In Vitro Interactions between Target of Rapamycin Kinase Inhibitor and Antifungal Agents against *Aspergillus* Species

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In vitro interactions of INK128, a target of rapamycin (TOR) kinase inhibitor, and antifungals, including itraconazole, voriconazole, posaconazole, amphotericin B, and caspofungin, against *Aspergillus* spp. were assessed with the broth microdilution checkerboard technique. Our results suggested synergistic effects between INK128 and all azoles tested, against multiple *Aspergillus fumigatus* and *Aspergillus flavus* isolates. However, no synergistic effects were observed when INK128 was combined with amphotericin B or caspofungin. No antagonism was observed for any combination.

The incidence of invasive aspergillosis, with high mortality rates, has increased significantly in recent decades, paralleling the increase in the number of immunocompromised patients (1). Although there has been an expansion of the antifungal armamentarium, resistance of *Aspergillus* spp. to azoles, polyenes, and echinocandins has been reported, especially the increasing emergence of azole resistance (2). Optimal treatments for aspergillosis remain elusive. Thus, novel therapeutic strategies are desperately needed.

The target of rapamycin (TOR) signaling cascade is conserved in eukaryotes from yeast to humans and responds to nutrients and growth factors to orchestrate cell growth and proliferation (3). In yeast cells, the TOR pathway has been implicated in the regulation of cellular responses to nutrients, including proliferation, translation, transcription, autophagy, ribosome biogenesis, lipid homeostasis, morphogenesis, and cellular aggregation, which has important implications for pathogenicity and virulence (3, 4). Thus, the TOR signaling cascade is an excellent target for the development of broad-spectrum antifungal drugs. INK128 is an ATP-competitive TOR kinase inhibitor that binds the TOR catalytic domain and selectively inhibits TOR (5). Therefore, it is tempting to speculate that INK128 might have antifungal effects or interactions with antifungals against *Aspergillus*.

In the present study, a total of 23 *Aspergillus* strains, including 15 strains of *Aspergillus fumigatus* and 8 strains of *Aspergillus flavus*, were studied. The *Aspergillus* strains were all clinical isolates from patients with invasive aspergillosis and were identified by morphological characteristics and molecular sequencing of the internal transcribed spacer (ITS) ribosomal DNA (rDNA) and the calmodulin gene (6–8). All strains were tested in duplicate via the microdilution checkerboard technique, adapted from the CLSI microdilution method (9). Serial dilutions of posaconazole (0.03 to 4 µg/ml; Selleck Chemicals, Houston, TX, USA), voriconazole (0.03 to 4 µg/ml; Selleck Chemicals), itraconazole (0.03 to 4 µg/ml; Selleck Chemicals), caspofungin (0.25 to 16 µg/ml; Selleck Chemicals), amphotericin B (0.03 to 4 µg/ml; Sigma Chemical Co., St. Louis, MO, USA), and INK128 (0.03 to 16 µg/ml; Selleck Chemicals) were prepared. The MIC, defined as the lowest concentration resulting in complete inhibition of growth, was used for the evaluation of itraconazole, voriconazole, posaconazole, amphotericin B, and INK128. The minimal effective concentration (MEC) and the MIC-2 were both used for caspo-

fungin alone and in the INK128-caspofungin combination. The MEC endpoint was taken as the lowest concentration at which the visual growth pattern change from granular to filamentous growth was detected, microscopically seen as restricted hyphal growth. The MIC-2 was defined as the lowest drug concentration that showed a prominent reduction of growth (approximately ≥50% reduction in turbidity), which was demonstrated to be comparable to the MECs for echinocandins against *Aspergillus* (10). Interpretation of results was performed after incubation at 35°C for 48 h. *Candida parapsilosis* ATCC 22019 was included to ensure quality control. The interactions of INK128 with azoles, amphotericin B, or caspofungin were assessed with the fractional inhibitory concentration index (FICI), which was classified as follows: FICI of ≤0.5, synergy; FICI of >0.5 to ≤4, no interaction (indifference); FICI of >4, antagonism (11).

The MIC ranges of individual tested drugs for *Aspergillus* isolates were >16 µg/ml for INK128, 0.5 to 2 µg/ml for itraconazole, 0.25 to 1 µg/ml for voriconazole, 0.25 to 1 µg/ml for posaconazole, and 0.25 to 1 µg/ml for amphotericin B (Tables 1 and 2). The MIC-2 values for caspofungin were 0.25 to 1 µg/ml (Table 2), which were in accord with MECs with favorable reproducibility in this study. INK128 individually did not exhibit any significant antifungal activity against the *Aspergillus* isolates tested. When INK128 was combined with itraconazole, however, the MICs of INK128 and itraconazole decreased to 0.5 to 8 µg/ml and 0.125 to 0.5 µg/ml, respectively. Favorable synergistic effects of INK128 and itraconazole (65%) were shown for 10 strains of *A. fumigatus* (10 of 15 strains) and 5 strains of *A. flavus* (5 of 8 strains) (Table 1). When INK128 was combined with voriconazole, the MIC ranges

