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# Alternation of Echinocandins with Liposomal Amphotericin B for Treatment of *Nakaseomyces glabratus* Candidemia: An Effective Strategy to Reduce Echinocandin Tolerant Pool and to Minimize Echinocandin Resistance

Liuyang Cai[1,3,4#], Xiaochun Xue[2,3,4#], Mostafa Salehi[5], David S Perlin[6,7,8], Martin Hoenigl[9,10,11], Wanqing Liao[1,3,4], Weihua Pan[1,3,4*], Amir Arastehfar[12,13*], and Wenjie Fang[1,3,4*]

[1] *Department of Dermatology, Shanghai Key Laboratory of Molecular Medical Mycology, Shanghai Changzheng Hospital, Naval Medical University, Shanghai, China*
[2] *Department of Pharmacy, 905th Hospital of People's Liberation Army Navy, Shanghai, China*
[3] *The Center for Fungal Infectious Diseases Basic Research and Innovation of Medicine and Pharmacy, Ministry of Education, Shanghai, China*
[4] *Oriental Institute of Infection and Inflammation, Guangzhou, Guangdong, China*
[5] *Department of Industrial Engineering, Faculty of K.N., Toosi University of Technology, Tehran, Iran*
[6] *Center for Discovery and Innovation, Hackensack Meridian Health, Nutley, New Jersey, USA*
[7] *Hackensack Meridian School of Medicine, Nutley, New Jersey, USA*
[8] *Georgetown University Lombardi Comprehensive Cancer Center, Washington, District of Columbia, USA*
[9] *Division of Infectious Diseases, Department of Internal Medicine, Medical University of Graz, Graz, Austria*
[10] *BioTechMed, Graz, Austria*
[11] *Translational Mycology Research Unit, ECMM Excellence Center for Medical Mycology, Medical University of Graz, Graz, Austria*
[12] *Division of Infectious Diseases, Massachusetts General Hospital, Boston, MA, USA*
[13] *Department of Medicine, Harvard Medical School, Boston, MA, USA*

**Abstract:** Echinocandins are frontline antifungal drugs, and the emergence of echinocandin-resistant (ECR) species, such as *Nakaseomyces glabratus*, complicates patient outcomes. Intriguingly, under laboratory conditions, we previously showed that echinocandin alternation with metabolic-independent antifungals, such as amphotericin B (AMB), more effectively kills and minimizes the ECR in *N. glabratus*. Building upon our previous observations, we examined the efficacy of echinocandin alternation to amphotericin B (EAMB) over echinocandin monotherapy using a systemic candidiasis mouse model to assess if EAMB warrants investigation with potential for clinical evaluation. Interestingly, we show that regardless of the mice's immune status (immunocompromised and immunocompetent) and the *N. glabratus* isolates [high and low echinocandin tolerance (ECT)] tested, EAMB more rapidly cleared the infection, and minimized ECR in all organs tested compared to caspofungin monotherapy. Pharmacokinetic data suggested that the superiority of EAMB is due to concentration-in-

# These authors contribute equally to this work: Liuyang Cai, Xiaochun Xue

* Corresponding author: Weihua Pan, E-mail: panweihua9@sina.com; Amir Arastehfar, E-mail: a.arastehfar.nl@gmail.com; Wenjie Fang, E-mail: fangwenjie1990@126.com

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dependent killing activity of liposomal AMB. Although biomarkers suggested higher kidney and liver damage in the EAMB group, histological analysis showed similar damage among both groups. Collectively, using comprehensive *ex vivo* and *in vitro*/*in vivo* experimental conditions, we introduce a novel antifungal therapeutic regimen, which effectively minimizes the ECT and ECR rate in *N. glabratus* and lays the foundation for in-human studies and clinical trials.

**Key words:** Echinocandin tolerance; Echinocandin resistance; Candidemia; Pharmacokinetics; Liposomal amphotericin B

## 1 Background

Pathogenic yeast *Candida* species are among the leading causes of bloodstream infections in clinical settings, associated with almost 1 million deaths annually [1] and pose a high economic burden [2]. Although antifungal drugs have improved survival of candidemia patients, antifungal resistance is an emerging crisis plaguing hospitals across the globe [3,4]. Azoles are static antifungal drugs, and their clinical use is notoriously impacted by a notable rate of azole resistance among non-*albicans Candida* pathogens [5]. Although polyenes have potent cidal activities, their use against candidemia is limited to recalcitrant *Candida* pathogens owing to their cytotoxic activities [5,6], which can be ameliorated by novel lipid-based formulations [7]. Echinocandins are the frontline antifungal drugs against candidemia [6] and possess high efficacy and safety profiles by selectively inhibiting β-1,3-glucan synthase, which produces a critical cell wall component [8]. Nonetheless, the increasing prevalence of *Candida* species, which may present echinocandin-resistant (ECR), i.e., *Nakaseomyces glabratus* [9], *C. parapsilosis* [10], and *C. auris* [11], is problematic. More troubling is that most of such ECR isolates are also resistant to azoles and therefore are multidrug-resistant (MDR) [9-11].

Echinocandin treatment failure is a multifaceted phenomenon mediated by host-, echinocandins-, and *Candida*-attributes [8]. Although ECR has been known as a major attribute underlying treatment failure from the *Candida* side, recent investigations have revealed that ECR colonies emerge from a very minor subpopulation of susceptible cells surviving therapeutic concentrations of echinocandins, which is known as echinocandin tolerance (ECT) [12]. Moreover, using systemic infection mouse models, we have recently shown that *N. glabratus* isolates exhibiting high ECT level *in vitro* have a higher burden and are more likely develop ECR under echinocandin treatment [13]. Therefore, effective treatment should ensure clearance or dramatically minimize the ECT subpopulation to prevent ECR emergence during echinocandin treatment.

