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In-vitro activity of nikkomycin Z alone and in combination with polyenes, triazoles or echinocandins against *Aspergillus fumigatus*

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ABSTRACT

The in-vitro activity of nikkomycin Z was investigated in combination with polyenes, triazoles or echinocandins against 20 clinical isolates of *Aspergillus fumigatus* with the fractional inhibitory concentration index (FICI) method. The drug interactions were classified as synergic (FICI ≤ 0.5), no interaction (FICI > 0.5, but FICI ≤ 4) or antagonistic (FICI > 4). The fungicidal activity of nikkomycin Z alone and in combination with a representative echinocandin (caspofungin) or triazole (voriconazole) was also examined with time-kill experiments and fungal cell viability assays. Two-drug combinations of nikkomycin Z with amphotericin B (FICI 3.59 ± 0.57), amphotericin B lipid complex (FICI 3.95 ± 0.74), liposomal amphotericin B (FICI 3.62 ± 0.98), itraconazole (FICI 2.0 ± 0.0), voriconazole (FICI 1.07 ± 0.37), posaconazole (FICI 2.20 ± 0.44) or ravuconazole (FICI 1.76 ± 0.44) showed no interactions, but the pairwise combination of nikkomycin Z with caspofungin (FICI 0.22 ± 0.19) or micafungin (FICI 0.35 ± 0.27) showed synergic activity against *A. fumigatus*. Time-kill studies and fungal cell viability assays showed that neither nikkomycin Z nor caspofungin alone possessed fungicidal activity against *A. fumigatus*, whereas a combination of these two drugs at concentrations ≥2 mg/L (≥ 0.031 × the concentration of drug that produced no visible growth) killed germinated conidia within 24 h in a concentration-dependent manner. These data suggest that two-drug combinations of nikkomycin Z with echinocandins, but not with polyenes and triazoles, have a synergic effect against *A. fumigatus*.

Keywords Antifungal combination, echinocandins, fungicidal activity, nikkomycin Z, polyenes, triazoles

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INTRODUCTION

The incidence of invasive aspergillosis has increased in the past decade as a result of more widespread use of aggressive chemotherapy regimens against cancer, an increasing population of patients suffering from AIDS, and the expansion of organ transplantation programmes. Although the incidence of fungal infection has increased significantly over the past decade, only modest progress has been made in the treatment and management of *Aspergillus* infection. Amphotericin B, itraconazole and voriconazole are the therapeutic options for invasive aspergillosis, but give less than optimal results. Since amphotericin B interferes with renal function, less nephrotoxic, but considerably more expensive, lipid formulations of amphotericin B have been introduced, with modest success [1–4].

The nikkomycins, echinocandins, triazoles and polyenes are different classes of antifungal drugs with distinct modes of action. Chitin is a polysaccharide found in most fungal cell walls, including *Aspergillus* spp. The nikkomycins inhibit chitin synthesis by acting as competitive analogues of the chitin synthase substrate UDP–*N*-acetylglucosamine [5–7]. Similarly, the echinocandins are cyclic lipopeptide compounds that inhibit glucan synthesis by inhibiting the enzyme 1,3-β-D-glucan synthase [8–12]. Lack of chitin and glucan in the cell wall often leads to osmotic lysis of the fungal cell. The triazoles and

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the polyenes interfere with the fungal plasma membrane function by either inhibiting the synthesis of ergosterol (triazoles) or binding to it (polyenes), thereby making the membrane a non-selective cellular barrier.

Since the current single-drug therapeutic approach against invasive aspergillosis gives sub-optimal results, the use of antifungal drugs in combination may be an alternative strategy. Combination treatment may have several advantages, namely enhanced efficacy, reduced toxicity by virtue of using lower doses of agents with adverse side effects, decreased incidence of in-vivo selection of drug-resistant variants, and a broader spectrum of activity. Although nikkomycin Z has an inhibitory effect on fungal growth, this compound by itself is not a potent antifungal drug against *Aspergillus* spp. [13,14]. However, because of its low toxicity and high antifungal specificity, it is suitable for use in combination with other antifungal drugs. The present study investigated the in-vitro activity of two-drug combinations of nikkomycin Z with echinocandins, triazoles, and conventional and lipid formulations of amphotericin B, against *Aspergillus fumigatus*.

