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In Vitro Antifungal Activity of Nikkomycin Z in Combination with Fluconazole or Itraconazole

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Nikkomycins are nucleoside-peptide antibiotics produced by *Streptomyces* species with antifungal activities through the inhibition of chitin synthesis. We investigated the antifungal activities of nikkomycin Z alone and in combination with fluconazole and itraconazole. Checkerboard synergy studies were carried out by a macrobroth dilution procedure with RPMI 1640 medium at pH 6.0. At least 10 strains of the following fungi were tested: *Candida albicans*, other *Candida* spp., *Cryptococcus neoformans*, *Coccidioides immitis*, *Aspergillus* spp., and dematiacious fungi (including *Exophiala jeanselmei*, *Exophiala spinifera*, *Bipolaris spicifera*, *Wangiella dermatitidis*, *Ochroconis humicola*, *Phaeoannellomyces werneckii*, and *Cladophialophora bantiana*), and 2 strains each of *Fusarium*, *Scedosporium*, *Paecilomyces*, *Penicillium*, and *Trichoderma* spp. A total of 110 isolates were examined. Inocula of fungal elements were standardized by hemacytometer counting or spectrophotometrically. MICs and minimum lethal concentrations (MLCs) were determined visually by comparison of growth in drug-treated tubes with growth in drug-free control tubes. Additive and synergistic interactions between nikkomycin and either fluconazole or itraconazole were observed against *C. albicans*, *Candida parapsilosis*, *Cryptococcus neoformans*, and *Coccidioides immitis*. Marked synergism was also observed between nikkomycin and itraconazole against *Aspergillus fumigatus* and *Aspergillus flavus*. No antagonistic interaction between the drugs was observed with any of the strains tested.

The types and frequencies of life-threatening fungal infections have increased dramatically in the past several years as a direct result of the increase in susceptible patients, which in turn is attributable to human immunodeficiency virus infection and iatrogenic immunosuppression. Treatment of deep-seated mycoses is complicated by the limited choice and spectra of existing antifungal agents as well as the toxicity often associated with antifungal chemotherapy.

One of the targets in the development of antifungal agents is cell wall chitin, a polysaccharide that is found in most medically important fungi. Since chitin does not exist in mammalian cells, compounds that inhibit chitin biosynthesis are considered potential antifungal drugs of low toxicity for humans. Nikkomycins are nucleoside-peptide antibiotics that inhibit chitin synthesis in fungi and insects (8, 9, 21). Like polyoxins, they act as competitive analogs of the substrate UDP-*N*-acetylglucosamine for chitin synthase (5, 19). Lack of chitin in the cell wall eventually leads to osmotic lysis (2).

The antifungal activity of nikkomycin Z, one of the naturally derived nikkomycins, has been described by many investigators (3, 5, 7, 11, 12, 19). In an animal model, nikkomycin Z was reported to prolong the survival of mice infected with *Coccidioides immitis* and *Blastomyces dermatitidis* (17). However, the antifungal efficacy of nikkomycin Z in experimental candidiasis was low (8), in spite of inhibition of chitin synthesis in vivo (6).

To increase the therapeutic index of antifungal drugs, one approach is to use these agents in combinations for synergistic interactions. Synergistic interactions of nikkomycin Z with azoles and with other compounds against *Candida albicans* have been reported by many investigators (15, 16, 20, 28). Combination therapy is of particular importance in infections involving immunocompromised patients where the infecting organisms are often resistant to antifungal therapy.

To explore possible applications of nikkomycin Z for treatment of mycoses, we conducted in vitro susceptibility testing of nikkomycin Z alone and in combination with fluconazole and itraconazole against a wide array of medically important fungi. Additive and synergistic interactions between nikkomycin Z and either fluconazole or itraconazole were observed for *C. albicans*, *Candida parapsilosis*, *Cryptococcus neoformans*, and *Coccidioides immitis*. Synergism was also observed between nikkomycin Z and itraconazole against *Aspergillus fumigatus* and *Aspergillus flavus*.