From the antifungal side, echinocandins have limited penetration into the gastrointestinal (GI) tract and intra-abdominal abscesses, which are known as the major reservoirs of ECR [14-16]. Additionally, echinocandins are known to be heavily bound to serum proteins, which reduces the active drug such that 97%–99.7% (depending on the echinocandins used) are serum bound [17,18], and therefore, favors a higher survival of *Candida* cells [8]. Furthermore, we have shown that the efficacy of echinocandins is heavily dependent on the metabolic activity of *Candida* pathogens [12]. This is of paramount importance as, unlike the high metabolic activity of *Candida* cells and higher efficacy of echinocandins in rich media used *in vitro*, during host infection, *Candida* cells become less metabolically active once trapped in phagosomes of innate immune cells, which antagonizes the efficacy of echinocandins [12,13]. Consistently, we have shown that macrophages provide a permissive environment for the emergence of ECT and ECR in *N. glabratus* during echinocandin treatment[12,13]. Moreover, observational multicenter studies across multiple European countries have shown that despite improving survival rates, echinocandins may increase hospitalization duration as guidelines require treatment for 14 days after the last positive blood culture, and echinocandins are only available as parenteral formulation [19]. While several clinical trials are now investigating shorter duration of antifungal treatment for candidemia, the success of such strategies will depend upon early clearance of *Candida* spp. from blood and affected organs [20,21]. Collectively, these lines of evidence suggest that even though echinocandins are uncontested as first-line treatment for candidemia, devising novel therapeutic regimens may help to overcome some issues of echinocandin therapy and may help to further preserve their clinical use by minimizing ECR rate in the clinic.

Interestingly, using *in vitro* and *ex vivo* models, we recently showed that alternation of echinocandins with am-

photericin B (AMB) can dramatically reduce the ECT and ECR rates compared to standard continuous echinocandin treatment [12]. The initial treatment with echinocandins kills most of the *N. glabratus* cells, and the rest of the ECT cells are effectively killed by switching echinocandins with AMB (Figure 1A), since AMB's efficacy is not metabolism-dependent [12]. Although this approach seems an attractive therapeutic strategy against candidemia, its applicability was not tested using systemic infection mouse models to examine if such a therapeutic strategy bears potential clinical use. Therefore, the objective of this study was to assess the efficacy of caspofungin alone and in alternation with liposomal AMB (LAMB) in burden clearance and ECR rate of *N. glabratus* using a mouse model of candidemia.

## 2 Materials and methods

### 2.1 Systemic infection, ECR colony capture, and *FKS1/2* sequencing

Specific pathogen-free 6-week-old female C57BL/6 immunocompetent mice (16.5±1.5 g) and standard rodent pellet diet were obtained from the Jingyuan Experimental Animal Technology Co., Ltd. (Zhejiang, China). All mice were freely provided with food and water. Mice were kept in a 12 h light/dark cycle room with relative humidity of 30%–70% and an appropriate temperature of 18–25 °C. Overnight-grown *N. glabratus* isolates were subjected to PBS wash, and 200 µL of $10^8$ CFU of *N. glabratus* isolates were used to infect mice via the tail vein route. Mice were categorized into untreated, continuously treated with caspofungin (5 mg/kg), and those receiving 3 caspofungin doses, followed by switching to LAMB (5 mg/kg). We included at least 3 mice in the untreated group and 4 mice in the treated group for each time point. Antifungals were administered intraperitoneally (i.p, 5 mg/kg) one day after infection, and treatment was continued every other day throughout the experiment. For immunosuppressed mice, we used cyclophosphamide pre-treatment as described previously [14]. At designated timepoints, mice were humanely euthanized prior to sacrifice, and the entire spleen, liver, and both kidneys were collected and homogenized, and after extensive homogenization, were plated on YPD agar plates with/without 0.25 µg/mL of caspofungin. The agar plates without caspofungin were incubated in a 37 °C incubator for 72 h, whereas those with caspofungin were incubated for up to a week. Burden data were reported as colony-forming units (CFU) per gram of each organ. The captured ECR colonies were subjected to PCR and Sanger sequencing using the exact methodology described previously [22].

### 2.2 Plasma, liver, spleen, and kidney in pharmacokinetics

Mice were infected with 200 µL of $10^8$ CFU of a clinical *N. glabratus* isolate exhibiting high ECT, followed by treatment with a single dose (i.p.) of 5 mg/kg caspofungin or LAMB one day post-infection (pi). Four mice from each group were sacrificed at each time point [0 (predose), 2, 4, 8, 12, 24, and 48 h]. Mice considered for pharmacokinetic analysis on days 14 and 20 received 3 doses of caspofungin on odd days, followed by either continuous caspofungin treatment or switching to LAMB until designated timepoints. Blood collected in EDTA anticoagulation tubes was subjected to plasma isolation. Liver, spleen, and kidney were respectively weighed and mixed with 50% methanol (ratio 1:10), and then homogenized. The concentrations of caspofungin and AMB in plasma, liver, spleen, and kidney were determined by liquid chromatography-tandem mass spectrometry. Detection was performed in the positive ionization mode on an AB Sciex API-4000 triple quadrupole mass spectrometer with selected multiple reaction monitoring mode. The chromatographic separation is performed using a Shimadzu Shim-Pack GIST-HP C18 AQ (2.1 mm×50.0 mm, 3.0 µm) with reverse-phase gradient elution. Mobile phase A consists of water containing 2 mmol/L ammonium acetate and 0.1% formic acid, while mobile phase B consists of acetonitrile containing 0.1% formic acid. The flow rate is set at 0.45 mL/min, the column temperature is maintained at 40 °C, and the injection volume is 5.00 µL. The calibration standard responses for both caspofungin and AMB were linear over the range between 0.100 and 100 µg/mL using a weighted ($W=1/\chi^2$) linear regression.