MATERIALS AND METHODS

Antifungal drugs

Voriconazole, itraconazole, posaconazole and ravuconazole were obtained from Pfizer Pharmaceuticals (New York, NY, USA), Janssen Pharmaceutica (Beerse, Belgium), Schering-Plough Research Institute (Kenilsworth, NJ, USA) and Bristol-Myers Squibb Institute for Medical Research (Princeton, NJ, USA), respectively. Amphotericin B was purchased from Sigma Chemical Company (St Louis, MO, USA). Amphotericin B lipid complex was obtained from Elan Pharmaceuticals (San Diego, CA, USA). Caspofungin was obtained from Merck (Rahway, NJ, USA), and micafungin and liposomal amphotericin B were obtained from Fujisawa Pharmaceuticals (Osaka, Japan). The triazoles and amphotericin B were dissolved in dimethylsulphoxide to make a stock solution of 1 g/L, and then stored as 0.25-mL aliquots at −20°C. Caspofungin, micafungin and nikkomycin Z were dissolved in sterile double-distilled water to a concentration of 10 g/L, and were stored as 0.25-mL aliquots at −70°C. Frozen stocks of the antifungal agents were thawed at room temperature and used within 24 h.

Clinical isolates

The 20 clinical isolates of *A. fumigatus* used in this study were selected randomly from our collection of cultures (n = 619), obtained from the Microbiology Laboratory of the Detroit Medical Center (Detroit, MI, USA) from 1994 to 2002. The original cultures, isolated from sputum, bronchial wash and lungs of immunocompromised patients, were subcultured on Sabouraud dextrose agar to check for purity and viability. Working cultures were maintained on Sabouraud dextrose agar slants at 4°C. For long-term preservation of the cultures, conidial suspensions were prepared in glycerol 25% v/v and stored at −80°C.

MIC determination

Conidial suspensions from 6-day-old *A. fumigatus* cultures were prepared, standardised by haemocytometry, and used as inocula for susceptibility testing. MICs of various antifungal agents for *A. fumigatus* isolates were determined in RPMI-1640 by the M38-A broth microdilution technique recommended by the National Committee for Clinical Laboratory Standards [15], except that the MIC was defined as the concentration of drug that produced no visible growth (MIC-0). Drug concentrations ranging from either 0.015 to 16 mg/L (triazoles, amphotericin B, amphotericin B lipid complex and liposomal amphotericin B) or 0.062 to 64 mg/L (nikkomycin Z and caspofungin) or 0.25 to 256 mg/L (micafungin) were used for MIC determinations. Where applicable, comparable concentrations of dimethylsulphoxide were used as controls. Each MIC determination was repeated at least once, with results that were either the same or ±1 two-fold dilution.

MEC determination

The MECs (minimum effective concentrations) of caspofungin and micafungin for *A. fumigatus* isolates were determined in RPMI-1640 with the M38-A broth microdilution technique [15]. The MEC was defined as the lowest concentration of the drug that produced a marked morphological change in the hyphae, from profuse mycelial growth producing straight, sparsely branching hyphal filaments to hyper-branching, crooked hyphae showing stunted growth, resulting in the production of microcolonies. The echinocandin-induced transition from the normal profuse hyphal growth to microcolonies was easily recognised with the aid of an MIC plate-viewing mirror. The concentrations of caspofungin and micafungin used for MEC determination ranged from 0.0019 to 2 mg/L. The MEC determination was repeated once, with results that were almost identical.

