

## ORIGINAL ARTICLE

# Synergistic effect of nikkomycin Z with caspofungin and micafungin against *Candida albicans* and *Candida parapsilosis* biofilms

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**Significance and Impact of the Study:** Antifungal lock therapy can be a potential therapeutic approach to eradicate the intraluminal *Candida* biofilms; however, there is no approved lock strategy against fungal species so far. The results of this study provide valuable evidence that nikkomycin Z acts synergistically in combination with caspofungin or micafungin against biofilms. In addition, this synergy was more pronounced for micafungin combined with nikkomycin Z. Therefore, nikkomycin Z can be considered as a potential agent in antifungal lock therapy especially with micafungin against *C. albicans* or *C. parapsilosis* biofilms.

## Keywords

biofilm, chitin synthase inhibitor, antifungal lock therapy, echinocandin, resistance.

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## Abstract

Antifungal lock therapy has received significant interest in the last few years because the frequently usage of intravascular devices is associated with an increasing number of catheter-related bloodstream infections caused by *Candida* species. Antifungal combinations with synergistic interaction can be a good choice for antifungal lock therapy; therefore, interactions were examined between two echinocandins (caspofungin and micafungin) and the chitin synthesis inhibitor nikkomycin Z against *Candida albicans* and *C. parapsilosis* biofilms. Susceptibility was evaluated using the XTT-based checkerboard microdilution method, while the nature of interactions was assessed by calculating fractional inhibitory concentration indices and using the Bliss independence model. Mathematic-based evaluations were supplemented with fluorescent LIVE/DEAD viability assay. The results obtained by statistical interaction analyses correlated well with the viability assay. The tested echinocandins with nikkomycin Z caused an extended cell death and the structure of the biofilm was sparse compared to the control, especially for *C. albicans*. The findings support the simultaneous usage of nikkomycin Z and caspofungin or micafungin in alternative therapies such as the antifungal lock therapy.

## Introduction

*Candida albicans* remains the most frequently isolated fungal species from catheter-associated infections followed by *C. parapsilosis* due to their high biofilm-forming ability (Bassetti *et al.* 2013; Soldini *et al.* 2018; Monfredini *et al.* 2018). A previous study revealed that *Candida* biofilm production is associated with significantly higher mortality compared to patients with bloodstream infections caused by non-biofilm-forming *Candida* species

(Rajendran *et al.* 2016). Echinocandins are a group of semisynthetic cyclic lipopeptides, which inhibit the synthesis of  $\beta$ -glucan in the fungal cell wall via noncompetitive inhibition of 1,3- $\beta$  glucan synthase (Patil and Majumdar 2017). They can be potential candidates for anti-biofilm alternative therapies such as antifungal lock therapy, where prolonged instillation of a solution containing high concentration of antifungal agent is utilized within and intravascular catheter (Walraven and Lee 2013). However, lock treatment can fail especially against

*C. parapsilosis* biofilms due to the previously well-documented intrinsic *fkS* mutation (Garcia-Effron *et al.* 2008). Nikkomycin Z is a nucleoside-peptide, which inhibits chitin synthesis by acting as competitive analogue of the chitin synthase substrate UDP-N-acetylglucosamine (Kim *et al.* 2002). *In vitro* synergistic interactions have been already described with various antifungals including echinocandins against *Candida* species; however, these studies focused on exclusively planktonic forms (Sandovsky-Losica *et al.* 2008; Szilágyi *et al.* 2012; Cheung and Hui 2017); therefore, the potential synergistically effect of nikkomycin Z with echinocandins against *Candida* biofilms remains unclear.

To extend our knowledge about *in vitro* interactions, we evaluated the effect of caspofungin and micafungin combined with nikkomycin Z against *C. albicans* and *C. parapsilosis* biofilms. We hypothesized that nikkomycin Z exerts a synergistic interaction with the tested echinocandins supporting the use of these combinations in the above-mentioned antifungal lock therapy.