MATERIALS AND METHODS

Organisms and cultural conditions. A total of 110 isolates, listed in Table 1, were tested. All fungi were clinical isolates obtained from the Fungus Testing Laboratory in the University of Texas Health Science Center at San Antonio and from the Fungal Reference Laboratory in the Audie L. Murphy Memorial Veterans Hospital. Yeast isolates were maintained and grown on Sabouraud dextrose agar at 37°C. Filamentous fungi were grown on potato dextrose agar slants at room temperature.

Drugs. Nikkomycin Z was obtained as a powder from Shaman Pharmaceuticals, South San Francisco, Calif. Fluconazole was received as a powder from Pfizer Inc., New York, N.Y.; itraconazole was received from Janssen, Piscataway, N.J. Stocks of nikkomycin Z and fluconazole and subsequent dilutions were prepared in sterile distilled water. A stock of itraconazole was prepared in polyethylene glycol (PEG) 400; subsequent dilutions were made in 50% PEG 400. Drug preparation and subsequent inoculation of yeast isolates were performed according to the macrobroth dilution protocol recommended by the National Committee for Clinical Laboratory Standards, M27-T (22). Aliquots of 50 μl of each drug at a concentration of 20 times the final concentration were dispersed in polystyrene tubes with caps (12 by 75 mm; Becton Dickinson, Lincoln Park, N.J.). In the checkerboard scheme, each tube contained different proportions of each drug tested. With single drug controls, 50 μl of the drug was mixed with 50 μl of sterile water. Drug preparations were stored at −70°C until used.

Antifungal susceptibility testing. Susceptibility tests were carried out in RPMI 1640 medium with L-glutamine, without sodium bicarbonate, and buffered with 0.165 M morpholinepropanesulfonic acid (MOPS). The pH of the medium was adjusted to 6 with 1 M HCl, as nikkomycin Z is more stable under acidic conditions. Yeast inocula (0.9 ml) were standardized spectrophotometrically and

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TABLE 1. Nikkomycin Z MICs and FICs for clinical fungal isolates

Species (no. of isolates)	Nikkomycin Z MIC (μg/ml)		FIC of nikkomycin Z with:	
	Range	MIC_{50}[a]	Fluconazole	Itraconazole
C. albicans (15)	<0.5–32	4	0.48	0.2
C. tropicalis (4)	>64	>64	>1	>1
C. krusei (4)	>64	>64	>1	>1
C. glabrata (3)	>64	>64	>1	>1
C. parapsilosis (4)	1–4	2	0.32	0.46
Cryptococcus neoformans (15)	<0.5–>64	>64	0.72	0.49
Scedosporium spp. (2)	>64	>64	>1	>1
Paecilomyces spp. (2)	>64	>64	>1	0.83
Trichoderma spp. (2)	64	64	>1	0.71
Penicillium spp. (2)	>64	>64	>1	0.83
Fusarium spp. (2)	>64	>64	>1	>1
A. fumigatus (13)	>64	>64	>1	0.35
A. flavus (13)	>64	>64	>1	0.33
A. niger (7)	>64	>64	>1	>1
A. versicolor (1)	>64	>64	>1	>1
A. nidulans (1)	>64	>64	>1	0.52
Coccidioides immitis (10)	1–16	4	0.44	0.47
E. jeanselmia (3)	>64	>64	>1	>1
E. spinifera (1)	>64	>64	>1	NA[c]
Bipolaris spp. (2)	>64	>64	0.64	NA
W. dermatitidis (1)	>64	>64	>1	0.56
O. humicola (1)	4	4	>1	>1
P. werneckii (1)	>64	>64	>1	>1
Cladophialophora bantiana (1)	>64	>64	>1	>1