### 2.3 Examination of liver and kidney damage on days 14 and 20

Blood samples were taken by puncturing the retro-orbital plexus while mice were under diethyl ether anaesthesia. Serum was collected from the centrifuged blood supernatant and used for liver and kidney function measurement and Enzyme-Linked Immunosorbent Assay (ELISA). Alanine aminotransferase (ALT) Kit, aspartate aminotransferase (AST) Kit, alkaline phosphatase (ALP) Kit, total bilirubin (TBIL) Kit, blood urea nitrogen (BUN)

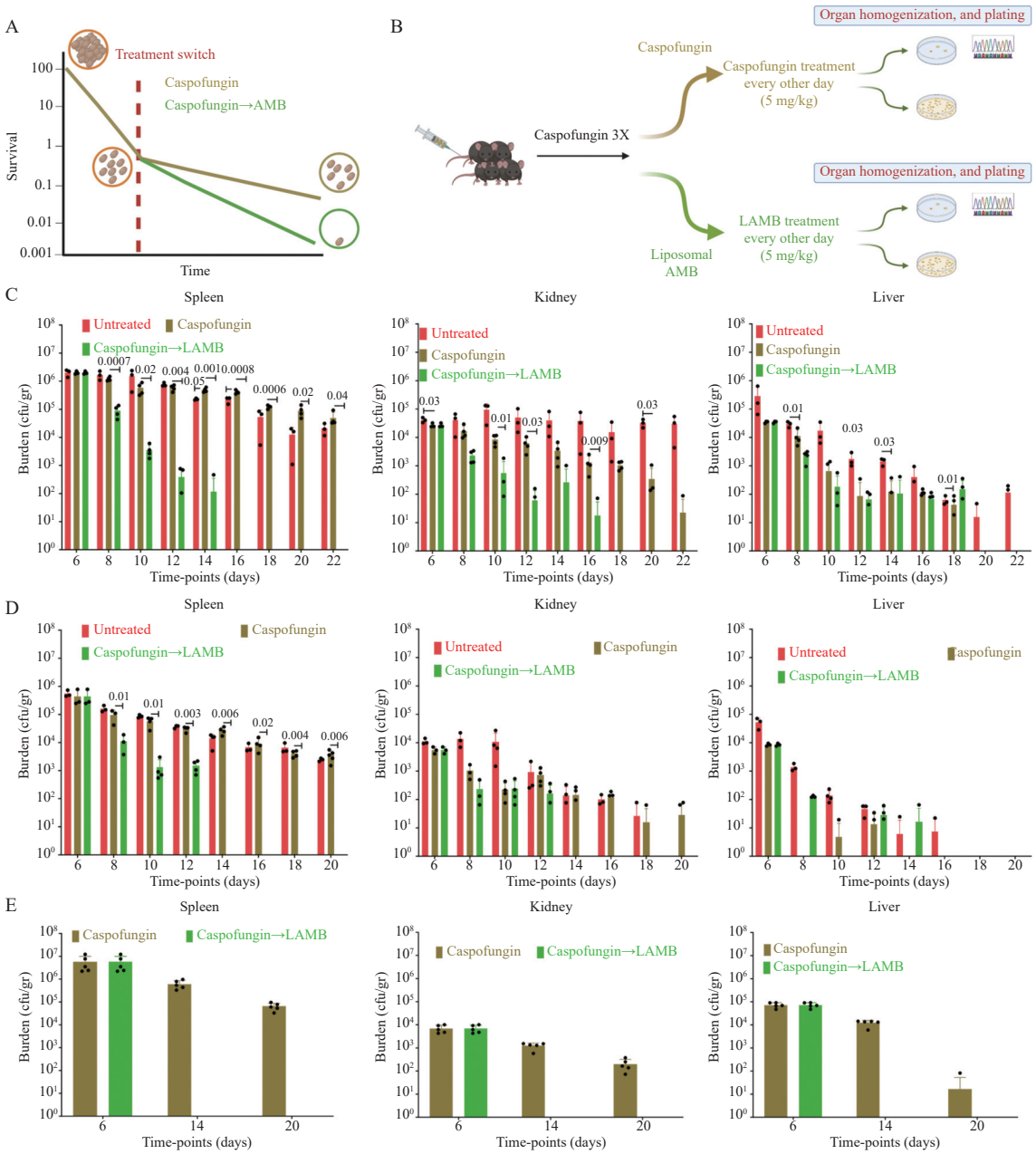


**Figure 1** Caspofungin alternation with LAMB shows superiority to caspofungin monotherapy regardless of the *Nakaseomyces glabratus* isolates and immune status. (A): Echinocandin-tolerant cells of *N. glabratus* can be more effectively killed once echinocandin treatment is switched to AMB. (B): Mice infected with *N. glabratus* isolates were treated with caspofungin for 5 days, followed by assigning them to continuous caspofungin or switching treatment to LAMB. Mice were sacrificed on designated timepoints, and spleen, kidney, and liver were homogenized and plated on YPD plates with/without caspofungin to determine burden and echinocandin-resistant colonies. (C): Mice infected with *N. glabratus* strain (#21) and treated with echinocandin alternation with LAMB had a significantly lower burden compared to conventional echinocandin treatment in all organs tested. (D): By repeating the entire experiment with the wildtype *N. glabratus* strain (CBS138), caspofungin alternation with LAMB also showed a higher efficacy in spleen and kidney when compared to standard caspofungin treatment, whereas such a trend was not observed in liver. (E): Immunosuppressed mice receiving LAMB alternation completely cleared the infection on day 14, whereas those receiving standard caspofungin failed to clear the infection on day 14. Similarly, except liver, caspofungin still failed to clear *N. glabratus* in the spleen and kidney of immunosuppressed mice on day 20. AMB = amphotericin B. LAMB = liposomal amphotericin B. CFU = colony forming unit.