## Results and discussion

Under planktonic conditions five out of six *C. albicans* isolates were susceptible to the tested echinocandins based on revised clinical laboratory standards institute break-points (Pfaller *et al.* 2011). As expected, isolate DPL18 was resistant both to caspofungin and micafungin (minimal inhibitory concentration was 2 mg l<sup>-1</sup> for caspofungin, as well as for micafungin) (Pfaller *et al.* 2011). For susceptible *C. albicans* clinical isolates, the planktonic MICs ranged from 0.015 to 0.03 mg l<sup>-1</sup>, from 0.015 to 0.06 mg l<sup>-1</sup>, and from 4 to 8 mg l<sup>-1</sup> for caspofungin, micafungin and nikkomycin Z respectively. Four out of five planktonic clinical *C. parapsilosis* isolates were susceptible to both echinocandins with a narrow range of MIC values (between 1 and 2 mg l<sup>-1</sup>), while isolate CP3 showed intermediate susceptibility (MIC of 4 mg l<sup>-1</sup>) both for caspofungin and micafungin (Pfaller *et al.* 2011). The examined nikkomycin Z concentrations resulted prominent inhibitory effect from 8 mg l<sup>-1</sup> concentration against planktonic *C. parapsilosis* isolates. The variability of the planktonic MICs across repetitions was low, the difference between repetitions were within one dilution in case of all isolates.

The median values of MIC for *C. albicans* and *C. parapsilosis* biofilms are presented in Table 1. Similar to planktonic forms, the variability of biofilm MIC between repetitions were within one dilution in case of all isolates. In case of echinocandin-susceptible *C. albicans* isolates, nikkomycin Z resulted in 2- to 16-fold and 16- to 128-fold decrease of the median MICs for caspofungin and micafungin respectively. Moreover, nikkomycin Z median

MIC values showed 8- to 512-fold decrease in combination with caspofungin or micafungin against susceptible *C. albicans* sessile cells. Nikkomycin Z exerted 64-fold and 4-fold MIC value decrease against isolate DPL18 for caspofungin and micafungin respectively. In case of *C. parapsilosis* biofilms, the median MIC values observed for caspofungin and micafungin in combination with nikkomycin Z demonstrated 2- to 4-fold and 2- to 64-fold decrease for caspofungin and micafungin respectively. The median MIC values of nikkomycin Z in combination with echinocandins showed 2- to 512-fold reduction for *C. parapsilosis* biofilms.

Table 1 summarizes the *in vitro* interactions between the tested echinocandins and nikkomycin Z based on FICI calculations and Bliss independence analysis. By FICI, synergy between nikkomycin Z and caspofungin or micafungin was observed against the majority of *C. albicans* isolates (Table 1). An indifferent effect was noticed exclusively for isolate CA2 and CA3 but these biofilms had lower MIC values against caspofungin or micafungin without nikkomycin Z (Table 1). The combination of caspofungin and nikkomycin Z yielded an indifferent interaction against all five *C. parapsilosis* isolates (FICI 0.502-1). Of note, FICIs of three out of five isolates were very close to threshold indicating synergy (FICI 0.502-0.508) (Table 1). Based on FICI values, a striking synergistic interaction was observed for four out of five *C. parapsilosis* isolates after micafungin with nikkomycin Z exposure (FICI 0.017-0.5), which was confirmed by the results of Bliss independence analysis (Table 1). Statistically significant synergy was revealed for most combinations with caspofungin and micafungin both against echinocandin-susceptible *C. albicans* clinical isolates and the resistant strain using Bliss analysis (Table 1, Fig. 1a, b). Although FICIs demonstrated indifferent interactions between caspofungin and nikkomycin Z against *C. parapsilosis* isolates, these interactions were synergistic for most combinations using MacSynergy calculations (Table 1). This discrepancy could be explained by the failure of FICI determination, which is too subjective and sensitive to experimental errors. The potential variability between various methods highlights the necessity to apply multiple analytic approaches in case of examinations of drug-drug interactions.

In accordance with FICI findings, highly significant synergy was detected using Bliss model for combinations of micafungin and nikkomycin Z against *C. parapsilosis* biofilms (Fig. 1c,d). It is noteworthy that clear antagonistic interactions were described for the tested species neither using FICI calculations nor using the Bliss independence model (Table 1).

