Natamycin and Azithromycin Are Synergistic *In Vitro* against Ocular Pathogenic *Aspergillus flavus* Species Complex and *Fusarium solani* Species Complex Isolates

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ABSTRACT The interaction of natamycin-azithromycin combination against 60 ocular fungal isolates was tested *in vitro*. The combination produced 100% synergistic interactions when natamycin was added to azithromycin 20, 40, and 50 $\mu\text{g/ml}$ against *Aspergillus flavus* species complex (AFSC) isolates and to azithromycin 50 $\mu\text{g/ml}$ against *Fusarium solani* species complex isolates. Addition of 50 $\mu\text{g/ml}$ azithromycin enhanced natamycin's effect against AFSC isolates by reducing natamycin MIC₉₀ from 64 to 0.031 $\mu\text{g/ml}$. No antagonism was observed.

KEYWORDS *Aspergillus flavus*, *Fusarium solani*, azithromycin, fungal keratitis, natamycin, synergistic activity

Keratomycosis is a major cause of blindness worldwide. *Fusarium solani* and *Aspergillus flavus* are two principal ocular isolates and are thought to have a particularly poor prognosis (1–8). Keratomycosis is proverbially difficult to treat. Natamycin is the only topical ocular antifungal agent approved by the U.S. Food and Drug Administration (FDA) and is the mainstay of treatment for keratomycosis in many countries (9, 10). However, some researchers have demonstrated that *Aspergillus* spp., especially *A. flavus*, are resistant to natamycin, and clinical responses in patients with *A. flavus* keratitis are poor (2, 5, 6, 8, 11–15). Otherwise, a study has shown that despite *Fusarium* spp. being sensitive to natamycin, with low mean MICs, 50% of patients need keratoplasty (8). Therefore, therapeutic methods to enhance the efficacy of natamycin are necessary. One possible approach is to combine natamycin with other antimicrobial agents. Through systematic screening of two-component combinations, we evaluated *in vitro* activities of natamycin associated with the antibiotics azithromycin, levofloxacin, gatifloxacin, chloramphenicol, tobramycin, clindamycin, and rifampin against *Aspergillus flavus* species complex (AFSC) isolates. Our results showed that only natamycin plus azithromycin obtained synergistic activity against natamycin-nonsusceptible AFSC isolates. We therefore investigated *in vitro* interactions between natamycin and azithromycin with clinically correlative concentrations for AFSC and *Fusarium solani* species complex (FSSC) strains.

A total of 30 AFSC and 30 FSSC strains isolated from cases with fungal keratitis seen at the Henan Eye Hospital in China were tested. These strains were identified by standard microbiological techniques (16). *Candida krusei* ATCC 6258 was tested as the quality control strain.

Antifungal susceptibility was assayed and drug interactions were tested as previously described (17). Simply, antifungal susceptibility was determined according to the microdilution method outlined in CLSI M38-A2 (18). The 30 AFSC and 30 FSSC strains were evaluated for susceptibility to natamycin (99% potency; Serva, New York) and

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TABLE 1 *In vitro* susceptibilities of ocular *Aspergillus flavus* species complex isolates to natamycin, azithromycin, levofloxacin, gatifloxacin, chloramphenicol, tobramycin, clindamycin, and rifampin alone and in combination

Organism (no. of isolates) and agent	MIC ($\mu\text{g/ml}$)		
	Range	MIC ₅₀	MIC ₉₀
<i>Aspergillus flavus</i> species complex (30)			
Natamycin	16–64	32	64
Levofloxacin	1,024	1,024	1,024
Gatifloxacin	4,096	4,096	4,096
Chloramphenicol	4,096	4,096	4,096
Tobramycin	4,096	4,096	4,096
Clindamycin	4,096	4,096	4,096
Rifampin	4,096	4,096	4,096
Natamycin + levofloxacin ^a	16–64	32	64
Natamycin + gatifloxacin ^b	16–64	32	64
Natamycin + chloramphenicol ^c	16–64	32	64
Natamycin + tobramycin ^d	16–64	32	32
Natamycin + clindamycin ^e	16–64	32	64
Natamycin + rifampin ^f	8–32	32	32
Natamycin + rifampin ^g	8–32	16	32

