Unraveling Drug Penetration of Echinocandin Antifungals at the Site of Infection in an Intra-Abdominal Abscess Model

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Intra-abdominal candidiasis (IAC) is a prominent invasive fungal infection associated with high mortality. Prompt antifungal therapy and source control are crucial for successful treatment. Echinocandin antifungal drugs are first-line agents. Yet, their clinical effectiveness is highly variable with known potential for breakthrough resistance, and little is known about drug exposure at the site of infection. Using matrix-assisted desorption/ionization mass spectrometry imaging technology, we investigated the spatial and quantitative distribution in tissue lesions for two echinocandin drugs, micafungin and CD101, in a clinically relevant IAC mouse model. Drug accumulation within lesions was observed with both drugs at their humanized therapeutic dose. However, CD101 but not micafungin, accumulated in lesions at levels above the mutant prevention concentration of the infecting strain. These findings indicate that current echinocandin drugs may be limited by penetration at the site of infection, which have implications for clinical outcomes and emergence of resistance in patients with IAC.

Key Words: intra-abdominal candidiasis; echinocandin; drug penetration; matrix-assisted desorption/ionization (MALDI) mass spectrometry imaging
INTRODUCTION

Candidemia and intra-abdominal candidiasis (IAC) are the two most common types of invasive candidiasis, which have an associated high mortality (1-3). However, unlike candidemia, which has been the focus of most studies of invasive candidiasis, IAC is poorly understood (4). Scattered abscesses and microlesions are the predominant histopathological findings within abdominal organs from humans with IAC. Prompt source control and institution of antifungal therapy are major determinants of successful outcomes among patients with IAC (2, 4). It has been postulated that restricted drug penetration into abscesses is the main cause of antifungal treatment failure and creates a hidden reservoir of resistance (5).

Echinocandins, the first class of antifungals to target the fungal cell wall (6), are recommended as first-line therapy for most types of invasive candidiasis (7, 8), but treatment failures occur in up to 40% of cases (9). In addition, despite the overall relatively low resistance frequency, widespread and expanding echinocandin usage has led to emergence of resistance, in particular among Candida glabrata (10-12). Limited data have suggested that echinocandin delivery to infection sites is often insufficient to achieve concentrations that eliminate Candida or suppress resistance (13, 14), which may account for a considerable amount of treatment failures. Yet, data on the infection site pharmacokinetic (PK) of echinocandins are extremely scarce and nothing is known about penetration into tissue lesions, although one population PK study reported that miconafungin exposure in peritoneal fluid was significantly lower than in plasma in IAC patients (14).

Sufficient penetration into infected tissue compartments is a key requirement for efficacy of all antimicrobial agents (15, 16). Taking the specific histopathology of IAC into account, perhaps the most clinically important and informative data is how drug distributes and penetrates into abscesses or other infected lesions within tissues, rather than drug concentrations in serum or
whole organs. Matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI) has emerged as a powerful tool to acquire spatially allocated molecular information about drug distributions in tissues (17, 18). MALDI-MSI has been used increasingly in drug discovery and development because of its ability to provide spatial distribution of target compounds without the need for special labels(19), as well as potential biomarkers of efficacy and toxicity (20, 21). Here, we took the initiative to apply this technology as well as standard analytical techniques to investigate echinocandin drug penetration at the site of infection in a clinically relevant IAC mouse model involving C. albicans (22).

Micafungin is a widely prescribed echinocandin drug used as a standard-of-care for patients with suspected or confirmed IAC. CD101 (Cidara Therapeutics, Inc.) is a next generation echinocandin agent in clinical development that features exceptional stability and long half-life PK properties relative to other drugs in this class (23, 24). These properties allow a single dose to be administered safely at a much higher level than conventional echinocandin drugs (25). The objective of this study was to critically evaluate the penetration of echinocandin drugs at the site of infection and assess whether drug levels in lesions help account for the observed clinical response and potential for later stage resistance emergence.

RESULTS

Tissue distribution and penetration after a single dose of micafungin and CD101.