Fractional inhibitory concentration index determination

The in-vitro susceptibility of *A. fumigatus* to two-drug combinations of nikkomycin Z with various polyenes, triazoles or echinocandins was evaluated with the fractional inhibitory concentration index (FICI) method. The FICI was determined with a two-dimensional chequerboard in a microtitre plate with the M38-A broth microdilution technique [15]. Pairwise combinations of the required concentrations of antifungal drug A and antifungal drug B were prepared in two-fold increments in 0.1-mL aliquots of RPMI-1640 containing 0.165 M MOPS buffer (pH 7.0). Appropriate drug-free growth controls were included. To each well, 0.1 mL of fresh conidial suspension (2×10^4 conidia/mL) was added. The contents of each well were mixed by repeated pipetting back and forth with a multichannel pipette. The microtitre plate was incubated at 35°C for 48 h, after which fungal growth was assessed by reduction of the tetrazolium

compound 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT assay). Briefly, after 48 h of growth, medium was removed from each well with a multichannel pipette, and 0.1 mL of fresh RPMI-1640 was added, followed by 0.025 mL of MTT solution (1 g/L) containing 0.2 mM (final concentration) menadione. Following incubation for 3 h at 35°C, fungal cells were stained blue following accumulation of the MTT–formazan insoluble salts in the mycelia. A well representing the lowest concentration(s) of the drug(s) with no visible colour was taken as the endpoint. The MIC was defined as the lowest concentration of the drug that showed no visible growth (MIC-0). The FICI was calculated with the formula:

$$\text{FICI} = (A_c/A_a) + (B_c/B_a)$$

where A_c and B_c are the MICs of drugs A and B in combination, and A_a and B_a are the MICs of drugs A and B.

The drug interactions were classified as synergic (FICI ≤ 0.5), no interaction (FICI > 0.5, but ≤4) or antagonistic (FICI > 4). In addition, the FICI was determined for caspofungin and micafungin in two-drug combinations with nikkomycin Z, using the MEC endpoints as described above.

Kill-curve study

The fungicidal activity of nikkomycin Z alone and in combination with caspofungin or voriconazole against *A. fumigatus* strains W73355 and H50246 was determined in kill-curve experiments. Aliquots (5 mL) of germinated (6 h at 35°C) conidia prepared in RPMI-1640 (1×10^6 conidia/mL) were incubated at 35°C in the presence of nikkomycin Z, caspofungin, or nikkomycin Z plus caspofungin, at concentrations ranging from 0.25 mg/L to 32 mg/L (0.004 ×MIC-0 to 0.5 × MIC-0). Similarly, to study the fungicidal activity of nikkomycin Z in combination with a triazole, conidia were incubated with either nikkomycin Z (1 mg/L and 16 mg/L; 0.0156 × MIC-0 and 0.25 × MIC-0, respectively), voriconazole (0.25 mg/L and 0.5 mg/L; 0.5 × MIC-0 and 1 × MIC-0, respectively), or nikkomycin Z plus voriconazole combinations, at the same concentrations. At 0, 3, 6, 9, 12 and 24 h, 0.1-mL aliquots of the conidial suspension were removed and diluted to obtain 10–10^4-fold dilutions, and 0.1-mL aliquots were spread in duplicate on Sabouraud dextrose agar plates. The plates were incubated at 35°C for 48 h, and the number of CFU/mL was determined. Kill-curves were constructed by plotting mean $\log_{10}$ CFU/mL against the time of exposure of conidia to antifungal drugs.

Fungal cell viability test

A. fumigatus conidia (1×10^6/mL) were germinated in RPMI-1640 for 6 h at 35°C in the wells of 96-well microtitre plates. At the end of the germination period, nikkomycin Z or caspofungin, or a combination of these drugs, was added to replicate wells to final concentrations ranging from 0.25 mg/L (0.004 × MIC-0) to 16 mg/L (0.25 × MIC-0). The germinated conidia were incubated with the drug(s) at 35°C for 24 h, the medium was removed, and the viability of the drug-treated germinated conidia was examined with the MTT assay as described above. The development of blue colour following the accumulation of formazan salt in the mycelia as a result of MTT reduction suggests the presence of viable cells.

