evaluate sensitivity to the compound. The biofilms were incubated with the compounds for 24h and 48h before assessment of the results with BLI. Fungal biomass was evaluated in parallel with cfu counting and xtt-reduction assay (see Supplementary Data online) as measures of fungal proliferation and viability in order to validate the imaging results.

Bioluminescent signal was reliably detected for biofilms formed by the bioluminescent SKCA23-*ACTgLuc* strain, whereas no signal (only background) was detected from the substrate (CTZ) alone or from biofilms formed by the wild-type SC5314 control *C. albicans*

strain after 24 and 48h (Figure 1). Biofilms treated with antifungal compounds elicited a less intense BLI signal, depending on the concentration of added compound. The BLI results pointed to anidulafungin as the compound with most efficient anti-biofilm activity, followed by the other echinocandins micafungin and caspofungin, for concentrations up to 4 and 8 μ g/mL, respectively. In this assay, the echinocandins showed superior anti-biofilm activity compared to amphotericin-B and fluconazole in terms of their efficiency after both 24h and 48h of incubation (Figure 1).

In general, BLI, cfu counts and xtt reduction assay yield similar results regarding anti-biofilm activity of the different compounds, both after 24h (Figure 2) and after 48h (Figure 3 and Supplementary Data online). More specifically, we observed a distinct BLI signal from bioluminescent biofilms compared to biofilms formed by wild-type cells, whereas the cfu and xtt-reduction assay data are similar for both gLuc-expressing as wild-type *Candida* cells. This validates the specificity of the bioluminescence reaction upon addition of substrate and excludes any potential background BLI signal generated by potential interaction between CTZ and the presence of biomolecules or antifungal compounds.

The BLI, cfu and xtt reduction assay data were highly congruent, especially concerning the echinocandins that were all found to act against mature biofilms in the highest concentration range, as confirmed by both BLI, cfu counts and xtt reduction analysis. Amphotericin-B and fluconazole were included as established examples of compounds with high and low activity against *C. albicans* biofilms, and this showed indeed in both the BLI, xtt and cfu data (Figure 1, 2 and 3, and Supplementary Data online). The test results after 48h of incubation with antifungal compounds (Figure 3) were largely similar to the results after 24h of incubation (Figure 2). Any differences between the results after 24h and 48h may reflect differences in compound stability at physiological conditions (pH and temperature). For 48h-long

amphotericin-B-treated biofilms, the BLI assay indicates inefficacy of the compound to eradicate cells completely after 48h compared to cfu counts and xtt-reduction assay, indicating impaired fungal proliferation capacity and viability. This may possibly be explained by the lower stability of AMB after 48h of incubation [10, 11]. Whereas for planktonic cells the labile character of AMB may not be an issue as cells die rapidly, when challenged within biofilms this may be different. Depending on the antifungal, 24h may therefore be the most reliable period for efficient screening of the anti-biofilm activity of a compound under *in vitro* conditions.

The results allowed us to observe that in general, the cfu data has a much larger standard deviation than the BLI data, indicating a favorable reproducibility of the BLI data. We can also observe a paradoxical growth effect after 48h of incubation for caspofungin, and to a lesser extent for micafungin but not for anidulafungin (Figure 2, 3 and Supplementary Data online). This same order was recently observed when screening a large collection of *C. albicans* strains [12]. This effect was picked up differently by BLI on the one hand and the cfu and xtt assays on the other hand. This type of differences in sensitivity to the *in vitro* biofilm behavior reflects how the different assays evaluate antifungal efficacy differently: where cfu analysis and the xtt reduction assay are both essentially measures of cell viability and their ability to proliferate, BLI measures the expression of luciferase on each cell, providing an immediate estimate of the number of cells.

3.2 BLI allows for efficient screening of antifungal activity against C. albicans biofilms under in vivo conditions

To evaluate BLI for antifungal activity testing against device-associated biofilms formed *in vivo*, we implanted inoculated catheters subcutaneously on the back of mice and left them to form mature biofilms for two days. Once mature biofilms had formed, treatment of the mice

was started with different antifungals on a daily basis during one week via intravenous administration. The mice were followed up longitudinally with BLI to monitor the fungal load of the mature biofilms formed inside the implanted catheter pieces. BLI scans were acquired just before treatment was started (2 days post implantation (p.i.)) to establish a baseline, and then 4 and again 3 days later (corresponding to 6 and 9 days p.i.) to evaluate the potential effect of treatment for each mouse individually.