Kit, and creatinine Kit were obtained from Sigma Chemicals (St. Louis, MO). Liver and kidney function were respectively performed on an autochemistry analyzer Chemray 800 (Rayto, Shenzhen, China) by using commercial Kits. All measurements were performed according to manufacturing instructions and standard methods recommended by the International Federation of Clinical Chemistry. Kim1 is one of the most reliable markers of acute kidney injury [23,24], and serum Kim1 concentrations were measured using the mouse Kim1 ELISA Kit (CSB-E08809m, CUSABIO, https://www.cusabio.com/) according to the manufacturer's instructions.

Harvested liver and kidney were fixed in 4% paraformaldehyde for 24 h. Liver and kidney sections were respectively paraffin-embedded, 5 µm sections were cut, and stained with hematoxylin and eosin (H&E) stain. Each section was scanned using a Panoramic 250 Flash III digital scanner (3DHISTECH Ltd., Budapest, Hungary) and scored blindly by pathologists. Liver and kidney sections were mainly examined for hepatic necrosis, and degeneration and necrosis of tubular epithelial cells, respectively. The score of tissue damage was based on the following standard: Grade 0 (no lesion), Grade 1 (0–10% affected by the lesion, slight lesion), Grade 2 (11%–20% affected by the lesion, small extent lesion), Grade 3 (21%–40% affected by the lesion, moderate lesion), and Grade 4 (41%–100% affected by the lesion, severe lesion).

### 2.4 Statistical analysis

SPSS software (v.24 for Windows; SPSS, Inc., Chicago, IL, USA) was used to carry out the statistical analysis, and $p$-values $\leq 0.05$ were considered statistically significant. The Shapiro test was used to determine the distribution of our data. Since our data were normally distributed, statistical analysis used a paired sample $t$-test to compare the average differences. Pearson correlation and simple regression analysis were used for the data presented in Figure 2I, and J. Wilcoxon test was used to compare non-parametric data (such as those presented in Figure 3E and I).

## 3 Results

### 3.1 Caspofungin alternation with LAMB shows superiority over caspofungin monotherapy against *N. glabratus* systemic infection

We used two isolates of *N. glabratus*, i.e., CBS138 and a clinical isolate (#21), which are fully susceptible to all antifungals tested and have similar minimum inhibitory concentration (MIC) against echinocandins (please see our previous study [25]). We have also previously shown that echinocandin-susceptible *N. glabratus* isolates can be categorized as high and low ECT through CFU counting under *in vitro* conditions. Consistently, using comprehensive *ex vivo* (within human macrophages) and systemic infection mouse models, the clinical isolate showed high ECT and ECR, whereas the WT strain CBS138 showed low ECT and did not bear ECR progenies [13] (Moreover, using multi-locus sequence typing, both isolates were found to belong to sequence type 15 [25]). Therefore, to better assess the ECR and the burden in various organs under caspofungin treatment, we initially used a clinical isolate (#21) to infect the C57BL/6 mice. This mouse model uses the humanized dose of caspofungin, which has been validated as a reliable model in various studies [12,13,26]. We chose caspofungin as it has been used as a comparator to novel antifungal drugs, such as rezafungin, in the context of randomized clinical trials of invasive candidiasis [27].

Mice were infected *N. glabratus* strain (#21) and allocated into 3 study groups, untreated, treated with humanized dose of caspofungin (5 mg/kg) for the entire study, and the third group comprised mice treated for 5 days with caspofungin, followed by changing treatment to LAMB (5 mg/kg) on day 7 (alternated group hereafter) (Figure 1B). Immediately upon changing the treatment and receiving a single LAMB dose, the alternated groups had a significantly lower burden compared to standard continuous caspofungin treatment in all organs tested (Figure 1C). Interestingly, fungal burden in spleen was similar between untreated and caspofungin-treated groups, whereas alternation with LAMB almost reduced the burden to the level of detection on day 14 (Figure 1C). Although caspofungin showed a higher efficacy in the kidney, the alternated group still showed superiority over caspofungin (Figure 1C). Both treatments effectively cleared the liver on the last days (Figure 1C).

To ensure that the observed results were not strain-specific and our observation was reproducible, we repeated the entire experiment with the wild-type *N. glabratus* strain (CBS138). As mentioned above, we have previously shown that this isolate has a low ECT and does not give rise to ECR under echinocandin treatment [13]. Interest-