LIVE/DEAD viability staining revealed that micafungin-exposed *C. albicans* and *C. parapsilosis* biofilms exhibited

**Table 1** Minimum inhibitory concentration of caspofungin (CAS) and micafungin (MICA) alone and in combination with nikkomycin Z (NZ) against *Candida albicans* (CA) and *Candida parapsilosis* (CP) biofilms (sMIC). Furthermore, *in vitro* interactions (INT) by fractional inhibitory concentration index (FICI) and Bliss independence analysis of caspofungin and micafungin in combination with nikkomycin Z against *C. albicans* and *Candida parapsilosis* biofilms

Isolates	Median sMIC values (mg l <sup>-1</sup> )										Type of interaction						
	sMIC alone					sMIC in combination					Median FICI			BLISS independence model			
	CAS	MICA	NZ	CAS	NZ	CAS	NZ	MICA	NZ	CAS	CAS	INT	MICA	INT	CAS Synergy/ Antagonism (log volume)	MICA Synergy/ Antagonism (log volume)	INT
<i>Candida albicans</i>																	
CA1	0.5	>1*	>8†	0.03	0.12	0.03	0.03	0.063	0.063	0.062	SYN	0.062	SYN	71.56/-2.18	162.72/-1.85	SYN§	SYN§
CA2	0.06	1	8	0.015	0.06	0.5	0.06	0.625	0.625	0.075	IND	0.075	SYN	28.36/-13.14	40.81/-5.43	SYN§	SYN§
CA3	0.06	>1*	>8†	0.03	0.015	2	0.015	0.75	0.75	0.061	IND	0.061	SYN	31.04/-6.27	205.45/-9.31	SYN§	SYN§
CA4	0.5	1	>8†	0.03	0.03	0.03	0.03	0.183	0.183	0.123	SYN	0.123	SYN	72.24/-8.02	114.55/-17.95	SYN§	SYN§
CA5	0.5	1	>8†	0.03	0.03	0.03	0.03	0.063	0.063	0.075	SYN	0.075	SYN	68.28/-5.08	98.45/-6.31	SYN§	SYN§
DPL18¶	8	4	>8†	0.12	1	1	2	0.245	0.245	0.037	SYN	0.037	SYN	83.75/-25.0	66.63/-3.61	SYN§	SYN§
<i>Candida parapsilosis</i>																	
CP1	128	>512‡	>8†	64	16	0.12	0.5	0.508	0.508	0.078	IND	0.078	SYN	33.87/-0.95	253.19/0	SYN§	SYN
CP2	128	>512‡	>8†	64	32	8	4	1	1	0.5	IND	0.5	SYN	33.05/-9.32	210.68/0	SYN§	SYN
CP3	256	>512‡	>8†	128	512	0.03	8	0.502	0.502	1	IND	1	IND	51.67/-0.3	93.15/0	SYN§	SYN
CP4	128	>512‡	>8†	64	16	8	0.03	1	1	0.017	IND	0.017	SYN	31.09/-6.12	239.42/0	SYN§	SYN
CP5	256	>512‡	>8†	64	16	0.03	0.03	0.502	0.502	0.017	IND	0.017	SYN	29.26/-5.38	437.95/0	SYN§	SYN

SYN, Synergy; IND, Indifferent.