In a first set of mice, we evaluated the antifungal activity against *in vivo* biofilms with BLI for micafungin, anidulafungin, caspofungin and fluconazole compared to sham-treatment (with vehicle) as a negative control. Treatment efficacy of *C. albicans* biofilms formed *in vivo* could be established in live animals by a lower BLI signal intensity, such as that observed for caspofungin-treated mice compared to sham-treated mice (Figure 4).

Quantification and analysis of the longitudinal BLI results, taking into account baseline and intermediate BLI signal intensities, revealed a significant treatment effect for all tested antifungals (compared to sham-treatment) after one week of treatment (Figure 5, panel A, left). From the same graph, a certain degree of variation in BLI signal intensity and therefore fungal load in biofilms at baseline became apparent. Repeated measures ANOVA returned indeed a significant effect for matching ($p = 0.0281$), thereby providing support for the relevance of establishing baseline values before treatment and the importance of longitudinal follow-up, in order to account for inherent variation between animals and biofilms.

When plotting BLI signal intensities relative to the baseline values before the start of treatment, the real treatment effect, *i.e.* the effect on limiting or diminishing biofilm growth relative to the extent of the fungal load when treatment was initiated, became apparent (Figure 5, panel B, left). The imaging results then showed a trend towards inhibition of biofilm growth compared to sham-treatment for micafungin, anidulafungin, caspofungin and

258 fluconazole, with the most pronounced effect to be observed for caspofungin that was
259 significant ($p = 0.0208$) after treatment for 7 days.

260 After the last imaging time point, we sacrificed the animals and compared the cfu counts from
261 biofilms inside individual explanted catheters to the *in vivo* BLI data for validation of the
262 imaging results. The cfu counts retrieved were consistent with the *in vivo* imaging results: for
263 all antifungals there were catheters retrieved that showed a lower fungal load compared to
264 sham-treatment (Figure 5 panel C, left). For caspofungin, the treatment effect based on cfu
265 counts reached significance and even some catheters could be retrieved that turned out to be
266 sterile. Of note, the modest treatment effect illustrated by several catheters with lower cfu
267 numbers was picked up by the longitudinal *in vivo* BLI data, demonstrating its sensitivity for
268 evaluating fungal load *in vivo* (compare Figure 5 panels A and B with panel C).

269 In order to assess the ability of longitudinal BLI to evaluate antifungal activity against
270 biofilms formed under different immune conditions, we included a second group of mice that
271 were immune suppressed with half the dose of dexamethasone during biofilm maturation and
272 subsequent treatment efficacy testing for amphotericin-B, anidulafungin and fluconazole
273 compared to placebo. BLI at two days p.i. confirmed mature biofilm formation under these
274 conditions, before treatment was started (Figure 5, panel A, right graph compared to left
275 graph).

276 Whereas the BLI signal intensities for all treatment groups were of similar order of magnitude
277 at the last time point, when taking into account the longitudinal data and the baseline values
278 for the biofilms before onset of treatment, a trend towards a positive treatment effect for
279 anidulafungin and even more for amphotericin-B could be observed – although none of the
280 differences reached significance (Figure 5, panel A, right).

Normalizing for differences at baseline by representing the BLI signal intensity relative to the initial values measured at baseline confirmed the trend for a modest but not significant treatment effect of amphotericin-B and anidulafungin (Figure 5, panel B, right), whereas fluconazole did not show activity against mature biofilms formed under these conditions.

The cfu counts from explanted catheters retrieved from the animals after the last imaging time point were all in concordance with the imaging results: higher BLI signal intensities seen for fluconazole and vehicle-treated catheters corresponded to higher average cfu counts from explanted biofilms (Figure 5, panels A & C). No significant treatment effect could be established for fluconazole based on the cfu counts, although the trend towards lower fungal load for amphotericin-B and anidulafungin-treated biofilms observed with BLI, was confirmed.