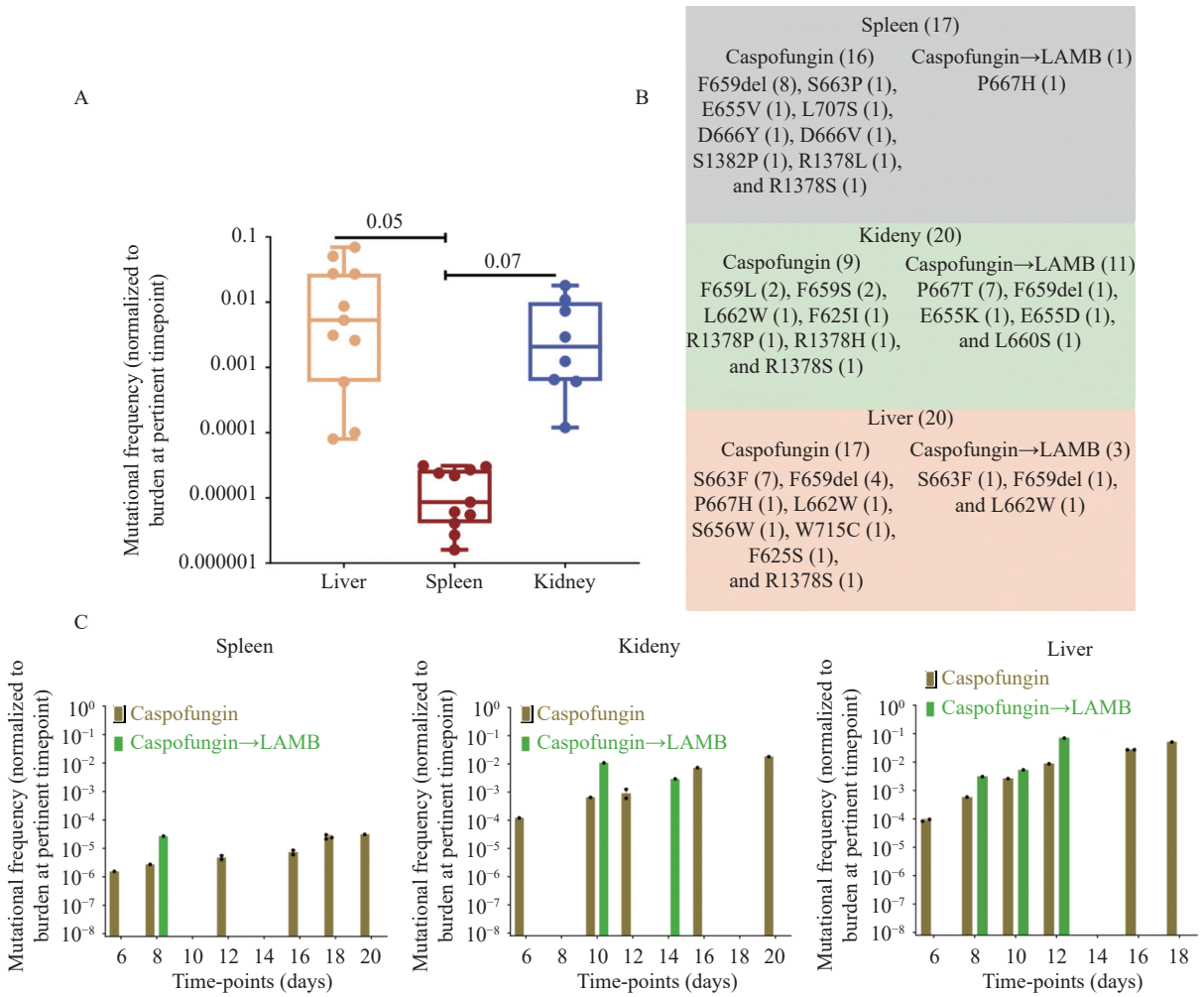


**Figure 2** Caspofungin alternation with LAMB shows superiority to caspofungin monotherapy in reducing the rate of ECR. (A and C): Although liver and spleen harbored the highest number of ECR colonies, the ECR rate (mutational frequency) was higher in liver and kidney compared to spleen. Note that each dot represents a single mouse. (B): Hotspot 1/2 of *FKS1* and/or *FKS2* mutations obtained from organs of mice. LAMB = liposomal amphotericin B. ECR = echinocandin resistant.

ingly, we observed the same trend, and LAMB alternation showed superiority in spleen and kidney (Figure 1D). We also assessed the applicability of this treatment strategy using an immunosuppressed systemic infection mouse model. Given that the burden difference was significant on days 14 and 20, we assessed organ burden on those days. Interestingly, whereas the caspofungin-treated group failed to clear yeast on day 14, all mice in the alternated groups cleared *N. glabratus* in all organs tested (Figure 1E). Except for the liver, fungal burden was still detectable on day 20 for caspofungin treated group. Collectively, these observations proved that irrespective of mouse immune system status and *N. glabratus* strains tested, caspofungin alternation with LAMB is superior to traditional caspofungin monotherapy.

### 3.2 Caspofungin alternation with LAMB minimizes the rate of ECR compared to traditional caspofungin monotherapy

Next, we investigated the rate of ECR in immunocompetent mice infected with the high ECT *N. glabratus* isolate (#21). The premise for using immunocompetent mice to assess ECR was that a combination of immune system activity alongside the high ECT is a prominent driver of ECR in *N. glabratus* [12,13]. The mechanism underlying ECR in *N. glabratus* is mostly driven by the acquisition of mutations in short stretches of catalytic subunit/s of β-1,3-glucan synthase, i.e., hotspot 1/2 (HS1/2) of *FKS1* and/or *FKS2*. Therefore, colonies growing on caspofungin plates were subjected to sequencing of all four loci, HS1 of *FKS1*/*FKS2* and HS2 of *FKS1*/*FKS2* (Figure 1B). Overall,