RESULTS AND DISCUSSIONS

In-vitro susceptibility

The in-vitro susceptibilities of 20 clinical isolates of *A. fumigatus* to ten antifungal drugs belonging to four classes are shown in Table 1. All of the isolates were highly susceptible to triazoles, with a narrow range (0.062–0.5 mg/L) of MIC-0 values. The conventional and lipid formulations of amphotericin B were less active (MIC-0 0.5–4 mg/L) against *A. fumigatus* than were the triazoles. Amphotericin B and amphotericin B lipid complex had similar MIC-0 values, whereas that of liposomal amphotericin B was 2–4-fold higher. The MIC-0 values of nikkomycin Z (16–64 mg/L) and the echinocandins (32 to >256 mg/L) for *A. fumigatus* were high compared to those of polyenes and triazoles. Since the MIC-0 values of echinocandins are many-fold above the levels of the drug achievable in blood and tissues, previous investigators have used an alternative clinically relevant endpoint to evaluate their effectiveness. However, in the present study, the MECs of caspofungin and micafungin were at least 250-fold (caspofungin) to 16 500-fold (micafungin) lower than the MIC-0 values.

Effect of two-drug combinations

Table 2 shows that two-drug combinations of nikkomycin Z with either conventional or lipid formulations of amphotericin B or triazoles had no specific synergic effects on *A. fumigatus*. However, the FICIs obtained for nikkomycin Z in combination with the polyenes (3.59 ± 0.57 and 3.95 ± 0.74) were *c.* two-fold higher than those obtained for nikkomycin Z in combination with

Table 1. In-vitro susceptibility of clinical isolates of *Aspergillus fumigatus* (n = 20) to antifungal drugs

Antifungal drug	MIC (mg/L) range	MIC_{50} (mg/L)	MIC_{90} (mg/L)
Nikkomycin	16–64	64	64
Amphotericin B	0.5–2	1	2
Amphotericin B lipid complex	0.5–2	1	2
Liposomal amphotericin B	2–4	2	4
Itraconazole	0.25–0.5	0.25	0.5
Voriconazole	0.25–0.5	0.25	0.25
Posaconazole	0.062–0.25	0.125	0.125
Ravuconazole	0.25–0.5	0.25	0.5
Caspofungin[a]	32–64 (0.125–0.25)	64 (0.25)	64 (0.25)
Micafungin[a]	64 to >256 (0.0019–0.0156)	> 256 (0.0019)	> 256 (0.0156)

[a]Values shown in parentheses for caspofungin and micafungin represent MECs.

Table 2. Interaction of nikkomycin Z in two-drug combinations with polyenes, triazoles and echinocandins against *Aspergillus fumigatus*

Drug combination	FICI ± SD	Drug interaction
NKZ + AMB	3.59 ± 0.57	No interaction
NKZ + ABLC	3.95 ± 0.74	No interaction
NKZ + L-AMB	3.62 ± 0.98	No interaction
NKZ + ITZ	2.00 ± 0.0	No interaction
NKZ + VCZ	1.07 ± 0.37	No interaction
NKZ + PCZ	2.20 ± 0.44	No interaction
NKZ + RCZ	1.76 ± 0.44	No interaction
NKZ + CFG	0.22 ± 0.19	Synergy
NKZ + MFG	0.35 ± 0.27	Synergy

NKZ, nikkomycin Z; AMB, amphotericin B; ABLC, amphotericin B lipid complex; L- AMB, liposomal amphotericin B; ITZ, itraconazole; VCZ, voriconazole; PCZ, posaconazole; RCZ, ravuconazole; CFG, caspofungin; MFG, micafungin.

triazoles (1.07 ± 0.37 and 2.20 ± 0.44). In contrast, the FICIs obtained for two-drug combinations of nikkomycin Z with caspofungin (0.22 ± 0.19) or micafungin (0.35 ± 0.27) were substantially lower than 0.5, indicating synergic action against *A. fumigatus*. Moreover, the FICIs obtained for nikkomycin Z in combination with either caspofungin or micafungin by the MEC-endpoint method were similar to those obtained when MIC-0 was used as an endpoint, suggesting that both MEC and MIC-0 endpoints could be used to study the interaction of two-drug combinations of nikkomycin Z with echinocandin compounds.