[a] MIC_{50}, MIC at which 50% of the isolates were inhibited.
[b] FICs were based on the MIC_{80}s for all isolates except those of *C. albicans*, *C. parapsilosis*, and *Cryptococcus neoformans*, for which MIC_{100}s were used (Table 2).
[c] NA, not applicable. Itraconazole MICs for these organisms were at the lowest concentration tested; therefore, the drug interaction could not be determined.

further diluted as described previously (22). For most filamentous fungi, hemacytometer conidia counts were used for inoculum standardization. Inocula of *Coccidioides immitis* were adjusted with a spectrophotometer to 95% optical transmission at 530 nm and further diluted 1/1,000 in medium. The final inoculum was approximately 0.5×10^3 to 2.5×10^3 cells/ml for yeast isolates and 1×10^4 conidia/ml for filamentous fungi. Final concentrations of fluconazole after inoculation ranged from 0.125 to 16 μg/ml, those of itraconazole ranged from 0.03 to 4 μg/ml (contained 2.5% PEG 400), and those for nikkomycin Z ranged from 0.5 to 64 μg/ml in twofold increments. Controls included drug-free inocula, blank media, and media containing 2.5% PEG 400 for itraconazole activity.

Yeasts were incubated at 35°C, and molds were incubated at 25°C. The turbidity (growth) of each tube was compared to that of the drug-free growth control tubes (or that of the PEG 400 control tube in the case of itraconazole) at 24 and 48 h for rapidly growing isolates or when obvious growth in the drug-free control tube was observed and at 24-h intervals thereafter. The reading at 48 h was used for determining the MIC. The MIC was defined as the lowest concentration of drug which produced a pronounced inhibition (80%) of growth compared to the level in the growth control tubes (MIC_{80}). The MIC_{100} was the lowest concentration of drug which resulted in total inhibition of growth. One hundred microliters of each of the growth-inhibited suspensions was plated onto one quadrant of Sabouraud dextrose agar plate and incubated at 37°C. The lowest concentration of drug which produced less than five colonies was defined as the MLC (minimum lethal concentration, >95% killing).

Definitions. Drug interactions were classified as synergistic, additive, indifferent, or antagonistic. The interaction was defined as synergistic if the MIC of each drug was at least fourfold lower when the drug was used in combination than when it was used alone. Additive interaction was defined as at least a twofold decrease in the MIC of each drug when two drugs were used in combination. The interaction was defined as indifferent when the MIC(s) of one drug (or both drugs) used in combination remained the same as when the drug was used alone. Antagonism was defined as a higher MIC for either drug when it was used in combination than when it was used alone.

Drug interaction was also categorized on the basis of the fractional inhibitory concentration (FIC) of a drug. The FIC index was calculated by the following formula based on the MICs of two drugs used alone and in combination: FIC = (MIC of *A* in combination ÷ MIC of *A* alone) + (MIC of *B* in combination ÷ MIC of *B* alone). Drug interactions were classified as follows. A drug was considered to be synergistic if its FIC was ≤0.5, additive if its FIC was >0.5 but ≤1, indifferent if its FIC was >1 but ≤2, and antagonistic if its FIC was >2.

RESULTS

When tested alone, nikkomycin Z MIC_{80} values ranged from ≤0.5 to 32 μg/ml for *C. albicans* (Table 1) and 1 to 4 μg/ml for *C. parapsilosis*, including azole-resistant isolates of both species. Other *Candida* species, including strains of *C. tropicalis*, *C. krusei*, and *C. glabrata*, were resistant to nikkomycin Z at the highest concentration tested (64 μg/ml) (Table 1). Two of 15 isolates of *Cryptococcus neoformans* were susceptible to nikkomycin Z (MIC_{80}s, 0.5 and 32 μg/ml); for the other 13 isolates MIC_{80}s were higher than 64 μg/ml. When nikkomycin Z was used alone against mycelial-phase *Coccidioides immitis*, its MIC_{80} was between 1 and 16 μg/ml (Table 1).