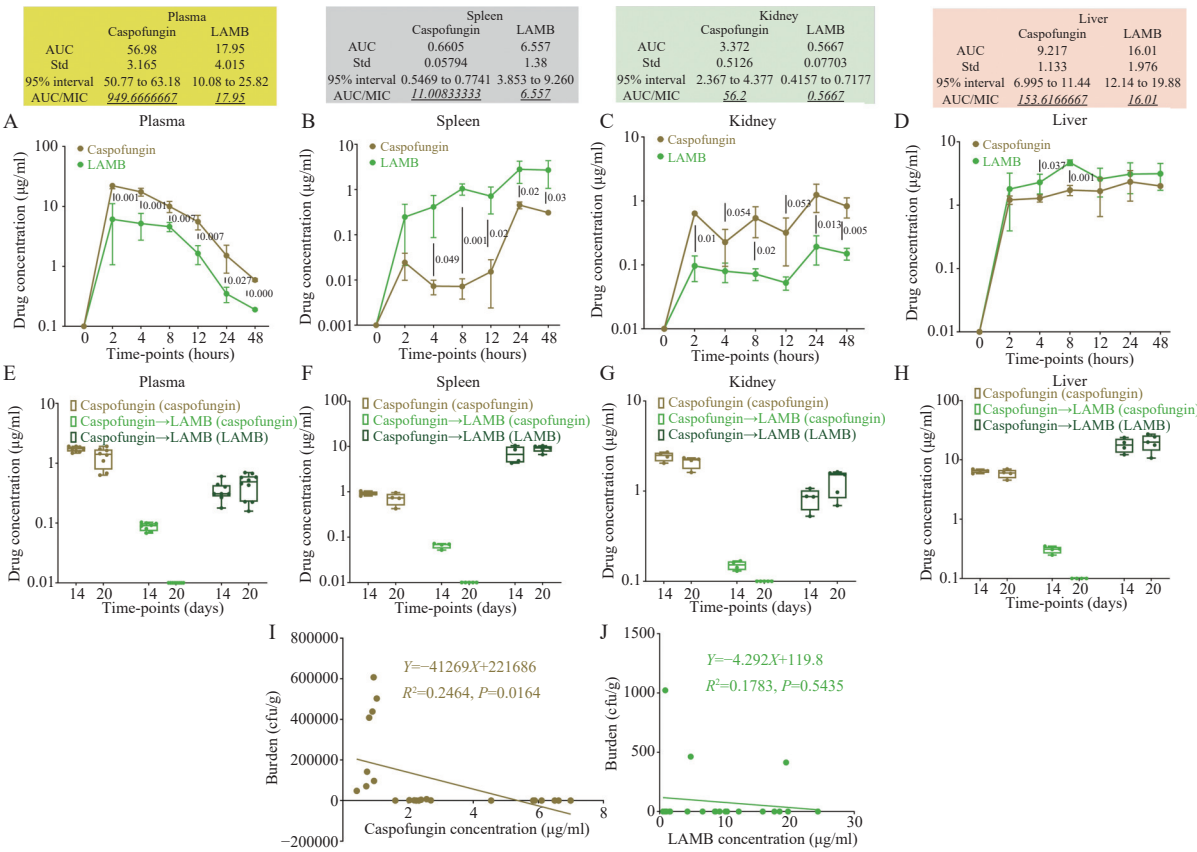


**Figure 3** Capofungin efficacy is associated with in-organ concentration, but LAMB effectively kills *Nakaseomyces glabratus* regardless of the in-organ concentration. (A-D): Our pharmacokinetic studies showed similar dynamics for caspofungin and LAMB, i.e., a rapid increase of drug concentration was followed by a rapid decline in plasma (A, lower panel), whereas the reverse trend was observed for other organs (B-D, lower panels). The AUC/MIC of caspofungin was higher than LAMB in all sample types (A-D upper panel). (E-H): The higher efficacy of caspofungin alternation with LAMB was not due to retention of residual caspofungin synergizing with LAMB, since caspofungin concentration was extremely low in the alternation arm on days 14 and 20 when mice within the caspofungin alternation with LAMB almost cleared the burden (light green and dark green boxes show the caspofungin and LAMB concentrations in the alternation arm, respectively). (I, J): Finally, whereas caspofungin organ concentration was negatively correlated with organ burden (I), such a trend was not observed for mice receiving caspofungin alternation with LAMB (J). LAMB = liposomal amphotericin B. AUC = area under the curve. MIC = minimum inhibitory concentration. CFU = colony-forming unit.

the number of mice harboring ECR mutants was higher in the liver and spleen compared to the kidney (Figure 2A and C). Additionally, ECR colonies were more steadily and frequently isolated from the organs of caspofungin treated group compared to those alternated with LAMB (Figure 2A and C). Interestingly, most of the mutations occurred in HS1-*FKS2*, followed by HS2-*FKS2* and HS1-*FKS1* (Figure 2B), which is consistent with the observation that almost 2/3 of clinical ECR isolates harbor *FKS2* mutations [28]. Moreover, with the exception of a few mutations, most of the mutations have already been described in clinical studies [28]. The detailed information on the mutations is listed in the Supplementary information (Table S1). Collectively, these observations suggested that, unlike the traditional caspofungin monotherapy, caspofungin alternation with LAMB minimizes the ECR rate in all organs tested.

### 3.3 Pharmacokinetic studies underline the superiority of caspofungin alternation with LAMB compared to traditional caspofungin monotherapy

The burden difference observed in organs of mice treated with caspofungin potentially indicated a differential caspofungin penetration. Therefore, to gain a deep insight on pharmacokinetics of caspofungin and LAMB, we treated mice with a single dose of caspofungin or LAMB and

monitored the dynamics of those antifungal drugs in plasma, spleen, kidney, and liver at multiple timepoints up to 48 h. Both caspofungin and LAMB had the highest plasma concentration (Cmax) at 2 h post-treatment (pt), followed by a rapid decline (Figure 3A to D). Contrarily, antifungal penetration gradually increased and either stayed constant after 2 h pt or continued to escalate, reaching a peak at 24 h in all organs (Figure 3A to D). Interestingly, caspofungin concentration was higher in plasma and kidney at all timepoints, whereas LAMB had a higher concentration in spleen, and both antifungal drugs showed almost similar concentration in liver (Figure 3A to D). Caspofungin concentration in organs was entirely consistent with the burden data; the liver exhibited the highest area under the curve (AUC)/MIC, followed by the kidney and spleen (Figure 3A to D). The higher killing of caspofungin in liver, therefore, can be explained by two factors, namely 1) a higher AUC/MIC of caspofungin in liver, and 2) a rapid Cmax attainment (at 2 h) and persistence in liver, which may have exposed *N. glabratus* to a significantly higher concentration for a longer period compared to kidney, and especially spleen. To rule out that the residual caspofungin concentration was sufficiently high to synergize with LAMB and therefore explain the higher efficacy of the alternated treatment, we measured caspofungin in the caspofungin monotherapy group and LAMB and caspofungin in the alternated group on days 14 and 20 pt. The caspofungin concentration of plasma, spleen, kidney, and liver was at least 10-fold lower in the alternated group compared to the caspofungin monotherapy group, and caspofungin was not detectable on day 20 in the alternated group (Figure 3E to H). Burden and caspofungin concentration measured on day 14 pt were negatively correlated (the higher the burden, the lower the drug penetration), whereas there was no association between LAMB organ concentration and burden (Figure 3I and J). Collectively, these observations suggest that the efficacy of the alternated strategy is due to in-organ concentration and metabolic independence of LAMB.