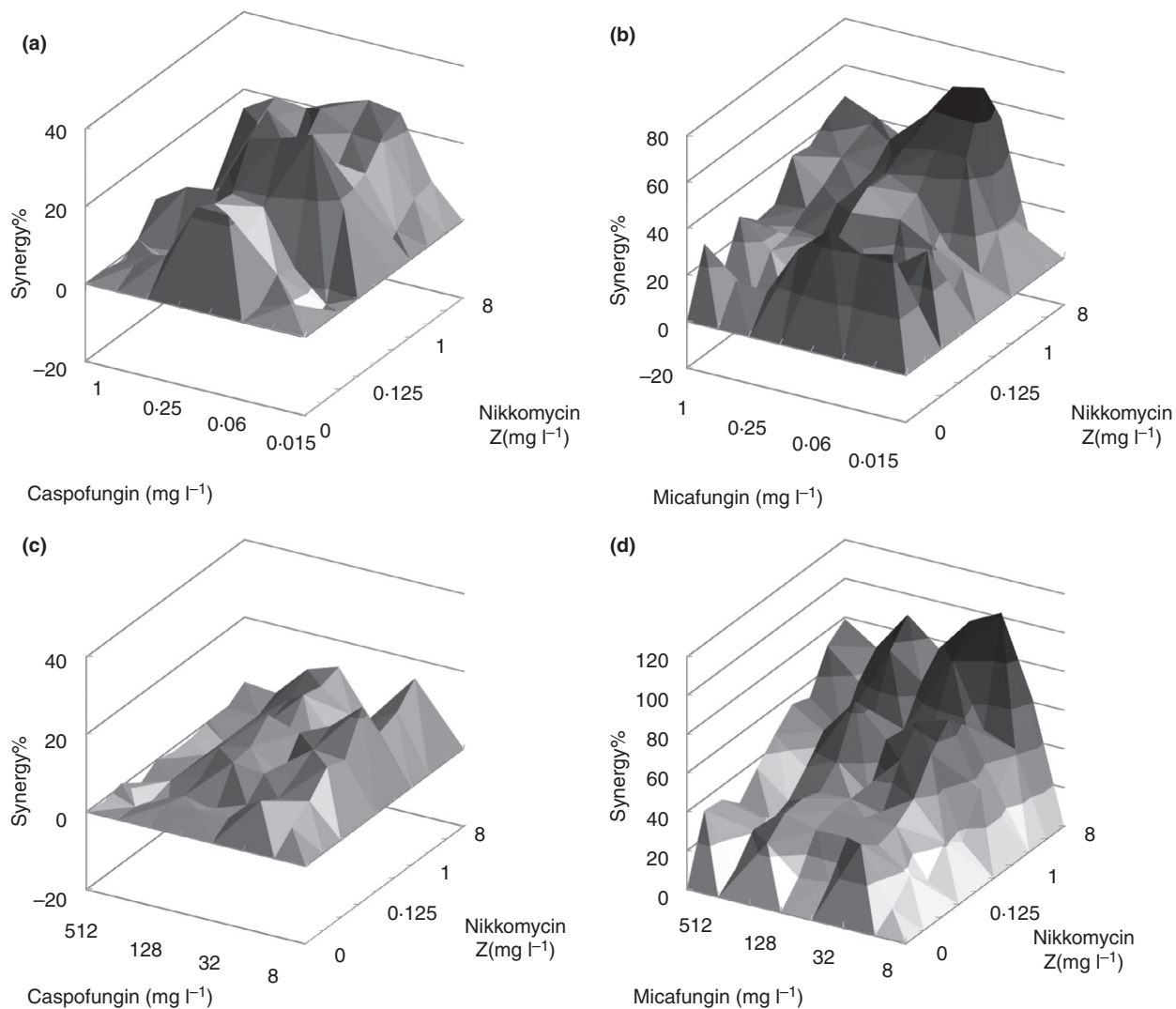
\*MIC is offscale at >1 mg l<sup>-1</sup>, 2 mg l<sup>-1</sup> (one dilution higher than the highest tested concentration) was used for analysis.

†MIC is offscale at >8 mg l<sup>-1</sup>, 16 mg l<sup>-1</sup> (one dilution higher than the highest tested concentration) was used for analysis.

‡MIC is offscale at >512 mg l<sup>-1</sup>, 1024 mg l<sup>-1</sup> (one dilution higher than the highest tested concentration) was used for analysis.

§Synergy for most combinations.

¶Echinocandin-resistant *C. albicans* isolate (FKS F641S).