4. DISCUSSION

We have introduced and evaluated a multi-temporal non-invasive imaging assay based on BLI for monitoring and quantifying the potential effect of antifungal compounds against *C. albicans* biofilms formed under *in vivo* conditions. The results from the longitudinal *in vivo* BLI assay were validated against the gold standard *ex vivo* methods to determine viable fungal load from explanted catheters by cfu counting. We demonstrated that the true efficacy of an antifungal compound is obscured by inherent variation of the fungal load in the mature biofilm at the onset of treatment, thereby pressing the need for the non-invasive establishment of baseline values to efficiently evaluate the true *in vivo* antimycotic activity of a compound. Solutions that enable longitudinal assessment of the fungal load in biofilms, such as the technique we propose here, are the only means of evaluating the baseline value at therapy onset and therefore of the true antifungal capacity of a compound under *in vivo* conditions,

and this for each animal individually. Thereby, variation between different animals and device-associated biofilms can be overcome, and the power and efficiency of preclinical therapy screenings increases significantly.

Longitudinal follow-up of the same animals and biofilm-loaded catheters over the course of mature biofilm formation and impact of treatment has important additional ethical, economical and statistical advantages. These are associated with the possibility to use repeated measures ANOVA to analyze the quantitative data and the resulting manifold reduction of the number of animals needed for this type of studies, in concordance with the 3R's of Russel and Burch for animal experimentation [13]. For the evaluation of antifungal efficacy against biofilms under different immune conditions, the longitudinal nature of the imaging data can reveal differences in biofilm growth dynamics and treatment outcome that are influenced by the immune status of the animals. Given the recently recognized importance of animal models for the discovery and screening of novel antifungals under *in vivo* conditions [3], this novel approach is likely to have an important impact on increasing the throughput and efficiency of animal studies.

In addition, we propose a BLI-based screening assay for the more efficient screening of antifungal activity against biofilms formed under *in vitro* conditions that is directly translatable to an *in vivo* experimental setting. This approach provides a more rapid, virtually instant, less variable and less labor-intensive method to evaluate fungal load in device-associated biofilms formed on the bottom of well plates compared to standard cfu counting after sonication and serial plating. It provides valuable information that can add to our insights regarding antifungal action, resistance development and the combined effect of compounds, as well as into additional phenomena such as paradoxical or trailing growth effects that are known to occur for *C. albicans* in the presence of azoles and echinocandins [14-17].

330 To evaluate our *in vitro* and *in vivo* imaging-based screening methods, we included three
331 different echinocandins, fluconazole and amphotericin-B. The activity of these compounds *in*
332 *vitro* and against systemic infections has been established before [18-21]. The antifungal
333 potential of echinocandins against *C. albicans* biofilms *in vivo* is less well documented. It has
334 been evaluated in guinea pigs and in a rat model similar to the mouse model used in this study
335 [22, 23]. Nett and Andes have recently summarized the different *in vivo* biofilm models and
336 treatment strategies [3]. The echinocandins and amphotericin-B show better efficacy against
337 biofilms, whereas biofilms are known to be more resistant against azoles [3]. Although our
338 primary aim was to establish an improved *in vivo* antifungal screening method, this is also the
339 first report of the use of different echinocandins for treatment of device-associated biofilms in
340 a mouse model. The dose we chose for treatment in our study was based on the dose range
341 evaluated in recently published reports on the use of echinocandins in rodents, albeit for
342 different models of infection, organisms and route of administration studied [22-27]. Our data
343 indicate that echinocandins, in particular caspofungin, have superior therapeutic efficacy at
344 the tested concentrations, consistent with earlier reports using rodent models [22, 23, 28].
345 More specifically, the efficacy of echinocandins against biofilms formed on medical devices
346 has been demonstrated before in a rat model similar to our mouse model: caspofungin (5
347 mg/kg/day), anidulafungin (10 mg/kg/day) and micafungin (30 mg/kg/day) significantly
348 decreased the number of *C. albicans* biofilm-forming cells retrieved from catheters. In
349 contrast, fluconazole (125 mg/kg/day) was ineffective in a rat model similar to our mouse
350 model [29, 30]. AMB was not tested before in a rodent catheter model, but did show efficacy
351 against biofilms formed on subcutaneous Teflon cages in a guinea pig model (2.5 mg/kg/day)
352 [31], in line with our results. In the same guinea pig model, echinocandins demonstrated
353 superior anti-biofilm activity with caspofungin (2.5 mg/kg/day), thereby outperforming
354 anidulafungin (20 mg/kg/day), again consistent with our findings. In the mouse central

venous catheter model, caspofungin was also demonstrated to be able to treat and prevent biofilm formation [32]. However, total efficacy was not reached at the doses tested in these studies and ours, illustrating the difficulty of eradicating *C. albicans* biofilms from implanted devices *in vivo* and the need for further optimization of treatment strategies. Our BLI approach is likely to have an important contribution towards assessing novel anti-biofilm strategies and hereby contributing to their development, as it has the potential to be extended to all these biofilm models.