Histopathology and MALDI imaging analysis. The intra-abdominal candidiasis model yielded abundant heterogeneous lesions (Fig. 1) at three days post infection. Echinocandin antifungal drugs were then introduced and the spatial distribution of micafungin and CD101 was visualized by MALDI mass spectrometry imaging (MALDI-MSI), delivering high-resolution heat maps of drug concentration in liver and kidney tissues. Side by side comparison of these images with histopathological staining (Hematoxylin and Eosin <H&E> and Gomori Methenamine Silver
of adjacent sections revealed drug penetration in different lesion structures, as well as the relationship between drug distribution and location of fungal cells. Upon histopathological analysis, we found that lesions formed in abdominal organs are characterized by large macrophage/neutrophil infiltrates surrounding a necrotic core of various sizes. Fungal-specific GMS staining further supported that fungal load in lesions appeared to correlate with necrotic severity of lesion. Predominantly, high fungal staining was observed in necrotic area (Supplementary Fig. 1).

After a single humanized dose, both echinocandins were quickly distributed into liver and kidney. However, the pharmacokinetics of tissue exposure and the pattern of lesion penetration were notably different for these two drugs. After a single dose administration of micafungin at 5 mg/kg, drug quickly distributed into liver tissues and reached peak intensity at 1h (Fig. 2a). Decreased drug intensities (relative drug abundance) over the entire tissue were observed at 3h and 6h; although, drug signal was barely detectable in lesions until 6h when the drug was observed at the edge of the lesion with little detected penetration into the necrotic core. Penetration of the drug into the necrotic lesion was clearly observed at 24h, when the micafungin signal was detected inside of the lesion with noticeably higher intensities in the outer rim of the lesion relative to the necrotic center, as well as the surrounding uninvolved tissue. An enlarged view of the 24h MALDI image and the adjacent GMS stained section (Fig. 3) showed that micafungin predominantly resided in the lesion edge, whereas fungal cells constitute a massive network throughout the entire lesion. Thus, interaction between the drug and fungi was limited in the outer part of the lesion. But within the necrotic center where the majority of the fungal population reside, there was no detectable drug exposure (Limit of detection <LOD> for MALDI-MSI analysis of Micafungin and CD101 was 500ng/g and 1µg/g of liver or kidney tissue respectively. Spot testing was performed for each drug to determine the LOD as previously described (19)). Moreover, without further dosing past 24h, such drug retention within the lesion was too low to
outcompete the quick drug clearance from liver, where drug levels dropped far below the limit of detection and no drug signals were detected at 48h. In kidneys, the same kinetic pattern of tissue distribution and lesion penetration was observed for micafungin (Supplementary Fig. 2a).

In liver, CD101 signal intensity was readily detected at the earliest time point investigated (1h postdose) and steadily increased at 3 h and 6h, with the highest drug signal detected at 6h postdose (Fig. 2b). Thereafter, CD101 drug intensity slowly declined but still persisted strongly even at 48h postdose. Closer examination of the ion map and GMS staining revealed detectable lesion penetration (CD101 signal appeared inside of lesion) as early as 3h and a gradient of drug distribution was observed within the lesion at 6h with higher drug intensity in the outer area and less signals in the necrotic center (Fig. 3). At later time points (24h and 48h), CD101 was primarily persisting with slow accumulation within the lesion while surrounding tissue drug levels were declining. The 48h enlarged view mapped out a more balanced distribution of CD101 with drug signals detected throughout the necrotic lesion (Fig. 3). Drug distribution and penetration of CD101 in kidneys were similar to what was observed in liver, whereas lesion formation and histopathology in kidneys (Supplementary Fig. 2b) was not as consistent as what was observed in livers with heterogeneous manifestations ranging from very tiny lesions to big or multifocal lesions.

Quantitative evaluation of drug exposure in liver lesions. MALDI imaging analysis provides valuable information on spatial distribution and lesion penetration. Yet, it is only semi-quantitative and not a measure of exact drug exposure at the site of infection. Hence, we next applied laser capture microdissection (LCM) followed by high-pressure liquid chromatography coupled tandem mass spectrometry (LC/MS-MS) to quantify absolute drug concentration in distinct compartments of involved tissues at two representative time points, 6h and 24h postdose. Only liver samples were analyzed due to the fact that kidney lesions were too small to...
dissect sufficient material to meet the LOD for LC/MS-MS analysis. Upon quantification (Fig. 4), the single dose of 5 mg/kg micafungin (at which dosage, experimental serum drug levels ranged approximately from 7 to 10 µg/g at 6h and around 2 µg/g at 24h (26, 27)) resulted in drug retention at 6.5 and 1 µg/g in uninvolved surrounding tissues and 4.9 and 3.4 µg/g in lesions at 6h and 24h, respectively. In contrast, remarkably high levels of CD101 were found in liver tissues. After therapeutic dosing (20 mg/kg) of CD101 (which resulted in serum drug levels of 43 and 22 µg/g at 6h and 24h, respectively (24)), the average drug level at 6h was 80.1 µg/g in non-lesion part and 31.6 µg/g in lesions. At 24h, the drug level in surrounding tissue dropped significantly compared to the 6h level (P=0.01) but were still high with a mean concentration of 38.7 µg/g. Moreover, the mean drug concentration within lesions increased to 44.5 µg/g at 24h even though the statistical significance of such increase was not achieved due to the small sample size. When the lower dose CD101 at 5 mg/kg was administered, a proportionally decreased but high level of drug was observed from both compartments at both time points. Drug concentration was 15.8 µg/g at 6h and 19.8 µg/g at 24h in surrounding tissues, and 6.6 and 12.7 µg/g in lesions at 6h and 24h, respectively.