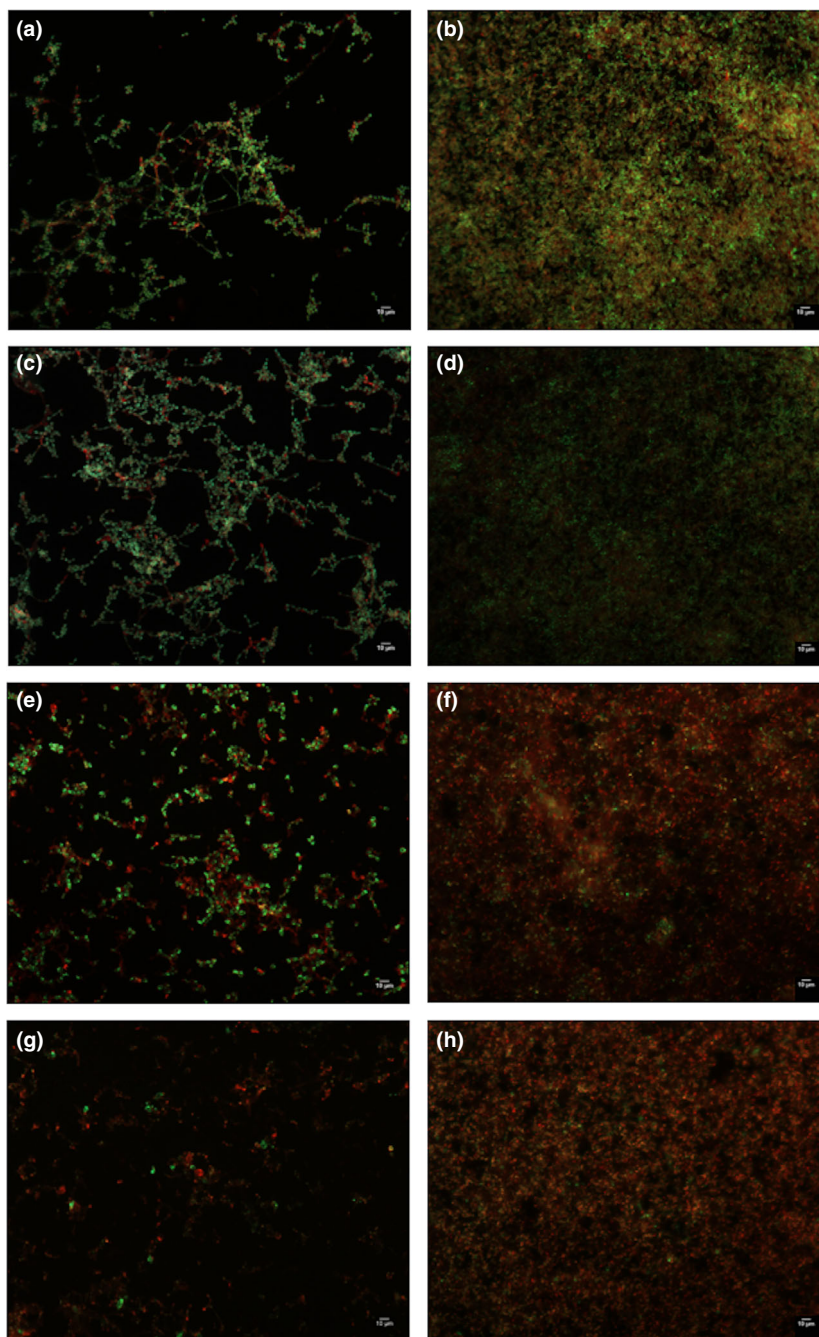


**Figure 1** Effect of nikkomycin Z in combination with caspofungin (a and c) and micafungin (b and d) against *Candida albicans* (a and b) and *Candida parapsilosis* (c and d) sessile cells of one-one representative isolate of *C. albicans* (CA1) and *Candida parapsilosis* (CP1) by MacSynergy II analysis. Peaks of positive values represent synergy, whilst negative values indicate antagonism at given concentrations (a and b: (■) 60–80, (■) 40–60, (■) 20–40, (■) 0–20; (□) –20–0; (□) –20–0; c and d: (■) 100–120, (■) 80–100, (■) 60–80; (■) 40–60; (■) 20–40; (□) 0–20).

increased cell death in the presence of nikkomycin Z (Fig. 2g,h) compared to untreated biofilms (Fig. 2a,b), micafungin-exposed sessile populations (Fig. 2e,f) or nikkomycin Z treated biofilms (Fig. 2c,d). Caspofungin showed highly similar pattern to micafungin both alone and in combination with nikkomycin Z. To confirm the metabolic activity-based experiments and LIVE/DEAD viability staining, the number of cultivable sessile cells was determined by quantitative culturing of one-one representative isolates from *C. albicans* (CA1) and *C. parapsilosis* (CP1) on Sabouraud dextrose agar as in our previous study (Nagy *et al.* 2018). The obtained CFU count in echinocandin-nikkomycin Z combinations showed significant CFU decrease ( $P < 0.05$ – $0.01$ )

compared to cell number derived echinocandin-exposed *Candida* sessile populations (data not shown).