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TABLE 1 MICs and FICIs for combinations of INK128 with azoles against *Aspergillus* strains^a

Strain	MIC (μg/ml)			INK128- ITC FICI	MIC (μg/ml)		INK128- VRC FICI	MIC (μg/ml)		INK128- POS FICI
	INK128	ITC	INK128/ITC		VRC	INK128/VRC		POS	INK128/POS	
<i>A. fumigatus</i>										
AF1	>16	1	1/0.125	S	0.5	1/0.125	S	0.5	0.5/0.25	I
AF2	>16	2	0.5/0.125	S	0.5	0.5/0.125	S	0.5	1/0.125	S
AF3	>16	0.5	4/0.5	I	0.25	4/0.25	I	0.25	4/0.25	I
AF4	>16	1	2/0.5	I	0.5	16/0.5	I	0.5	16/0.5	I
AF5	>16	1	1/0.25	S	0.5	16/0.25	I	0.5	16/0.25	I
AF6	>16	1	1/0.25	S	0.5	8/0.25	I	0.5	0.5/0.125	S
AF7	>16	0.5	8/0.5	I	0.5	4/0.125	S	0.5	4/0.25	I
AF8	>16	2	0.5/0.25	S	1	2/0.25	S	1	1/0.25	S
AF9	>16	1	1/0.125	S	0.5	0.5/0.125	S	0.5	1/0.125	S
AF10	>16	2	0.5/0.25	S	0.5	1/0.125	S	0.5	1/0.125	S
AF11	>16	0.5	2/0.25	I	0.25	2/0.25	I	0.25	2/0.25	I
AF12	>16	1	0.5/0.25	S	0.5	2/0.125	S	0.5	2/0.125	S
AF13	>16	1	2/0.25	S	0.5	1/0.125	S	0.5	2/0.25	I
AF14	>16	2	1/0.25	S	0.5	1/0.125	S	1	0.5/0.25	S
AF15	>16	0.5	1/0.25	I	0.25	0.5/0.25	I	0.5	0.5/0.25	I
<i>A. flavus</i>										
AFL1	>16	1	1/0.5	I	0.25	8/0.125	I	0.25	4/0.25	I
AFL2	>16	1	1/0.25	S	0.5	0.5/0.125	S	0.5	8/0.25	I
AFL3	>16	2	0.5/0.25	S	1	1/0.25	S	1	1/0.125	S
AFL4	>16	1	4/0.5	I	0.25	8/0.25	I	0.25	16/0.25	I
AFL5	>16	1	1/0.125	S	0.5	1/0.125	S	0.5	1/0.125	S
AFL6	>16	1	1/0.25	S	0.5	0.5/0.125	S	0.5	1/0.125	S
AFL7	>16	1	0.5/0.125	S	1	2/0.125	S	0.5	0.5/0.125	S
AFL8	>16	0.5	4/0.25	I	0.25	1/0.25	I	0.5	1/0.25	I

^a MICs were the concentrations achieving 100% growth inhibition. ITC, itraconazole; VRC, voriconazole; POS, posaconazole. S, synergy (FICI of ≤0.5); I, indifference (no interaction) (FICI of >0.5 to ≤4).

for INK128 and voriconazole decreased to 0.5 to 16 μg/ml and 0.125 to 0.5 μg/ml, respectively, demonstrating synergy (61%), against 9 strains of *A. fumigatus* (9 of 15 strains) and 5 strains of *A. flavus* (5 of 8 strains) (Table 1). When INK128 was combined with posaconazole, the MIC ranges for INK128 and posaconazole decreased to 0.5 to 16 μg/ml and 0.125 to 0.5 μg/ml, respectively. However, synergy between INK128 and posaconazole (50%) was observed for only 7 strains of *A. fumigatus* (7 of 15 strains) and 4 strains of *A. flavus* (4 of 8 strains) with this combination (Table 1). No synergistic effect was observed when INK128 was combined with amphotericin B or caspofungin (Table 2). Importantly, no antagonism was observed with these combinations.

The TOR signaling pathway has been widely investigated and has been recognized as a central controller of cell growth in eukaryotes (3). TOR kinases are the central elements of this signaling cascade. Rapamycin, the classic allosteric inhibitor, was initially identified as a potent antifungal compound against diverse species, including *Candida* spp., *Cryptococcus* spp., *Aspergillus* spp., *Fusarium* spp., *Penicillium* spp., and dermatophytes (12). Rapamycin exhibits a drawback in its potent immunosuppressive properties, however, and it failed to be used as an antifungal. In this study, we tested the *in vitro* antifungal activity of INK128, a highly potent, orally active, TOR kinase inhibitor (5), alone and in combination with antifungal agents, against *A. fumigatus* and *A. flavus*. Although INK128 alone was inactive against *Aspergillus* in this study, synergistic activity was observed for INK128 with itraconazole (65%), voriconazole (61%), and posaconazole (50%); the effective working range of INK128 was mostly within 0.5 to 1

μg/ml. No interaction between INK128 and amphotericin B or caspofungin was observed. Moreover, no antagonism was observed.