In the combinations of nikkomycin Z with azoles, synergistic or additive interaction was observed with most strains of *C. albicans* (11 of 15 for the nikkomycin Z and fluconazole combination and 15 of 15 for the nikkomycin Z and itraconazole combination based on MIC_{100} results) (Fig. 1A) and all isolates of *C. parapsilosis* (Fig. 1B). Synergistic or additive interactions of nikkomycin Z and azoles were also observed with some isolates of *Cryptococcus neoformans* (Table 2). The azole MIC_{80} for some isolates of *C. albicans*, *C. parapsilosis*, and *Cryptococcus neoformans* was at the lowest concentration tested. Therefore, the interaction (additive or synergistic) could not be determined based on MIC_{80} results. Consequently, drug interactions with these species were determined based on the MIC_{100} results.

A striking synergistic interaction was observed between nikkomycin Z and either fluconazole or itraconazole against essentially all isolates of *Coccidioidis immitis* tested (Table 2 and Fig. 1C). Against *Aspergillus* species, nikkomycin Z by itself was ineffective in growth inhibition (MIC > 64). However, nikkomycin Z in combination with itraconazole showed marked synergy when the drugs were tested against virtually all isolates of *A. fumigatus* and *A. flavus* (Tables 1 and 2). This synergistic interaction of nikkomycin Z and itraconazole was observed in growth inhibition (lowered MIC_{80}s and MIC_{100}s) as well as in fungicidal effect (lowered MLCs) (Fig. 2). Similar effects were not observed against *Aspergillus niger*, *Aspergillus versicolor*, and *Aspergillus nidulans*, nor were they observed for the nikkomycin Z and fluconazole combination.

Isolates of *Scedosporium*, *Paecilomyces*, *Trichoderma*, *Penicillium*, and *Fusarium* species were resistant to nikkomycin Z (Table 1). The combination of nikkomycin Z and itraconazole resulted in moderate growth inhibition (Table 2). Among the dematiacious fungi tested (*Exophiala*, *Bipolaris*, *Wangiella*, *Ochroconi*, *Phaeoannellomyces*, and *Cladophialophora* spp.), MICs of nikkomycin Z alone were higher than 64 μg/ml except for the one isolate of *Ochroconi humicola* (Table 1). Moderate additive or synergistic interactions based on MIC_{80}s were recorded when nikkomycin Z and fluconazole were used in combination against one isolate each of *Exophiala jeanselmia* and *Bipolaris* sp. An additive interaction was also observed between nikkomycin Z and itraconazole against one isolate of *Wangiella dermatitidis* (Table 2).

DISCUSSION

Chitin and glucans are major components of the fungal cell wall and play important roles in maintaining the structural integrity of fungal cells (13, 26). Compounds which interfere with synthesis of the fungal cell wall may result in growth inhibition, analogous to the effect of beta-lactams on bacteria. Nikkomycins have long been known to inhibit chitin synthesis in fungi and insects (3, 9, 11). Among the medically important fungi, *Coccidioidis immitis* and *B. dermatitidis* are susceptible to