Previous studies revealed the potential synergizing effect of nikkomycin Z in combination with traditional antifungal agents against *Candida* species (Sandovsky-Losica *et al.* 2008; Szilágyi *et al.* 2012; Cheung and Hui 2017). However, these works examined exclusively the anti-planktonic effect of these combinations and recently there is no information about susceptibility of biofilms formed by *C. albicans* or non-albicans species. A combination treatment with nikkomycin Z and an echinocandin have several advantages such as the prevention of echinocandin-induced paradoxical growth, which can cause concerns if high doses of echinocandins are used



**Figure 2** LIVE/DEAD fluorescence imaging of one *Candida albicans* (CA1) (a, c, e and g) and one *Candida parapsilosis* (CP1) (b, d, f and h) representative isolates after micafungin exposure with (g and h) and without (e and f) nikkomycin Z. Pictures a and b show the untreated *C. albicans* and *Candida parapsilosis* biofilms respectively; while picture c and d demonstrate the nikkomycin Z-exposed biofilms for *C. albicans* (c) and *C. parapsilosis* (d) respectively. Live cells (green) and nonviable cells (red) were stained with Syto9 and propidium iodide respectively. All images show typical fields of view. Scale bars represent 10  $\mu\text{m}$ . [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

against *C. albicans* or *C. parapsilosis* in antifungal lock therapy (Melo *et al.* 2007; Shields *et al.* 2011). Furthermore, *C. parapsilosis* isolates have intrinsic reduced susceptibility to echinocandins (Garcia-Effron *et al.* 2008); therefore, compounds with echinocandin-potentiator effect are needed against biofilms formed by this species.

*C. albicans* cells with decreased chitin content are associated with increased susceptibility against echinocandins; which can explain the synergizing effect of nikkomycin Z (Plaine *et al.* 2008). The observed synergistic interaction

between echinocandins and nikkomycin Z against *C. albicans* biofilms may be attributable to the simultaneous inhibition of synthesis of glucan and chitin components in cell wall leading to higher osmotic stress and cell lysis (Plaine *et al.* 2008; Cheung and Hui 2017). Our fluorescent images support this hypothesis because there were no filamentous elements following exposure to echinocandins with nikkomycin Z. Furthermore, the number of viable cells was low, and the structure of the biofilm was sparse compared to the control, especially for *C. albicans*.

Concerning echinocandin-resistant *C. albicans* biofilms, synergy was observed similar to the planktonic results by Cheung and Hui (2017). Their study described a synergistic effect between nikkomycin Z and micafungin or anidulafungin (FICI 0.38) in line with the findings of our reported FICIs (FICIs were 0.245 and 0.037 for caspofungin and micafungin respectively) (Cheung and Hui 2017).

Unfortunately, the lack of data dealing with detailed molecular changes in echinocandin- or nikkomycin Z-exposed *C. parapsilosis* cell wall precludes any explanation regarding the background of synergism between echinocandins and nikkomycin Z. However, we hypothesize that the synergistic effect observed against *C. parapsilosis* may be caused by understudied specific cell wall stress pathways of *C. parapsilosis*.

Interestingly, micafungin showed higher synergism combined with nikkomycin Z both against *C. albicans* and *C. parapsilosis* biofilms compared to the combination of caspofungin and nikkomycin Z. This striking synergy observed can be explained by micafungin-induced extended production of reactive oxygen species and increased apoptosis as reported by Shirazi and Kontoyiannis (2015). Our study was subject to two limitations. First, we included only one *fls* mutant *C. albicans* isolates. Second, we did not evaluate the effect of anidulafungin combined with nikkomycin Z. Despite these limitations, the potentiator effect of nikkomycin Z in combination with caspofungin or micafungin against *C. albicans* or *C. parapsilosis* biofilms is unquestionable, which supports their common use in alternative treatment even in antifungal lock therapy.

In conclusion, the present study is the first analysis examining the interaction of echinocandins and nikkomycin Z against biofilms. We have shown that nikkomycin Z is capable of causing a synergy in combination with caspofungin or micafungin against *C. albicans* and *C. parapsilosis* biofilms. This synergy was more pronounced for micafungin combined with nikkomycin Z. It is highlighted that synergistic interaction was observed not only for echinocandin-susceptible strains but also for the echinocandin-resistant *C. albicans* isolate. Our results support the simultaneous use of nikkomycin Z and caspofungin or micafungin in alternative therapies as antifungal lock therapy. Nevertheless, further animal experiments are needed in the future to confirm these promising *in vitro* results.