Liver burden assessment. To understand the relationship between tissue drug exposure and efficacy, we measured liver burdens at 0, 6, and 24h post single dose treatment of CD101 at 20 or 5 mg/kg and micafungin at 5 mg/kg (Fig. 5). An average of 5.4 log_{10} CFU/g burden was recovered from livers at day 3 post-infection before treatment. Mean burden counts at 6h postdose were 4.9, 4.2, 4.0, and 4.8 log_{10} CFU/g for vehicle control, 20 mg/kg CD101, 5 mg/kg CD101, and 5 mg/kg micafungin, respectively. Although the burden reduction resulting from echinocandin treatment was not statistically significant, it is noteworthy that at 6 h no liver burden (effective sterilization) was observed in 2 out of 5 mice in each echinocandin treatment group but not in the vehicle control. By 24h postdose, liver burdens showed an average of 4.3 log_{10} CFU/g for vehicle control, 3.7 and 4.3 log_{10} CFU/g for 20 and 5 mg/kg CD101, and 4.7 log_{10} CFU/g for 5 mg/kg micafungin.
log\(_{10}\) CFU/g for 5 mg/kg micafungin treated mice. Mice treated with 20 mg/kg CD101 had significantly lower liver burdens compared to mice treated with micafungin \((P = 0.047)\), largely due to the fact that liver sterilization was achieved in 4 out of 5 mice in the 20 mg/kg CD101 group but none from micafungin group. The CD101 5 mg/kg treatment also led to liver sterilization in one mouse, yet burden counts in the rest of mice from the same group were not much different from either vehicle control or micafungin treatment group.

Comparison of drug accumulation at site of infection after multiple doses of micafungin and single dose of CD101.

MALDI imaging. Given the fact that the standard micafungin regimen in the clinical setting is daily dosing, a multiple-dose experiment was designed to examine drug accumulation of micafungin at steady-state. We analyzed drug distribution at 24h following 2 and 3 therapeutic doses of micafungin. MALDI imaging analysis (Fig. 6a) showed that multidosing had limited impact on partitioning into liver tissue, as drug signals were barely captured in the non-lesion part of tissues even after 3 doses of micafungin. In contrast to marginally detectable drug levels in the surrounding tissues, a noticeably increased drug intensity was observed inside lesions after multiple doses, indicating micafungin was accumulating somewhat within lesion at above normal tissue drug level at the steady state. Given the extended PK properties of CD101, we were driven to assess whether the quick and favorable lesion penetration after single dose CD101 could persist for a long time and/or lead to drug accumulation at least comparable to the steady state of micafungin. For this purpose, a single therapeutic dose CD101 arm was established in parallel with the multidosing micafungin arm. Tissue samples from the CD101 arm were collected at 48h and 72h postdose, equivalent to 24h post 2 and 3 doses of micafungin, respectively. Consistent with the previous single dose experiment, at 48h robust CD101 signal was detected from the entire tissue and drug diffused into lesions more effectively.
Drug accumulation within the necrotic region of the lesions became more visible at 72h, when drug intensities in the surrounding tissue were reduced.

**Quantifying drug levels.** Compared to single dose micafungin, additional daily dosing, which reached steady-state after 3 doses, promoted drug retention within lesion even though drug level in surrounding non-lesion tissues was low at only ~ 0.5 µg/g and not much different from that at 24h after single dosing (Fig. 7). Micafungin accumulated in lesions slowly but continuously, retaining 3.5 µg/g and 4.9 µg/g at 24h post 2nd and 3rd drug dose, respectively. In comparison, the extensive tissue distribution and lesion penetration after single dose CD101 was confirmed once again when samples were assessed at an extended time point to match steady-state micafungin sample collection. A mean CD101 concentration of 37.7 µg/g and 29.7 µg/g was reported from dissected lesions at 48h and 72h, respectively, and corresponding drug levels in surrounding tissues were measured at 42.2 µg/g and 19.1 µg/g (Fig. 7), significantly higher than that of micafungin in corresponding sites at each time point. These results are consistent with the MALDI imaging data.