Fungicidal activity

Since the echinocandins in two-drug combinations with nikkomycin Z produced a synergic action against *A. fumigatus*, the fungicidal activity of pairwise combinations of nikkomycin Z and caspofungin were examined at concentrations ranging from 0.25 to 32 mg/L (0.004 × MIC-0 to 0.5 × MIC-0) against *A. fumigatus*. Neither nikkomycin Z nor caspofungin alone showed fungicidal activity against *A. fumigatus* at concentrations as high as 64 mg/L (1 × MIC-0) compared to the drug-free control. However, a combination of the two drugs at ≥ 2 mg/L (0.031 × MIC-0) killed *A. fumigatus* conidia in a concentration-dependent manner within 24 h, whereas no substantial killing of conidia was obtained with combinations of these drugs below 2 mg/L. For example, nikkomycin Z and caspofungin at 32 mg/L each (0.5 × MIC-0) killed >99% of conidia within 24 h of exposure to the drugs (Fig. 1A), whereas a combination of caspofungin at 0.25 mg/L and nikkomycin Z at 1 mg/L showed no fungicidal activity (Fig. 1B).

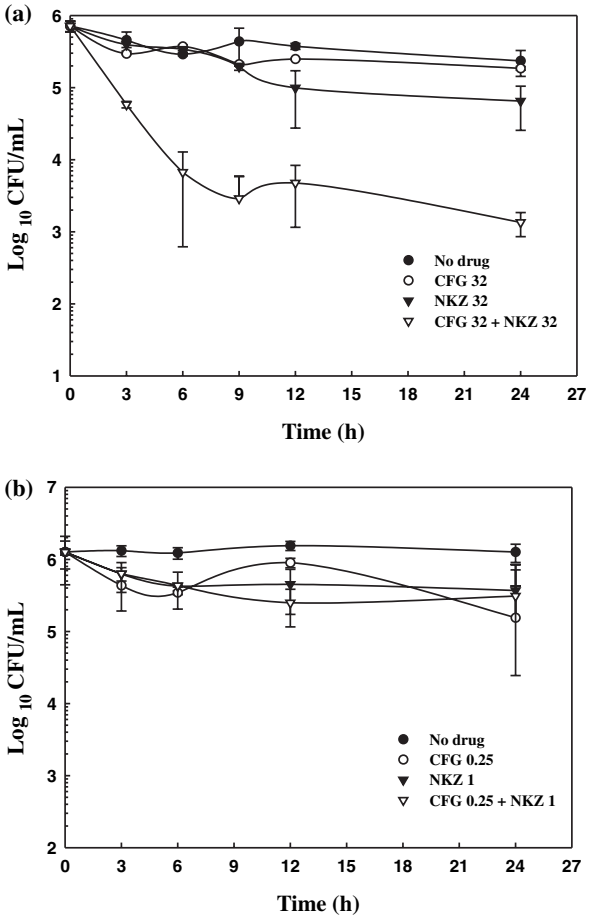

Fig. 1. In-vitro fungicidal activities of (A) high and (B) low concentrations of nikkomycin Z in combination with high and low concentrations of caspofungin against *Aspergillus fumigatus*. Each point represents the mean of two independent experiments in which *A. fumigatus* strains W73355 and H50246 were used. The vertical bars denote twice the standard deviation. CFG 0.25, caspofungin 0.25 mg/L; CFG 32, caspofungin 32 mg/L; NKZ 1, nikkomycin Z 1 mg/L; NKZ 32, nikkomycin Z 32 mg/L.

In addition to the time-kill experiments, the fungicidal activities of nikkomycin Z and caspofungin were investigated, both alone and in combinations, on germinated conidia of *A. fumigatus* with the fungal cell viability test. Neither nikkomycin Z nor caspofungin, alone or in two-drug combinations at concentrations ≤2 mg/L (0.031 × MIC-0), showed fungicidal activity against *A. fumigatus* conidia germinated for 6 h. Combinations of drugs, but not individual drugs, at concentrations ≥8 mg/L (0.125 × MIC-0) killed germinated conidia completely, as these cells were unable to reduce MTT. The number of CFUs obtained from each well was found to correlate with the presence of blue colour.