### 3.4 Caspofungin alternation with LAMB shows a promising safety profile

AMB is known to exert liver and kidney cytotoxicity, and LAMB is currently the polyene drug used in clinical practice to minimize the AMB-induced damage [7]. However, liver and kidney damage in antifungal therapy remains an important aspect in evaluating treatment regimens. Therefore, we sought to examine liver (ALT, AST, ALP, TBIL) and kidney (BUN, creatinine, and Kim1) damage on days 14 and 20 pt. We also morphologically assessed liver and kidney damage using H&E, using the scoring system described in the methods section. Liver panel data suggested a higher level of ALT for the alternated group on day 20 compared to other groups (Figure 4A). Similarly, AST level was significantly higher at day 20 for the alternated group compared to the caspofungin monotherapy group (Figure 4B). However, as for ALP and TBIL, there was no significant difference between alternated and caspofungin monotherapy groups. The TBIL level was higher in uninfected animals compared to the rest at both timepoints (Figure 4C and D). H&E on liver sections suggested similar levels of damage across all infected groups regardless of the timepoints (Figure 4E; Figure S1). These observations may suggest that despite an initial liver damage potentially induced by LAMB at day 14 pt, liver damage at day 20 pt is similar among the infected groups and may have been induced by infection. Regarding the kidney panel, BUN and creatinine levels were similar across the timepoints and groups (Figure 4F and G), whereas Kim1 was significantly higher for the alternated group compared to the rest only at day 20 pt (Figure 4H). Nonetheless, H&E on the kidney section suggested similar levels of damage across all infected groups (Figure 4I; Figure S2). Collectively, the combination of histological and biomarker analysis suggests that caspofungin alternation with LAMB shows a desirable safety profile, especially one week after LAMB treatment (day 14).

## 4 Discussion

ECR and MDR *Candida* pathogens pose a relevant threat to the clinical utility of echinocandins, and devising novel strategies to mitigate this issue is highly desirable [29,30]. Herein, we investigated a novel alternating treatment strategy, which effectively clears a systemic infection of *N. glabratus* regardless of immune status and the isolate used. Importantly, it minimized the ECR rate in all organs tested.

Echinocandins are highly dependent on the metabolism of *Candida* cells, and ECT cells exploit their lower metabolic activities to survive and to fuel the ECR. Polyenes, on the other hand, are not metabolically dependent, but their long-term use confers hepatic and renal cytotoxicity [12]. Therefore, alternate use of echinocandins

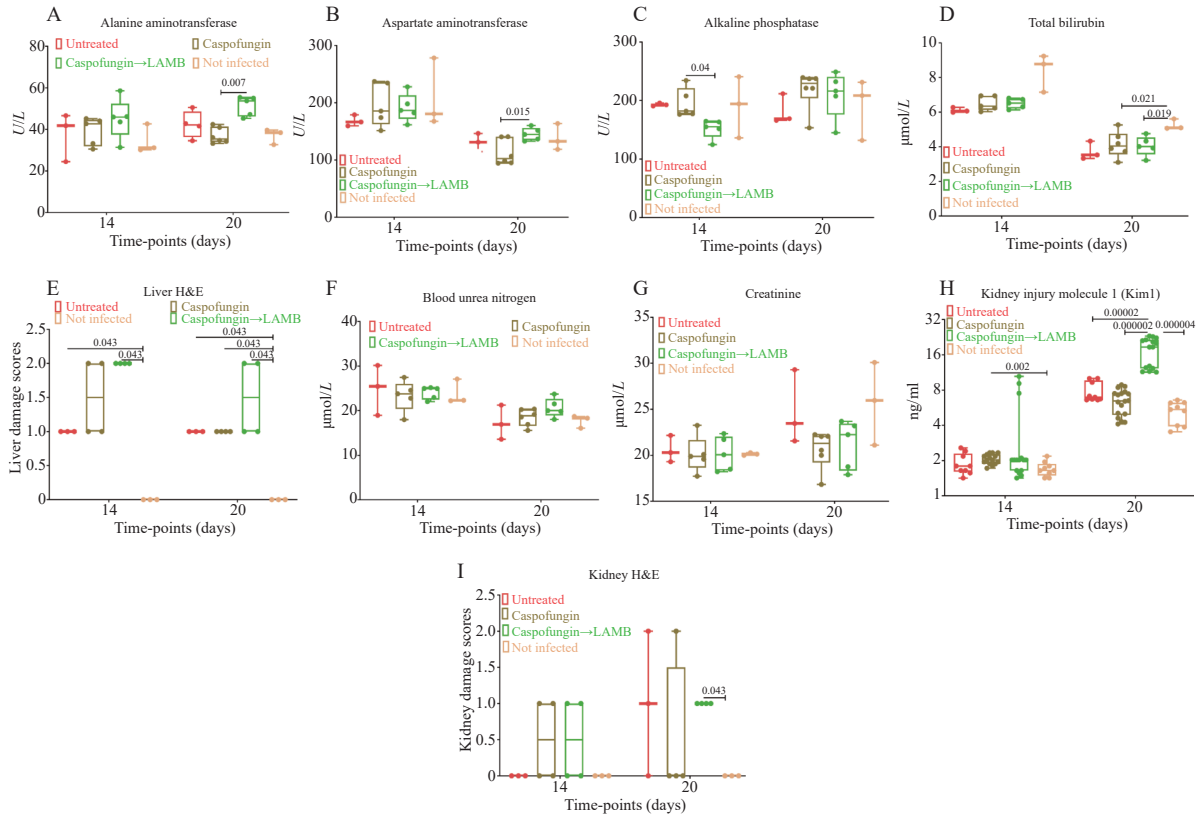


**Figure 4** Comparing liver and kidney damage using a panel of biomarkers and H&E stain. (A-C): Alanine- and aspartate aminotransferases were higher for mice treated with LAMB alternation (A and B), whereas alkaline phosphatase was similar across all groups (C). (D): Total bilirubin was similar across all the timepoints among infected groups. (E): H&E staining of liver sections showed similar damage across all infected groups. (F and G): Blood urea nitrogen and creatinine were similar across the mice within the infected groups. (H): Kim1 biomarker showed a significantly higher kidney damage in mice treated with LAMB alternation on day 20. (I): H&E staining of kidney sections showed similar damage across all infected groups. LAMB = liposomal amphotericin B. H&E = hematoxylin and eosin.