**DISCUSSION**

Despite widespread knowledge that tissue penetration is crucial for efficacy of antifungal agents, there is no definitive information on the extent that these drugs penetrate into the site of infection for various invasive fungal infections (28). This important therapeutic issue has been largely ignored experimentally because of long standing technical obstacles to estimate drug concentration in various tissue subcompartments. Current understanding of tissue distribution of antifungal agents has been primarily based on drug concentration measurement in whole tissue homogenates (29-32). Drug concentration in tissue homogenates is a useful measure of exposure. Yet, it can be misleading due to the loss of key information on the spatial distribution of drugs in distinct subcompartments, particularly when abscesses or other forms of lesions are present.
formed. Since infecting organisms are often residing within these subcompartments, it would be most valuable if the drug and targeting organism can be colocalized within tissue beds. Herein, by employing MALDI imaging technology and LCM-directed drug quantification in a clinically relevant IAC mouse model, we have successfully evaluated for the first time drug exposure within intra-abdominal abscesses.

IAC is difficult to treat and outcome is poor even after proper source control and adequate antifungal treatment (2, 4). Moreover, a recent study reported that IAC, particularly cases due to Candida glabrata, was a hidden reservoir for emergence of echinocandin resistance (5). These observations have raised concern about insufficient drug penetration during therapy for IAC.

One of the very few studies examining infection site PK of micafungin reported that there was a moderate drug penetration into the peritoneal cavity in IAC patients, with a median AUC$_{0-24}$ peritoneal fluid/plasma ratio of 0.3 after the first dose and of 0.3 at steady-state (14). Interestingly, our data revealed that micafungin was gradually penetrating into liver and kidney abscesses, but only reached detectable levels inside lesions at 6h after the first dose. The penetration improved upon multiple doses of treatment, and only at steady-state were drug signals observed from the necrotic core where large amount of fungal cells proliferate. Absolute drug quantification from lesions at different postdose time points further confirmed this drug accumulation pattern.

CD101 is a next-generation echinocandin drug candidate in clinical development. Due to substantially reduced toxicity due to chemical stability and enhanced PK properties, CD101 can be safely dosed at much higher levels compared to other echinocandin drugs (25, 33, 34). Based on mouse PK and human phase I clinical trial data, 20 mg/kg in mice is considered equivalent to human therapeutic dose. At this dosing level, CD101 has demonstrated better or at least comparable efficacy in vivo against invasive candidiasis relative to micafungin (24). In
the present study, CD101 showed an extensive tissue distribution with an impressive drug level of 80.1 µg/g in non-lesion part of liver at 6h after a single dose treatment at 20 mg/kg. More notably, the drug was observed to quickly penetrate into abscesses as early as 3h and rapidly reach the necrotic core interacting with the main fungal population at 6h, with an average of 31.6 µg/g drug in lesions. Given the long half-life of CD101 ($t_{1/2}$ was 38.9h in mice at 20 mg/kg (24)), it is not surprising that sustained drug penetration and accumulation within lesion was continuously observed for all remaining time points included in the study. Even at 72h following a single dose, drug levels inside lesions were still close to 30 µg/g, about 6-fold higher than that for micafungin at steady-state. The outstanding penetration of CD101 at the site of infection is dose-dependent. In the low dose (5 mg/kg) CD101 experiment, we observed the same penetration pattern, but proportionally lowered drug levels both in and outside lesions at selected time points compared to the 20 mg/kg treatment. At 24h after a single low dose of CD101, the mean drug concentration within lesions was 12.7 µg/g, still about 4-fold higher than what was seen with micafungin (3.4 µg/g) at the same dosage, indicating a true superior lesion penetration feature of CD101.