## Materials and methods

### Isolates

Five clinical *C. albicans* and five *C. parapsilosis sensu stricto* isolates derived from bloodstream infections were

examined. All were identified using MALDI/TOF (matrix-assisted laser desorption/ionization/time of flight) analysis (Microflex, Bruker Daltronics, Bremen, Germany). Furthermore, identification of *C. parapsilosis sensu stricto* isolates was also confirmed using molecular biology method (Tavanti *et al.* 2005). One echinocandin-resistant *C. albicans* isolate (DPL18, FKS F641S) was also included in the study.

### Minimal inhibitory concentration (MIC) determination of planktonic cells

Planktonic MIC determination was performed in accordance with the protocol M27-A3 of Clinical Laboratory Standards Institute (Clinical and Laboratory Standards Institute 2008). MICs of caspofungin (Sigma, Budapest, Hungary), micafungin (Abmole Bioscience Inc., Houston) and nikkomycin Z (Sigma, Budapest, Hungary) were determined in RPMI-1640 (with L-glutamine and without bicarbonate, pH 7.0 with MOPS; Sigma, Budapest, Hungary). The tested drug concentrations ranged from 0.015 to 8 mg l<sup>-1</sup> for caspofungin and micafungin, while nikkomycin Z concentrations ranged from 0.06 to 32 mg l<sup>-1</sup>. MICs were determined as the lowest drug concentration that produces at least 50% growth reduction compared to the growth control. MICs represent three independent experiments per isolate and are expressed as median.

### Biofilm development

*Candida albicans* and *C. parapsilosis* 1-day-old biofilms were prepared as described previously (Kovács *et al.* 2016). Briefly, isolates were suspended in RPMI-1640 broth in concentration of 1 × 10<sup>6</sup> cells per ml and aliquots of 100 µl were inoculated onto flat-bottom 96-well sterile microtitre plates (TPP, Trasadingen, Switzerland) and then incubated statically at 37°C for 24 h (Kovács *et al.* 2016).

### Antifungal susceptibility testing of biofilms

The examined echinocandin concentrations for MIC determination ranged from 0.015 to 1 mg l<sup>-1</sup>, from 0.25 to 16 mg l<sup>-1</sup> and from 8 to 512 mg l<sup>-1</sup> for echinocandin-susceptible *C. albicans* isolates, echinocandin-resistant *C. albicans* strain and *C. parapsilosis* isolates respectively. The examined nikkomycin Z concentrations ranged from 0.03 to 8 mg l<sup>-1</sup> in case of both species. The biofilms were washed three times with sterile physiological saline. Afterwards, MIC determination was performed in RPMI-1640 using the XTT-assay as described previously (Kovács *et al.* 2016). The percentage change in metabolic activity

was calculated on the basis of absorbance ( $A$ ) as  $100\% \times (A_{\text{well}} - A_{\text{background}}) / (A_{\text{drug-free well}} - A_{\text{background}})$ . MICs of biofilms were defined as the lowest drug concentration resulting in at least 50% metabolic activity reduction compared to growth control cells (Kovács *et al.* 2016). MICs represent three independent experiments per isolate and are expressed as median value.

#### Evaluation of interactions by fractional concentration index (FICI) and Bliss independence model

Interactions between echinocandins and nikkomycin Z were assessed using two-dimensional broth microdilution checkerboard assay for sessile cells. Interactions were then analysed using FICI determination and Bliss independence model (Van Dijck *et al.* 2018). The tested concentration ranges were the same as described above for biofilm MIC determination. FICIs were calculated using the following formula:  $\Sigma\text{FIC} = \text{FIC}_A + \text{FIC}_B = \text{MIC}_A^{\text{comb}} / \text{MIC}_A^{\text{alone}} + \text{MIC}_B^{\text{comb}} / \text{MIC}_B^{\text{alone}}$ , where  $\text{MIC}_A^{\text{alone}}$  and  $\text{MIC}_B^{\text{alone}}$  stand for MICs of drugs A and B when used alone, and  $\text{MIC}_A^{\text{comb}}$  and  $\text{MIC}_B^{\text{comb}}$  represent the MIC values of drugs A and B in combination at isoeffective combinations respectively (Kovács *et al.* 2016; Van Dijck *et al.* 2018). FICI was determined as the lowest  $\Sigma\text{FIC}$ . MIC values of the drugs alone and of all isoeffective combinations were determined as the lowest concentration resulting in at least 50% metabolic activity reduction compared to the untreated control biofilms. If the obtained MIC value is higher than the highest tested drug concentration, the next highest twofold concentration was considered as MIC. FICI values of  $\leq 0.5$  were defined as synergistic, between  $>0.5$  and 4 as indifferent, and  $>4$  as antagonistic. FICIs were determined in three independent experiments and median values were presented (Kovács *et al.* 2016; Van Dijck *et al.* 2018).