5. CONCLUSION.

In this manuscript, we present a longitudinal imaging-based approach to monitor fungal biofilms, forming an important step forward for the evaluation of the therapeutic efficacy of antifungal compounds in rodent models of device-associated *C. albicans* biofilm formation. It will greatly improve cost and time efficiency of preclinical therapy studies, enhance the scientific value of outcome measures, enable the evaluation of antifungal potential under different immune conditions and increase the power of animal experimentation, thereby significantly reducing the ethical burden associated with this type of studies.

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9. FIGURE LEGENDS

Figure 1: BLI allows *in vitro* screening of antifungal activity against mature biofilms treated for 24 or 48 hours. Graphs of BLI signal intensity of biofilms formed by SKCA23-*ACTgLuc* and SC5314 (wt control) *C. albicans* cells on the bottom of 96-well plates, after addition of CTZ (or CTZ alone as a control for background luminescence), untreated ('no AF') and treated with decreasing concentrations of antifungal compounds (as indicated above the graphs) during 24h and 48h. (Error) bars indicate the mean and SD of triplicate samples. A typical bioluminescence image of the *in vitro* setup is shown on the center left.

Figure 2: Comparison of BLI and cfu screening for antifungal activity against mature biofilms treated during 24 hours. Graphs of BLI signal intensity (left) and cfu counts (right) quantified for mature biofilms formed by SKCA23-*ACTgLuc* and SC5314 (wt control) *C. albicans* cells on the bottom of 96-well plates, after addition of CTZ (or CTZ alone as a control for background luminescence), untreated ('no AF') and treated during 24h with different concentrations of antifungal compounds (as indicated above the graphs). (Error) bars indicate the mean and SD of triplicate samples.

Figure 3: Comparison of BLI and CFU screening for antifungal activity against mature biofilms treated during 48 hours. Graphs of BLI signal intensity (left) and CFU counts (right) quantified for mature biofilms formed by SKCA23-*ACTgLuc* and SC5314 (wt control) *C. albicans* cells on the bottom of 96-well plates, after addition of CTZ (or CTZ alone as a control for background luminescence), untreated ('no AF') and treated during 48h with different concentrations of antifungal compounds (as indicated above the graphs). (Error) bars indicate the mean and SD of triplicate samples.

Figure 4: Antifungal activity against biofilms can be evaluated under *in vivo* conditions with BLI. Typical *in vivo* BLI results for a mouse under high immune suppressive conditions treated with caspofungin and a mouse treated with placebo for a week, imaged 9 days after catheter implantation.

Figure 5: BLI-based quantification of *in vivo* antifungal activity against biofilms. Panels A: graphs of *in vivo* BLI signal intensity quantification from biofilms formed by SKCA23-*ACTgLuc*-expressing *C. albicans* cells on implanted catheter pieces, before (2 days p.i., 'in vivo baseline') and after (6 and 9 days p.i.) of treatment with antifungals or placebo (vehicle control), for two independent experiments carried out under high (graphs on the left) and moderate (graphs on the right) immune suppression conditions. The longitudinal data was

analyzed with two-way (repeated measures) ANOVA followed by Bonferroni tests, with the significance results only for the effect of treatment indicated on the graphs. Panel B: graphs of *in vivo* BLI signal intensity from panel A expressed as n-fold increase respective to baseline, analyzed with one-way ANOVA and Dunnett tests for the last time point (day 9). Panel C: Graphs of CFUs quantified from biofilms formed by SKCA23-*ACTgLuc* *C. albicans* cells explanted from mice after 9 days from the two independent experiments, analyzed with one-way ANOVA and Dunnett tests. (Error bars indicate SEM of replicate samples; *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.005$, ns: not significant).

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Figure1

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