Drug exposure is one of the most important determinants for efficacy and we assessed liver burdens in different treatment groups at 6h and 24h after a single dose of CD101 or micafungin. Consistent with drug levels, at 6h postdose, both 20 and 5 mg/kg CD101 had greater burden reduction than 5 mg/kg micafungin (0.7 and 0.9 vs. 0.1 log$_{10}$CFU/g), although the differences were not statistically significant and all treatment groups had two mice with complete resolution of liver infection. Burden reduction efficacy of CD101 was further observed at 24h with 20 mg/kg treatment, with which 4 of 5 mice livers were sterilized, which was consistent with accumulating drug in the lesions. In comparison, no liver sterilization was observed for micafungin treated mice, and resolved liver infection was achieved in one mouse but not others in the 5 mg/kg CD101 group. These data suggest that a prominent drug level (to be defined) at the infection.
sites may be required for resolution of organ invasion in treating IAC with echinocandins. Hence, at recommended therapeutic dose, currently approved echinocandins may have limited efficacy in IAC treatment.

The ability to quantify kinetically the level of drug at the site of infection has important implications for emergence of drug resistance. Insufficient penetration and/or drug accumulation in lesions may create temporal or spatial windows in specific niches, allowing acquisition of mutations in major drug target genes, and eventually facilitating emergence of resistance. The mutant prevention concentration (MPC) derived from the mutant selection window hypothesis, which was raised to address the need of dosing strategy to restrict emergence of resistance to antibacterial agents (35-37). MPC is the minimal concentration that inhibits drug-susceptible mutant subpopulation. The MPC-based PK/PD measurement, AUC$_{24}$/MPC, is suggested to be more accurate than AUC$_{24}$/MIC in predicting resistance occurrence (36, 38, 39), and in vivo experiments support its usefulness to control the frequency of resistant mutant development by maintaining drug levels above MPC for certain time of period (40, 41). The MPCs for both CD101 and micafungin are reported as 16 µg/ml against wildtype strains of C. albicans and C. glabrata (24). In this study, we found that at steady-state micafungin diffused into abscesses at just under 5 µg/g (equal to 5 µg/ml of tissue homogenate), which was above the minimum inhibitory concentration (MIC=0.03 µg/ml) but below the reported MPC. This result may help account for echinocandin treatment failures and emergence of resistance observed with some IAC patients, as drug fall below critical levels. In contrast, CD101 penetrated into the lesions as early as 6h after a single dose at 20 mg/kg, and drug levels were maintained exceptionally high throughout a 72h endpoint with a mean concentration of 29.7 µg/g (equal to 29.7µg/ml), which was well above the MPC. A tentative interpretation for this observation is that, under well-determined and properly designed dosing regimen, CD101 may have the potential to overcome or limit resistance development induced by insufficient drug penetration of currently approved
Nevertheless, studies examining detailed relationship between resistance development and drug penetration and exposure in the context of IAC are warranted. It should be noted that even with LCM the usefulness of absolute drug quantification may still be limited when lesions are small (< 1 mm$^2$). Due to the current limit of detection of LC/MS, we pooled 6 consecutive LCM sections from the same lesion for drug quantification. For small lesions, such sample pooling method resulted in measuring both inner and outer areas of the lesion rather than just the necrotic core. In this situation, MALDI imaging along with histopathological analyses are the most useful tools to elucidate drug location within lesions. One example is that the average drug concentration within liver lesions of micafungin at steady-state was not significantly higher than that at 6h after single dose administration. However, MALDI imaging demonstrated remarkably different profiles at these two time points. Drug signals only appeared in the outer layer within the lesion at 6h post single dose whereas fungal hyphae were scattered all over inside of the lesion with largest amount of cells clumping in the center. In contrast, at steady state, micafungin was mostly seen in the center area of lesion where the majority of fungal cell population were residing.

In summary, our study represents the first successful application of MALDI imaging in the antifungal field. It opens up a new path to study drug penetration at the site of infection and establishes a cornerstone for advanced antifungal drug research. With this new strategy, we have demonstrated differential spatial distribution of lesion penetration for two echinocandin drugs. The new member of this drug class, CD101, displays extraordinary penetration attributes at the site of infection relative to micafungin. Insights gained in this study have important clinical implications for both treating IAC and preventing emergence of resistance more effectively. Moreover, our findings are potentially relevant to the other predominant type of invasive
candidiasis, candidemia, in which seeding of target organs like liver and kidney is also characterized by microabscess formation.

Finally, more broadly, these types of studies are relevant to a wide range of infectious and non-infectious disease pathologies (e.g. tumors) that require effective drug levels for clinical response. It is apparent that some drugs are narrowly dosed to their pharmacodynamic target level and monitoring serum drug levels may not adequately predict drug exposure at the disease source, especially when necrotic tissue is involved.