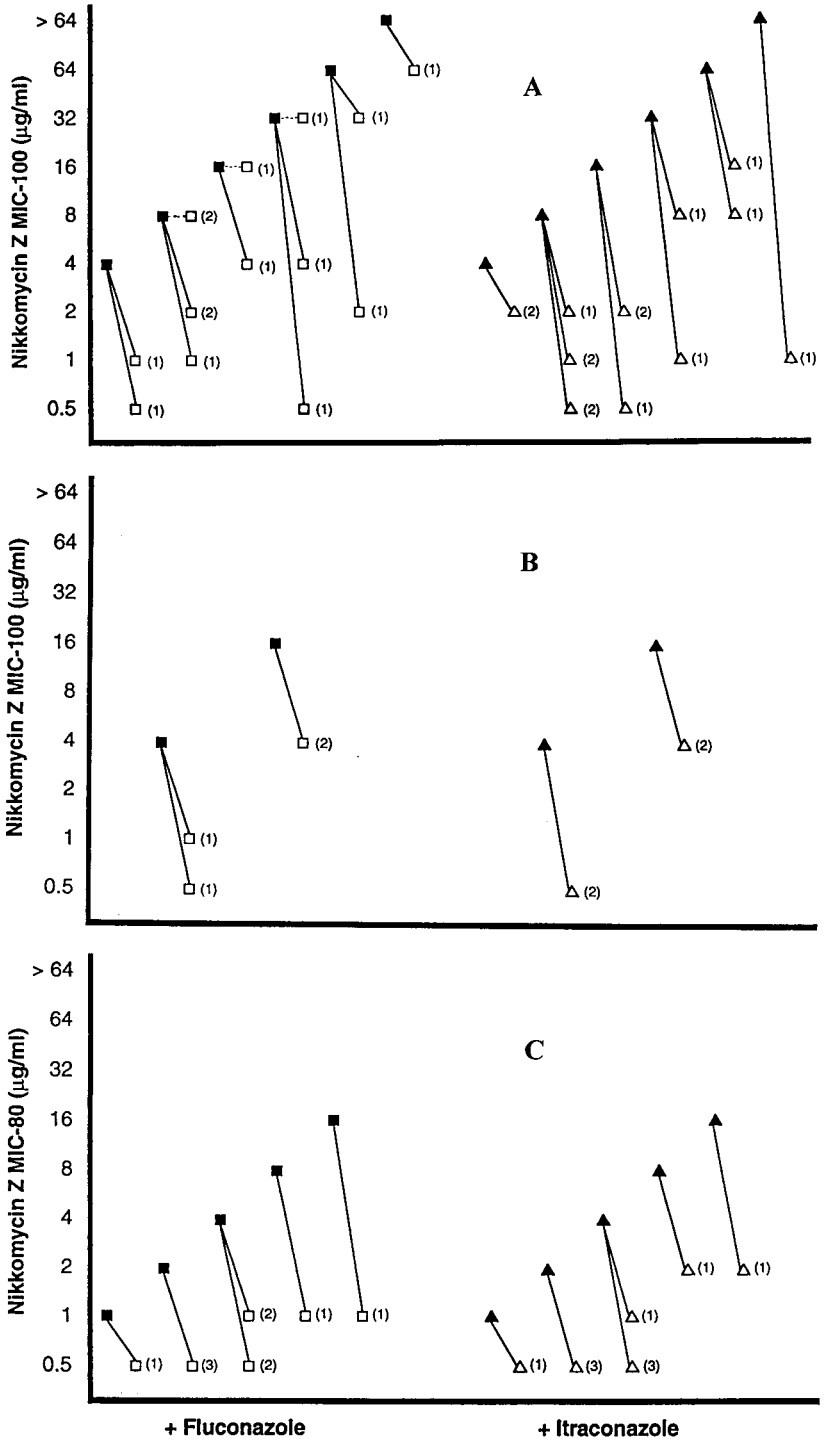

FIG. 1. MICs of nikkomycin Z for 15 *C. albicans* (A), 4 *C. parapsilosis* (B), and 10 *Coccidioides immitis* (C) isolates when nikkomycin Z was used alone (■ and ▲) and in combination with fluconazole (□) and itraconazole (△). Values in parentheses are numbers of isolates. Note that MIC_{80}s and MIC_{100}s were used for *Coccidioides immitis* and *Candida* species, respectively.

nikkomycin Z both in vitro and in vivo (17, 23). However, other fungi such as *Cryptococcus neoformans*, *Aspergillus* spp., and *Fusarium* spp. have been reported to be resistant to nikkomycins (18). Although isolates of *C. albicans* are moderately susceptible (6, 15), other Candida species are resistant to nikkomycin Z in vitro (14). The mechanisms for nikkomycin resistance are not clear.