Previous studies reported positive interactions between rapamycin and caspofungin against *A. fumigatus* isolates with the disc diffusion assay (13) and synergistic interactions between rapamycin and amphotericin B (70%), itraconazole (50%), and posaconazole (40%) against *Mucorales* (previously referred to as zygomycetes) with the broth microdilution checkerboard procedure (14). However, there were no interactions between INK128 and caspofungin or amphotericin B against the tested *Aspergillus* spp. in the present study. Although antagonism of rapamycin-itraconazole and indifference to significant antagonism of rapamycin-posaconazole against *Mucorales* (previously referred to as zygomycetes) have also been observed (14, 15), there was no antagonism between INK128 and posaconazole or itraconazole in this study. The difference in the profiles of interactions with antifungals for INK128 and rapamycin might due to differences in the evaluation methods, the species tested, and/or the mechanisms through which INK128 and rapamycin interact with fungi and antifungals. This study extends previous findings on combination interactions between TOR kinase inhibitors and antifungals. It would be of significance to investigate whether INK128 would also enhance the *in vitro* susceptibility of azole-resistant *Aspergillus* isolates, as well as the underlying mechanism.

INK128, which was originally developed for cancer treatment, is now currently in clinical trials (5, 16) and may be used in the near future for cancer patients. Given that cancer patients under-

TABLE 2 Results for combinations of INK128 with amphotericin B or caspofungin against *Aspergillus* strains

	MIC (μg/ml) ^a			INK128- AMB FICI ^b	MEC/MIC-2 (μg/ml) ^c		INK128- CAS FICI ^b
Strain	INK128	AMB	INK128/AMB		CAS	INK128/CAS	
<i>A. fumigatus</i>							
AF1	>16	0.5	1/0.5	I	0.5	>16/0.5	I
AF2	>16	0.25	1/0.25	I	1	>16/1	I
AF3	>16	0.5	4/0.5	I	1	>16/1	I
AF4	>16	1	2/1	I	0.5	>16/0.5	I
AF5	>16	1	1/1	I	0.25	>16/0.25	I
AF6	>16	0.5	5/0.5	I	0.25	>16/0.25	I
AF7	>16	0.5	2/0.5	I	0.5	>16/0.5	I
AF8	>16	1	2/0.5	I	0.25	>16/0.25	I
AF9	>16	0.5	0.5/0.5	I	0.5	>16/0.5	I
AF10	>16	1	0.5/1	I	0.25	>16/0.25	I
AF11	>16	1	0.5/1	I	1	>16/1	I
AF12	>16	0.5	0.5/0.5	I	0.25	>16/0.25	I
AF13	>16	0.5	1/0.5	I	0.5	>16/0.5	I
AF14	>16	1	0.5/1	I	0.25	>16/0.25	I
AF15	>16	0.5	0.5/0.5	I	0.5	>16/0.5	I
<i>A. flavus</i>							
AFL1	>16	0.25	2/0.25	I	0.5	>16/0.5	I
AFL2	>16	0.5	1/0.25	I	0.25	>16/0.25	I
AFL3	>16	1	0.5/1	I	1	>16/1	I
AFL4	>16	1	2/1	I	0.5	>16/0.5	I
AFL5	>16	0.5	1/0.5	I	0.25	>16/0.25	I
AFL6	>16	0.5	1/0.5	I	0.5	>16/0.5	I
AFL7	>16	1	1/1	I	0.25	>16/0.25	I
AFL8	>16	1	0.5/1	I	0.25	>16/0.25	I

^a MICs were the concentrations achieving 100% growth inhibition. AMB, amphotericin B.^b I, indifference (no interaction) (FICI of >0.5 to ≤ 4). For INK128-CAS FICI calculations, the concentration of 32 $\mu\text{g/ml}$ was used as the MIC for INK128.^c The MIC-2 used for caspofungin was the lowest drug concentration that showed a prominent reduction of growth, which was comparable to the MEC in this study. CAS, caspofungin.

going chemotherapy are predisposed to invasive aspergillosis, this *in vitro* antifungal interaction profile might help clinicians choose proper antifungal treatments for patients receiving INK128, without concerns regarding antagonism. It has been demonstrated that orally administered INK128 has high absorption and bioavailability in mice, with doses of 3 mg/kg yielding a maximal concentration (C_{max}) of 0.599 $\mu\text{g/ml}$ in plasma (5). In addition, daily administration of INK128 at doses of up to 5 mg/kg over a 2-week period showed no obvious toxicity in mice (17), suggesting that effective antifungal levels of INK128, as observed in our study, could be achieved. However, further studies are needed to investigate the potential for concomitant use of these agents in humans.

In conclusion, INK128 could enhance the *in vitro* antifungal activities of itraconazole, voriconazole, and posaconazole against *Aspergillus* isolates, suggesting that the combination of azoles with a TOR kinase inhibitor might provide a potential strategy for the treatment of aspergillosis. However, further animal studies are needed.

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