**MATERIALS AND METHODS**

**Ethics statement**

Mice were housed in Public Health Research Institute’s Animal Biosafety Level-2 Research Animal Facility (ICPH RAF), a center of the New Jersey Medical School, Rutgers University (NJMS-Rutgers). Our animal facility follows the Public Health Service and National Institute of Health Policy of Humane Care and Use of Laboratory Animals. All experimental protocols were approved by Rutgers Institutional Animal Care and Use Committee (IACUC).

*Candida albicans* strain and antifungal drugs. *C. albicans* strain SC4315 was grown in yeast extract peptone dextrose broth at 37°C with shaking overnight. Cells were washed, counted and prepared to 1x10^8 CFU/ml for inoculation as previously described (24). CD101, CD101-D9 (Cidara Therapeutics, Inc., San Diego, CA, USA) and micafungin (Astellas Pharma Inc., Tokyo, Japan) were obtained as standard powders from their manufacturer. ^13^C-micafungin was purchased from ALSACHIM, France.

Mouse model of intra-abdominal candidiasis and tissue sample collection. A mouse model of IAC established by Cheng et al. was used for this study (22). Female 6-8 week old CD1 mice
(Charles River Laboratories) weighing 18-22 g were infected intraperitoneally (IP) with $1 \times 10^7$ CFU of *C. albicans* SC5314 mixed with sterile stool matrix as previously described. Single IP doses of CD101 at 20 mg/kg (equivalent to humanized therapeutic dose) or micafungin at 5 mg/kg (therapeutic dose) were administered to groups of 15 mice at day 3 post-inoculation. Mice were sacrificed at just before antifungal treatment (n=1), and at 1, 3, 6, 24, and 48 h post-dose (3 mice per group per time point). Livers and kidneys were explored for abscesses > 1mm in diameter (*Fig. 1*), dissected, placed on a cryohistology tray, and snap-frozen in liquid nitrogen and stored at -80°C for tissue sectioning for MALDI imaging. We also performed an experiment where low dose of CD101 at 5 mg/kg was administered and micafungin was given at the same dose. Liver and kidney samples were collected at 6h and 24h postdose for both MALDI imaging and absolute drug quantification. Moreover, infected livers were collected from 45 mice (5 mice per time point per group), and measured for burden counts at 0, 6, and 24h post single dose treatment of 20 or 5 mg/kg CD101, 5 mg/kg micafungin, or vehicle control. Treatment was started at day 3 post-infection for all groups. In another separate experiment that aimed at comparing therapeutic level of single dose CD101 and multiple doses of micafungin, single dose of CD101 at 20 mg/kg was given at day 3 post-inoculation, and once daily treatment of micafungin at 5 mg/kg started from day 3 post-inoculation and a total of 3 doses of micafungin was administered. Livers and kidneys were collected at 48 and 72h post first dose of each drug.

**Tissue sectioning and matrix application.** Tissues were sectioned at 12 µm thickness using a Leica CM1850 cryostat (Buffalo Grove, IL) and mounted onto stainless steel slides (for MALDI-MSI analysis) or frosted glass microscope slides (for H&E staining) as previously described(42). Tissue sections were stored at -80°C until analysis. Prior to MALDI-MSI analysis, tissue sections were thawed and applied with ionization matrix. For CD101, 2,5-dihydroxybenzoic acid (DHB) (20 mg/ml in 50% methanol) containing 267 fmol/µl CD101-D9 (Cidara Therapeutics, Inc) was applied to the surface using a HTX TM_sprayer (Chapel Hill, NC) operating with 50µL/min
flow rate, 60°C nozzle temperature and 5 p.s.i. Twenty-five passes over the tissue were performed. For micafungin, 1,5-diaminonaphthalene (1,5-DAN) matrix (5 mg/ml in 50% acetone) containing 5nmol/µl $^{13}$C$_6$-micafungin (Alsachim, France) was coated onto tissue surface by the TM-sprayer at flow rate of 60 µl/min, 50°C nozzle temperature and 5 p.s.i. Twenty-five passes over the tissue were performed.