The clinically useful aspect of nikkomycin may come from its additive or synergistic interactions with other antifungal drugs (15, 16, 20, 28). In this study, nikkomycin Z worked synergistically with fluconazole and itraconazole against *C. albicans*, *C. parapsilosis*, *Cryptococcus neoformans*, and *Coccidioides immitis*. Synergism was also observed between nikkomycin Z and itraconazole against *A. fumigatus* and *A. flavus*. Although the

TABLE 2. Summary of interactions between nikkomycin Z and fluconazole and itraconazole against clinical fungal isolates

Species (no. of isolates)	FIC[a]							
	Nikkomycin Z + fluconazole				Nikkomycin Z + itraconazole			
	Synergistic	Additive	Indifferent	NA[b]	Synergistic	Additive	Indifferent	NA
C. albicans (15)	7 (9)	2 (2)	4 (4)	2 (0)	3 (13)	5 (2)		7 (0)
C. tropicalis (4)		1	3			1	3	
C. krusei (4)			4				4	
C. glabrata (3)		1	2			1	2	
C. parapsilosis (4)	1 (4)	3 (0)			0 (3)	3 (1)		1 (0)
Cryptococcus neoformans (15)	1 (6)	3 (1)	10 (8)	1 (0)	1 (8)	3 (3)	4 (4)	7 (0)
Scedosporium spp. (2)			2				2	
Paecilomyces spp. (2)			2			1	1	
Trichoderma spp. (2)			2		1		1	
Penicillium spp. (2)			2			1	1	
Fusarium spp. (2)			2				2	
A. fumigatus (13)			13		11	2		
A. flavus (13)			13		10	3		
A. niger (7)			7				7	
A. versicolor (1)			1				1	
A. nidulans (1)			1			1		
Coccidioides immitis (10)	8	2			8	2		
E. jeanselmia (3)		1	2				3	
E. spinifera (1)			1					1
Bipolaris spp. (2)	1		1					2
W. dermatitidis (1)			1			1		
O. humicola (1)			1				1	
P. werneckii (1)			1				1	
Cladophialophora bantiana (1)			1				1	

[a] Drug interactions were based on MIC_{80}s. Values in parentheses were based on MIC_{100}s.
[b] NA, not applicable. When the MIC for either drug used alone is lower than the lowest concentration tested, synergistic or additive interaction cannot be determined. For the same reason, FICs cannot be determined for these isolates.

mechanism for this positive interaction between nikkomycin and azoles is not known, it is possible that the loss of membrane integrity caused by azoles facilitates the cellular uptake of nikkomycin Z. Alternatively, azoles themselves may interrupt chitin synthesis or precursor transport to the cell wall (20, 24, 27). Since chitin synthase is located at the plasma membrane, it is reasonable to speculate that changes in the cell membrane affect enzyme activity (1).

Nikkomycin Z inhibits the growth of *C. albicans* in our assay system at concentrations from 0.5 to 32 μg/ml. It is also fungicidal for most (12 of 15) *C. albicans* isolates tested, with MLCs between 4 and 64 μg/ml (data not shown). Previous reports showed low efficacy of nikkomycin Z for candidiasis in animal models (6, 8), possibly due to low intracellular concentrations of the drug or the low percentage of chitin in the cell wall of *C. albicans*. Our data confirm the observation made by others of the synergistic effect of nikkomycin Z and azoles in inhibition of *C. albicans* in vitro and in vivo (16, 20). We also showed a similar effect of the drug combination against *C. parapsilosis*. The fungicidal nature of nikkomycin Z and its synergistic interaction with azoles indicate possible usefulness of nikkomycin Z in combination therapy for candidiasis.