^aNatamycin at 10 concentrations in combination with 5, 20, 60, and 200 $\mu\text{g/ml}$ levofloxacin.^bNatamycin at 10 concentrations in combination with 5, 10, and 15 $\mu\text{g/ml}$ gatifloxacin.^cNatamycin at 10 concentrations in combination with 10 and 50 $\mu\text{g/ml}$ chloramphenicol.^dNatamycin at 10 concentrations in combination with 20, 30, 40, 50, and 100 $\mu\text{g/ml}$ tobramycin.^eNatamycin at 10 concentrations in combination with 40 and 60 $\mu\text{g/ml}$ clindamycin.^fNatamycin at 10 concentrations in combination with 4 and 8 $\mu\text{g/ml}$ rifampin.^gNatamycin at 10 concentrations in combination with 16 $\mu\text{g/ml}$ rifampin.

azithromycin (99% potency; Ruibang Pharmaceutical Co. Ltd., Zhejiang, China) alone and in combinations. The AFSC strains were evaluated for susceptibility to levofloxacin and gatifloxacin (99% potency; Beide Pharmaceutical Co. Ltd., Zhejiang, China), chloramphenicol (99% potency; Baijiingyu Pharmaceutical Co. Ltd., Nanning, China), tobramycin (99% potency; Xinbeijiang Pharmaceutical Co. Ltd., Lizhu Bloc, China), clindamycin (99% potency; Pukang Pharmaceutical Co. Ltd., Nanning, China), and rifampin (99% potency; Alfa Aesar) alone and in combinations. The following concentrations were used to test each drug alone: natamycin, 0.125 to 64 $\mu\text{g/ml}$; gatifloxacin, 0.5 to 256 $\mu\text{g/ml}$; levofloxacin, 1 to 512 $\mu\text{g/ml}$; and azithromycin, chloramphenicol, tobramycin, clindamycin, and rifampin, 4 to 2,048 $\mu\text{g/ml}$. The interactions of the drugs were evaluated by the limited checkerboard method (19, 20), where natamycin at concentrations of 0.125 to 64 $\mu\text{g/ml}$ was combined with azithromycin at 10, 20, 40, and 50 $\mu\text{g/ml}$; levofloxacin at 5, 20, 60, and 200 $\mu\text{g/ml}$; gatifloxacin at 5, 10, and 15 $\mu\text{g/ml}$; chloramphenicol at 10 and 50 $\mu\text{g/ml}$; tobramycin at 20, 30, 40, 50, and 100 $\mu\text{g/ml}$; clindamycin at 40 and 60 $\mu\text{g/ml}$; and rifampin at 4, 8, and 16 $\mu\text{g/ml}$. MIC was defined as the lowest concentration that showed 100% inhibition of growth. Interaction was decided by computing the fractional inhibitory concentration index (FICI). Synergy was defined as FICI ≤ 0.5 , indifference as FICI > 0.5 and ≤ 4 , and antagonism as FICI > 4 (19, 20).

The MIC ranges, MIC₅₀s, and MIC₉₀s for natamycin and azithromycin alone and in combination against all of the strains were estimated using SPSS software. For calculation off scale, MIC was changed to the next higher dilution.

In vitro susceptibilities of AFSC strains to natamycin, levofloxacin, gatifloxacin, chloramphenicol, tobramycin, clindamycin, and rifampin alone and in combination are outlined in Table 1, and *in vitro* susceptibilities of AFSC and FSSC strains to natamycin and azithromycin alone and in combination are outlined in Table 2. Our results showed that only natamycin plus azithromycin obtained significant activity against AFSC and FSSC isolates. The MIC₉₀s of natamycin against FSSC and AFSC were 8 and 64 $\mu\text{g/ml}$, respectively. The activity of natamycin against FSSC was 8 times greater than that against AFSC. This result was consistent with our earlier study (2). A noteworthy finding was that natamycin-azithromycin combinations exhibited greater activity against AFSC than against FSSC. In combination with 10, 20, 40, and 50 $\mu\text{g/ml}$ azithromycin, the