To further analyse echinocandin-nikkomycin Z interactions, MacSynergy II was used, which employs the Bliss independence algorithm to determine synergy. The Bliss independence model is defined by the equation  $E_{ab} = E_a + E_b - (E_a E_b)$ , where  $E_{ab}$  is the additive effect of compounds  $a$  and  $b$  as predicted by their individual effects ( $E_a$  and  $E_b$ ) (Kovács *et al.* 2016; Lai *et al.* 2016). The obtained E values of each combination were presented on the  $z$ -axis in the three-dimensional plot. Synergy and antagonism were expressed in units of  $M^2\%$  which are analogous to the units for area under a dose-response curve in the two-dimensional graph. Synergy or antagonism is significant if the interaction volumes are greater than  $25 M^2\%$  (log volume  $>2$ ) or lower than  $25 M^2\%$  (log volume  $<2$ ) respectively (Kovács *et al.* 2016; Lai *et al.* 2016). When a small number of drug concentration pairs show indifferent or antagonistic interaction in a generally

synergistic combination, the applied terminology is 'synergy for most combinations'. The synergy volumes were calculated at the 95% level of confidence (Kovács *et al.* 2016; Lai *et al.* 2016).

#### Biofilm viability assay with and without nikkomycin Z

All *C. albicans* and one *C. parapsilosis* isolates were examined for LIVE/DEAD<sup>®</sup> BacLight<sup>™</sup> viability assay and pictures from one-one representative isolate were presented (CA1 and CP1 for *C. albicans* and *C. parapsilosis* respectively). Biofilms were grown on the surface of 8 well Permax slide for 24 h (Lab-Tek<sup>®</sup> Chamber Slide<sup>™</sup> System, VWR, Debrecen, Hungary). After a one-day incubation period, the preformed biofilms were washed three times using sterile physiological saline and various drug concentrations were added to the samples as follows: *C. albicans*: 8 mg l<sup>-1</sup> nikkomycin Z, 0.25 mg l<sup>-1</sup> caspofungin, 0.25 mg l<sup>-1</sup> micafungin, 0.25 mg l<sup>-1</sup> caspofungin + 8 mg l<sup>-1</sup> nikkomycin Z, 0.25 mg l<sup>-1</sup> micafungin + 8 mg l<sup>-1</sup> nikkomycin Z; *C. parapsilosis*: 8 mg l<sup>-1</sup> nikkomycin Z, 128 mg l<sup>-1</sup> caspofungin, 128 mg l<sup>-1</sup> micafungin, 128 mg l<sup>-1</sup> caspofungin + 8 mg l<sup>-1</sup> nikkomycin Z, 128 mg l<sup>-1</sup> micafungin + 8 mg l<sup>-1</sup> nikkomycin Z. These concentrations were chosen based on the checkerboard results. After 24 h of antifungal treatment, biofilms were washed with sterile physiological saline and the ratio of viable and dead cells was evaluated using fluorescent LIVE/DEAD<sup>®</sup> BacLight<sup>™</sup> viability kit (Thermo Fisher Scientific, Waltham, MA) as described our previous work (Nagy *et al.* 2018). Biofilms were stained for 15 min in darkness at 37°C using Syto 9 (3.34 mmol l<sup>-1</sup> solution in DMSO) and propidium iodide (20 mmol l<sup>-1</sup> solution in DMSO) to examine viable and dead *Candida* cells respectively (Basas *et al.* 2016). Fluorescent cells were studied using a Zeiss AxioSkop 2 mot microscope (Jena, Germany) coupled with a Zeiss AxioCam HRc camera (Jena, Germany). Analysis of images was performed using Axiovision 4.8.2 (Jena, Germany).

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#### Conflict of Interest

L. Majoros received conference travel grants from MSD, Astellas and Pfizer. All other authors declare no conflicts of interest.

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