and polyenes can a) more effectively kill the ECT and reduce ECR, and b) shorten the polyenes use duration and therefore minimize their potential cytotoxicity. Additionally, adaptation and acquiring resistance against two classes of antifungal drugs with entirely different modes of action is much more challenging compared to a single antifungal drug [31]. Importantly, our experiences with patients infected with MDR *C. parapsilosis* have also proven that the polyene AMB is the preferred choice to effectively tackle such infections [32]. Nonetheless, frequent use of any antifungal drugs will eventually lead to antifungal resistance, but this can be minimized by alternating use of two antifungal drugs as mentioned. Finally, AMB resistance among prevalent *Candida* species, i.e., *C. albicans*, *C. parapsilosis*, *N. glabratus*, and *C. tropicalis*, is extremely rare, which is attributed to the high fitness cost carried by AMB-resistant isolates [33]. Therefore, our treatment strategy should also be extended to test whether it has the potential to improve the outcome of patients with concomitant invasive candidiasis or those requiring prolonged and repeated echinocandin treatment, which favors ECR emergence. Additionally, although novel antifungal drugs, such as newly approved rezafungin, have shown similar efficacy to other echinocandins, single and widespread use of such antifungal drugs can drive the emergence of resistance and deteriorate their clinical utility in the future [34,35]. Therefore, our therapeutic strategy, once validated in clinical trials and in-human studies, may also be applied and used in tandem with other antifungal drugs to minimize resistance and preserve their efficacy.

In fact, clinical studies using human samples have noted that, except for *C. parapsilosis*, effective clearance of candidemia requires reaching an AUC/MIC of ≥ 3 000

and < 12,000 [36]. Nonetheless, the humanized dose used in our study failed to reach this recommended range in all specimens tested. Consistently, some studies have advocated increasing echinocandin dosage [36-38] even up to 900 mg/day for several weeks or a single dose of 1 400 mg/day to attain a higher clinical success [38]. This concept may seem reasonable since antifungal resistance is also proposed to be driven by exposure to sublethal concentrations of antifungals [14-16]. However, our *in vivo* data do not agree with the direct correlation between echinocandin concentration and ECR. Indeed, the AUC/MIC of liver was almost 14 times higher than spleen; nonetheless, the number of mice harboring ECR colonies between these organs was similar, and the mutational frequency (ECR colonies/organ burden) was significantly higher in liver compared to spleen (up to 1 000-fold higher). Importantly, such studies failed to take this important criterion into account, as the final goal was set at burden.

Although LAMB is a less toxic polyene, it exerts some degree of liver and kidney damage as judged by measuring various kidney and liver biomarkers, especially at the last time point. Nonetheless, H&E liver and kidney sections showed similar levels of damage for both therapeutic regimens. These differences could be explained by technical differences inherent to biomarkers and pathologic analysis, and we believe that, consistent with previous studies, a higher proportion of patients treated with LAMB may suffer from nephrotoxicity compared to caspofungin monotherapy [39]. Despite extensive use of animal experiments and promising observations, our therapeutic measure potentially would not be optimal for some species, such as *C. auris*, as it has a high rate of AMB resistance [11]. Secondly, future studies are warranted to examine the efficacy of our treatment strategy in the context of a) intra-abdominal candidiasis and GI-tract models, and b) a mouse model of candidiasis induced by other *Candida* species.

Collectively, we showed that alternate use of caspofungin with LAMB offers therapeutic advantages compared to caspofungin monotherapy by shorter therapy to reach near fungal clearance and minimizing ECR rate, while reducing the LAMB treatment duration to decrease polyene-induced kidney and liver damage. The promising findings found herein suggest that our therapeutic regimen would be an ideal candidate to be tested against caspofungin monotherapy in the context of clinical trials as targeted treatment. Moreover, patients undergoing allogeneic hematopoietic cell transplantation prophylactically treated with micafungin may also experience breakthrough infection without the emergence of ECR *C. parapsilosis* isolates [40]. Indeed, such isolates are shown to exhibit a higher ECT [40], which further highlights the importance of drug exposure levels and minimization or elimination of the entire microbial subpopulation within the host to reach therapeutic success. Therefore, our strategy may also be beneficial for prophylactic measures, which again requires clinical trials.

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## Conflicts of interest

D.S.P. serves as an advisor to Merck, Scynexis, and MundiPharma. M.H. received research funding from Gilead, Astellas, MSD, IMMY, Pulmocide, Shionogi, Melinta, Mundipharma, Scynexis, F2G, and Pfizer. All other authors declare no financial or non-financial competing interests.

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## Author's contributions

W.P. and A.A. carried out the conception and design of

the study. L.C. and X.X. conducted the experiments and acquired the data. L.C., A.A., and M.S. analyzed and interpreted the data. L.C., W.P., and A.A. drafted the article. D.S.P., M.H., W.L., and W.F. revised it critically for important intellectual content. All authors reviewed the manuscript.

## Data availability

Accession numbers of HS1/2 of *FKS1* and *FKS2* are deposited in NCBI under the following accession numbers PV930070-PV930078 and PV940282-PV940329.

## Ethical approvals statement

All the animal experiments used in the current study followed the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and EU Directive 2010/63/EU, and received ethical approval from the Naval Medical University (82204470).

## Electronic supplementary material

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