TABLE 2 *In vitro* susceptibilities of ocular *Aspergillus flavus* species complex and *Fusarium solani* species complex isolates to natamycin and azithromycin alone and in combination

Organism (no. of isolates) and agent ^a	MIC ($\mu\text{g/ml}$)		
	Range	MIC ₅₀	MIC ₉₀
<i>Aspergillus flavus</i> species complex (30)			
NAT	16–64	32	64
AZI	128	128	128
NAT + AZI 10 $\mu\text{g/ml}$	4–32	16	16
NAT + AZI 20 $\mu\text{g/ml}$	1–8	4	8
NAT + AZI 40 $\mu\text{g/ml}$	0.063–4	2	4
NAT + AZI 50 $\mu\text{g/ml}$	0.031	0.031	0.031
<i>Fusarium solani</i> species complex (30)			
NAT	4–32	8	8
AZI	128	128	128
NAT + AZI 10 $\mu\text{g/ml}$	4–8	8	8
NAT + AZI 20 $\mu\text{g/ml}$	4–8	8	8
NAT + AZI 40 $\mu\text{g/ml}$	1–4	2	4
NAT + AZI 50 $\mu\text{g/ml}$	0.031	0.031	0.031
<i>Candida krusei</i> ATCC 6258 (1) ^b			
Amphotericin B	0.5–1.0		
Ketoconazole	0.125–0.25		

^aNAT, natamycin; AZI, azithromycin.^bStandard quality control strain for antifungal susceptibility tests.

concentrations of natamycin against AFSC were 4-, 8-, 16-, and 2,065-fold lower, respectively, than with natamycin alone; but the concentrations of natamycin against FSSC were 0-, 0-, 2-, and 258-fold lower, respectively, than with natamycin alone. More strikingly, the combination of azithromycin 50 $\mu\text{g/ml}$ and natamycin increased the antifungal activity of natamycin against AFSC by decreasing natamycin MIC₉₀ from 64 $\mu\text{g/ml}$ (nonsusceptible level) to 0.031 $\mu\text{g/ml}$, a level easily achieved in cornea. For the FSSC strain, the MIC of natamycin decreased from 32 to 0.031 $\mu\text{g/ml}$ when combined with 50 $\mu\text{g/ml}$ azithromycin. Interactions of natamycin-azithromycin combinations determined by FICI are shown in Table 3. The combination produced 100% synergistic interactions when natamycin was combined with azithromycin 20, 40, and 50 $\mu\text{g/ml}$ against AFSC and when natamycin was combined with azithromycin at 50 $\mu\text{g/ml}$ against FSSC. Importantly, no antagonism was observed.

Some studies have shown that higher MICs are significantly related to poor performances (6), and lower MICs are particularly related to successful clinical results in keratomycosis (21). Susceptibility breakpoints of natamycin have not yet been delineated in the CLSI guidelines, but ≤ 16 $\mu\text{g/ml}$ of the MIC suggests susceptibility of a fungal strain (8, 15). According to this criterion, most of the FSSC isolates were susceptible and 96.7% of the AFSC isolates were nonsusceptible to natamycin in our study. Our data sustain the clinical evidence that *A. flavus* is often resistant to natamycin. In this study, 3.3% of FSSC and 96.7% of AFSC strains exhibited natamycin MICs of ≥ 32 $\mu\text{g/ml}$, which are not achievable levels in cornea with the present natamycin

TABLE 3 Interactions of natamycin-azithromycin combinations against ocular *Aspergillus flavus* species complex and *Fusarium solani* species complex isolates