**MALDI-MSI analysis.** MALDI-MSI analysis was performed using a MALDI LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) with a resolution of 60,000 at m/z 400, full width at half maximum. The resolution was sufficient to resolve CD101, CD101-D9, micafungin, and $^{13}$C$_6$-micafungin peaks from background without the requirement from MS/MS and subsequent loss of signal. However, drug peak identities were confirmed by acquiring several MS/MS spectra directly from the dosed tissues. Standards of CD101 and micafungin were analyzed both direct from the stainless steel target plate and spiked into drug-naïve liver tissue to optimize instrument parameters. Limit of detection (LOD) for MALDI-MSI analysis of Micafungin and CD101 was 500ng/g and 1µg/g of liver or kidney tissue respectively, calculated as described (19). For CD101, spectra were acquired in the m/z 1000-1500 range, using positive ionization with a laser energy of 25 µJ and 15 laser shots were fired at each position. Spectra for micafungin were acquired in the same m/z range under negative ionization mode with a laser energy of 10 µJ and 5 laser shots at each position. The laser step size was set at between 50-75 µm, at which small necrotic areas within lesions could easily be resolved and no overlapping of the laser spot on adjacent acquisitions was observed.

Data visualization was performed using Thermo ImageQuest software. Normalized ion images of CD101 were generated by dividing CD101 [M+H]$^+$ signal (m/z 1225.603 ± 0.005) by CD101-
Laser-capture microdissection. Necrotic lesion and surrounding tissue areas totaling 2-6 million µm² were dissected from between 3 and 6 serial liver or kidney biopsy tissue sections using a Leica LMD6500 system (Buffalo Grove, IL). Lesion areas were identified optically from the brightfield image scan and by comparison to the adjacently-sectioned H&E reference tissue. Pooled dissected lesion tissues were collected into 0.25ml standard PCR tubes and immediately transferred to the -80°C freezer for storage.

Prior to analysis, the tubes were thawed at room temperature for 30 minutes. Fifty microliter of extraction solution (ACN/MeOH (1/1) with 100 ng/ml CD101-D9 and 100ng/ml ¹³C₆-micafungin) was added to each tube, which were then sonicated for 5 min and centrifuged at 10000 RPM for 5 min at room temperature. Forty microliter of supernatant was transferred for LC/MS-MS analysis and diluted with an additional 40 µl of MilliQ water.

Neat 1mg/ml DMSO stocks for all compounds were serial diluted in 50/50 Acetonitrile water to create standard curves and quality control spiking solutions. Three microliter of neat spiking solutions were added to 2 µl of lesion homogenate and extraction was performed by adding 50 µl of extraction solution (ACN/MeOH (1/1) with 100 ng/ml CD101-D9 and 100ng/ml ¹³C₆-micafungin). Extracts were vortexed for 5 minutes and centrifuged at 10000 RPM for 5 min. A 40 µl of supernatant was transferred for LC/MS-MS analysis and diluted with an additional 40 µl of MilliQ water. Previously optimized LC/MS-MS parameters were used for analysis (see LC/MS-MS section).

Drug quantitation by LC/MS-MS. LC-MS analysis was performed on a Q Exactive high resolution mass spectrometer (Thermo Fisher Scientific, Waltham, MA) coupled to a Thermo Scientific Dionex UltiMate 3000 binary system. Chromatography was performed with a Kinetex
C18 column (2.1 x 50 mm; particle size 1.7µm, Phenomenex, Torrance, CA) using a reverse phase gradient elution, ACN:H₂O (60:40) and 10 mM ammonium acetate for mobile phase A and IPA:ACN:MeOH (80:10:10) and 10 mM ammonium acetate for mobile phase B. A flow rate of 300 µl/min was used, with a gradient consisting of 20% B held for 0.5 min, followed by linear increase to 95% in 3.5 min, held for 2.2 min and return to the initial 20% B in 0.3 min. The column was equilibrated for 1.5 min before the next injection, and the temperature of column and sample tray were held at 50 and 4°C, respectively. The column retention time for CD101 and micafungin was 3.32 min and 3.2 min respectively.

Key MS parameters were as follow: the spray voltage, 3.5 kV; capillary temperature, 320°C; HESI probe temperature, 400°C; S-lens RF level, 50. The sheath gas and auxiliary gas were set to 45 and 10 units, respectively. External mass calibration was performed before each sequence. For CD101, full scan was applied in positive ionization mode with a mass range of m/z 250-1500 at resolution power 70,000, AGC target 3e6 for a maximum IT of 100ms. For micafungin, full scan was applied in negative ionization mode with a mass range of m/z 250-1500 at resolution power 70,000, AGC target 3e6 for a maximum IT of 100ms. CD101 [M+H]+ signal was normalized to CD101-D9 [M+H]+ and micafungin [M+H]- signal was normalized to 13C₆-micafungin [M+H]-.