The efficacy of nikkomycin Z against *Coccidioides immitis* has been reported (18, 23). In a microdilution assay, the MIC of nikkomycin Z was 0.125 μg/ml for the spherule-endospore phase (17). Our results indicated a higher MIC_{80} (4.9 μg/ml) for the mycelial phase, although a different method was employed (macrobroth dilution). Combinations of nikkomycin Z and azoles showed synergism in our study, based on growth inhibition of the mycelial phase (Fig. 2C). Given the high content of chitin (5 to 22%) in mycelial *Coccidioides immitis* (29, 30), the growth inhibition may have been due to blockage of septum formation as a result of reduced chitin synthesis. However, under the conditions of these assays, nikkomycin Z

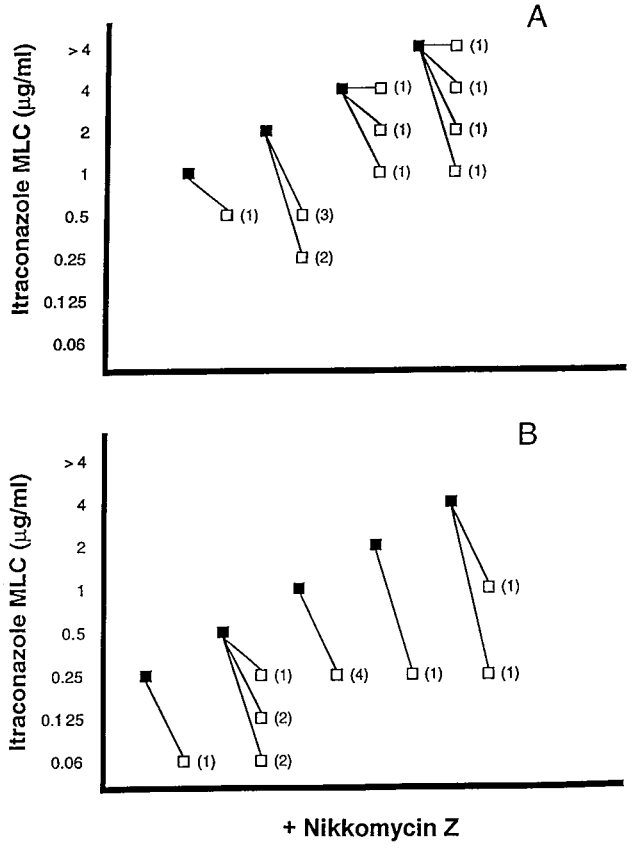

FIG. 2. Itraconazole MLCs for 13 isolates of *A. fumigatus* (A) and 13 isolates of *A. flavus* (B) when itraconazole was used alone (■) and in combination with nikkomycin Z (□). Values in parentheses are numbers of isolates.

by itself is not fungicidal for most mycelial isolates of *Coccidioides immitis* at the concentrations tested and it does not readily affect the MLCs of azoles for most isolates.

As previously reported, the MIC of nikkomycin Z for *Aspergillus* species was greater than 64 μg/ml (18). When it was used in combination with itraconazole, synergistic interaction was observed for most strains of *A. fumigatus* and *A. flavus*, resulting in at least a fourfold decrease in the MIC_{80}s of both drugs (Table 1). Nikkomycin Z also dramatically reduced the MLCs of itraconazole for these species (Fig. 2). It is not clear why similar effects were not observed with the fluconazole-nikkomycin Z combination or against other species such as *A. nidulans*, as chitin and glucans are essential polymers of the cell wall of *A. nidulans* (4, 31). There may be chemical-structural differences between the plasma membranes and cell walls of species of *Aspergillus* that are responsible for the observed differences in drug susceptibility.

It is well known that the determination of meaningful MICs of antifungal compounds is difficult, especially for filamentous fungi for which inoculum size is hard to standardize. The interpretation of MICs is further complicated by the frequent lack of in vitro-in vivo correlations (10, 25). While many host factors need to be considered for clinical efficacy of a particular compound, in vitro susceptibility testing remains an invaluable tool for antifungal drug development. Results of our experiments indicate that the combinations of nikkomycin Z and azoles are inhibitory or fungicidal for several clinically important fungi. These drug combinations should be further studied in animal models for in vivo efficacy and toxicity.

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