Combination ^a	FICI score ^b and % of isolates showing interaction in:					
	<i>Aspergillus flavus</i> species complex ($n = 30$)			<i>Fusarium solani</i> species complex ($n = 30$)		
	FICI (mean $\pm$ SD)	Synergy	Indifference	FICI (mean $\pm$ SD)	Synergy	Indifference
NAT + AZI 10 $\mu\text{g/ml}$	0.42 $\pm$ 0.15	56.7	43.3	1.05 $\pm$ 0.14	3.3	96.7
NAT + AZI 20 $\mu\text{g/ml}$	0.29 $\pm$ 0.07	100	0	1.13 $\pm$ 0.14	3.3	96.7
NAT + AZI 40 $\mu\text{g/ml}$	0.39 $\pm$ 0.04	100	0	0.74 $\pm$ 0.17	3.3	96.7
NAT + AZI 50 $\mu\text{g/ml}$	0.39 $\pm$ 0.00	100	0	0.40 $\pm$ 0.00	100	0

^aNAT, natamycin; AZI, azithromycin.^bInteractions: synergy, FICI of ≤ 0.5 ; indifference, FICI of > 0.5 and ≤ 4 ; antagonism, FICI of > 4 .

formulation. The pharmacokinetic results of natamycin show that the corneal peak concentrations of natamycin are $0.276 \pm 0.158 \mu\text{g/g}$ in the intact rabbit cornea and $1.22 \pm 0.356 \mu\text{g/g}$ in the debrided rabbit cornea after one 50- μl drop of 5% natamycin ophthalmic suspension is topically applied (22). Natamycin barely penetrates an intact corneal epithelium. However, although debridement increases corneal penetration of natamycin, debridement cannot result in an optimal drug concentration after one drop of natamycin is topically applied. The current natamycin dosage regimen is topical natamycin (5%) applied to the affected eye every half hour for the first day, followed by every hour for 1 week, and then every 2 h for 3 to 6 weeks (5, 8, 23). The frequent dosing schedule may enhance drug absorption substantially and achieve therapeutic concentrations more efficiently for susceptible fungal isolates, but the concentrations of natamycin in corneas for nonsusceptible organisms are significantly lower than the MICs of natamycin, inducing failure of therapy and occurrence of drug-resistant keratomycosis. In our study, the combination with 50 $\mu\text{g/ml}$ azithromycin can significantly reduce natamycin MIC_{90s} against AFSC from 64 to 0.031 $\mu\text{g/ml}$; therefore, the corneal peak concentrations of natamycin ($1.22 \pm 0.356 \mu\text{g/g}$) are at least 39 times higher than the MIC values of natamycin, which improve its fungicidal effect.

Azithromycin 50 $\mu\text{g/ml}$ is an achievable level in cornea. The results from pharmacokinetic analyses of azithromycin show that significant antibacterial azithromycin concentration in the cornea ($252.00 \pm 86.56 \mu\text{g/g}$) is seen as early as 1 h after instillation and sustained for 6 days postdosing following the FDA-approved dosing regimen (1 drop twice daily for 2 days, then single instillation for 5 days) for treating bacterial conjunctivitis (24). Azithromycin, a broad-spectrum macrolide antibiotic, is valid for Gram-positive, Gram-negative, and atypical bacteria. Azithromycin acts by combining with the 50S subunit of the bacterial ribosome to restrain RNA-dependent protein synthesis (25). Azithromycin ophthalmic solution 1% is available for the treatment of bacterial conjunctivitis. The mechanism of synergy between natamycin and azithromycin against AFSC and FSSC isolates is unclear. We postulated that azithromycin has no antifungal effect on its own because of a defect in intracellular penetration. Natamycin binds to sterols in the fungal cell membrane and enhances permeability, allowing azithromycin to enter the fungal cell and inhibit protein synthesis. Their different modes of antimicrobial effect may indicate the potential for the synergy.

No studies emphasizing natamycin-azithromycin combination against *A. flavus* and *F. solani* isolates have been reported so far. We believe that our results are clinically significant. The susceptibility of *A. flavus* to natamycin is particularly poor, and patients with this infection may require natamycin-azithromycin combination as salvage therapy. Considering the synergism results, the lack of antagonism between natamycin and azithromycin in this study, and that azithromycin is known to be a safe drug, we believe the natamycin-azithromycin combination regimen may prove to be an important therapy against keratitis with *A. flavus* and *F. solani* in humans. However, because current data are derived only from *in vitro* conditions, the *in vivo* effect needs to be studied in detail.

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