**Statistical analysis.** Absolute drug concentrations were graphed and statistically analyzed in the GraphPad software (Prism 7; GraphPad Software, Inc., San Diego, CA). Drug levels in different tissue compartment at different time points were compared by the one way analysis of variance (ANOVA) and Dunn’ multiple comparison was used for the post hoc analyses. Statistical significance was defined as \( P < 0.05 \). Prompt antifungal therapy and source controls are crucial for successful treatment.

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FIGURE LEGENDS

Figure 1. Liver and kidney abscesses at necropsy on day 3 post infection. Neon yellow arrows point out multiple lesions formed in liver (left) and kidney (right).

Figure 2. Drug distribution in infected liver tissues after single dose micafungin and CD101. (a) Upper row: Ion maps of micafungin in representative liver tissues collected at 1, 3, 6, 24, and 48h post a single dose of micafungin at 5 mg/kg; signal intensity color bar is fixed for micafungin, with gradually increased intensity from blue (no signal) to red (max signal); H&E and GMS staining of adjacent sections are shown below each set of ion maps. Outlines highlight the lesion area on each tissue section. Scale bars, 3 mm. (b) Upper row: Ion maps of CD101 in representative liver tissues collected at 1, 3, 6, 24, and 48h post a single dose of CD101 at 20 mg/kg; signal intensity color bar is fixed for CD101, with gradually increased intensity from blue (no signal) to red (max signal). Matched H&E and GMS staining results are shown in the middle and bottom rows, respectively. Scale bars, 3 mm.

Figure 3. Close up examination of drug penetration for micafungin at 24h, and CD101 at 6 and 48h post single dosing. Enlarged view of drug distribution in a single lesion at pixel level. Matched GMS staining of adjacent sections was placed on the bottom. Signal intensity is fixed for CD101 and micafungin, respectively. Scale bars, 5 mm.

Figure 4. Quantification of drug exposure in liver lesions and surrounding tissues. Drug concentration was measured in lesions and surrounding uninvolved tissues dissected from liver sections collected at 6h and 24h post single dose of micafungin at 5 mg/kg or CD101 at 20 and 5 mg/kg. Error bars, mean ± s.d. of 3-5 liver pieces or distinct lesions.
Figure 5. Liver burden comparison at 0 (predose), 6 and 24h post single dose treatment of CD101 at 20 or 5 mg/kg, micafungin at 5 mg/kg, and vehicle control. Each symbol represents liver burden of a single animal. Short lines are mean burdens determined for 5 mice in each treatment group. Symbols on the X-axis represent mice with no liver burden (sterilization). Percentage of mice with liver sterilization were plotted as bars on the right Y-axis underneath burdens of each corresponding group. Compared to micafungin, the 20 mg/kg CD101 resulted in significant burden reduction in liver at 24h postdose with a $P$ value of 0.047, as well as percent of sterilized animals.

Figure 6. Drug penetration after (a) multi-dosing micafungin and (b) single dosing CD101. Micafungin is steadily being accumulated in abscesses upon 2 and 3 doses. Micafungin signal was only detected from lesion enters at steady state (after 3 doses). CD101 diffused into lesions thoroughly at 48h post single dosing, and accumulated in necrotic area of each lesion at 72h. H&E and GMS staining of adjacent sections are shown below each set of ion maps. Outlines highlight the lesion area on each tissue section. Scale bars, 3 mm.

Figure 7. Drug accumulation comparison between multiple doses of micafungin (5 mg/kg) and single dose of CD101 (20 mg/kg). Absolute drug level was measured for lesions and surrounding uninvolved tissues from liver samples collected at 48h and 72h post the first dose of micafungin, and those treated with a single dose of CD101 and collected at the matched time points. Error bars, mean ± s.d. of 3-5 liver pieces or distinct lesions. *** $P < 0.001$, drug levels of CD101 were significantly higher than micafungin in corresponding sites (lesion or uninvolved tissue) at specified time point.
Lesion (6h) involved (6h)
Lesion (24h) involved (24h)

CD101 (g/g)

0 20 40 60 80 100

Micafungin (g/g)

0 5 mg/kg Micafungin

0 20 40 60 80 100

5 mg/kg Micafungin
Log_{10} CFU/g liver

% Sterilization

Predose
Vehicle 6h
CD101 20 mg/kg 6h
Micafungin 5 mg/kg 6h
Vehicle 24h
CD101 20 mg/kg 24h
Micafungin 5 mg/kg 24h

P = 0.047

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