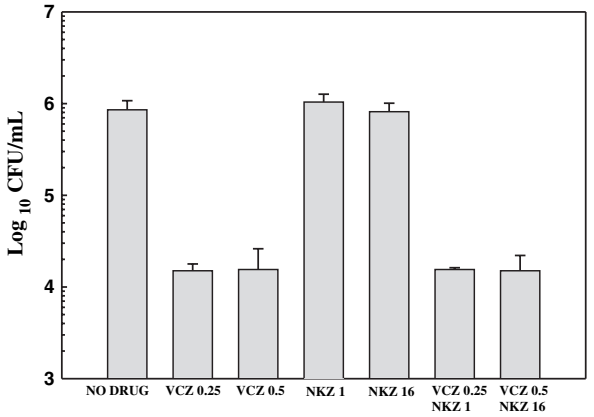

Fig. 2. In-vitro fungicidal activity of nikkomycin Z alone and in combination with voriconazole against *Aspergillus fumigatus*. The vertical bar on each histogram represents twice the standard deviation for two independent experiments. VCZ 0.25, voriconazole 0.25 mg/L; VCZ 0.5, voriconazole 0.5 mg/L; NKZ 1, nikkomycin Z 1 mg/L; NKZ 16, nikkomycin Z 16 mg/L.

The fungicidal activity of nikkomycin Z was also investigated in combination with voriconazole, a representative of the triazoles. Conidia exposed to nikkomycin Z at 1 mg/L and 16 mg/L for 24 h at 35°C showed no reduction in CFU/mL compared to the drug-free control. Voriconazole at 0.25 mg/L and 0.5 mg/L ($0.5 \times$ MIC-0 and $1 \times$ MIC-0) reduced the viable count by >95% within 24 h. No enhanced killing was obtained when nikkomycin Z at 1 mg/L and 16 mg/L was added to voriconazole at 0.25 mg/L and 0.5 mg/L, respectively (Fig. 2).

Combinations of antifungal drugs belonging to different classes with distinct modes of action have the highest potential for synergic interaction. The in-vitro synergic action of nikkomycin Z in two-drug combinations with itraconazole [16], micafungin [17] and the echinocandin anidulafungin (formerly known as LY303366) [14,18] against *A. fumigatus* has been studied previously with the FICI method. With the use of a nikkomycin Z MIC endpoint defined on the basis of partial growth inhibition, it was shown in these studies that anidulafungin plus nikkomycin Z, or micafungin plus nikkomycin Z, showed synergic action against *A. fumigatus*; these results are in agreement with the present data obtained for two-drug combinations of nikkomycin Z with caspofungin or micafungin. On the other hand, Li and Rinaldi [16] reported that a combination of itraconazole and nikkomycin Z showed synergic action against *A. fumigatus*. In the present study, the FICI value (2.00 ± 0.0) obtained for the nikkomycin Z plus itraconazole combination indicated no drug interaction.

The fungicidal activity of nikkomycin Z plus caspofungin against *A. fumigatus* is intriguing, since neither nikkomycin Z nor caspofungin alone show fungicidal activity against this filamentous fungus. Inhibition of glucan or chitin synthesis in itself, by caspofungin or nikkomycin Z, appears to be inadequate to inhibit the formation of cell walls completely in *A. fumigatus*, and the resulting microcolonies are metabolically functional, although colonies grown in the presence of caspofungin have profoundly altered hyphal morphology [19]. However, a combination of nikkomycin Z and caspofungin will inhibit the synthesis of glucan and chitin simultaneously, resulting in complete inhibition of cell wall synthesis. In the absence of an intact functional cell wall, the fungal cell is highly susceptible to osmotic pressure and lysis, resulting in death. Overall, the present results show that two cell wall-specific fungistatic drugs with weak activity against *A. fumigatus* exert potent fungicidal activity when combined.

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