In Vitro Antifungal Activities of Bis(Alkylpyridinium)Alkane Compounds against Pathogenic Yeasts and Molds[▽]

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Ten bis(alkylpyridinium)alkane compounds were tested for antifungal activity against 19 species (26 isolates) of yeasts and molds. We then determined the MICs and minimum fungicidal concentrations (MFCs) of four of the most active compounds (compounds 1, 4, 5, and 8) against 80 *Candida* and 20 cryptococcal isolates, in comparison with the MICs of amphotericin B, fluconazole, itraconazole, voriconazole, posaconazole, and caspofungin, using Clinical Laboratory and Standards Institutes broth microdilution M27-A3 (yeasts) or M38-A2 (filamentous fungi) susceptibility protocols. The compounds were more potent against *Candida* and *Cryptococcus* spp. (MIC range, 0.74 to 27.9 µg/ml) than molds (0.74 to 59.7 µg/ml). MICs against *Exophiala* were 0.37 to 5.9 µg/ml and as low as 1.48 µg/ml for *Scedosporium* but ≥25 µg/ml for zygomycetes, *Aspergillus*, and *Fusarium* spp. Compounds 1, 4, 5, and 8 exhibited good fungicidal activity against *Candida* and *Cryptococcus*, except for *Candida parapsilosis* (MICs of >44 µg/ml). Geometric mean (GM) MICs were similar to those of amphotericin B and lower than or comparable to fluconazole GM MICs but 10- to 100-fold greater than those for the other azoles. GM MICs against *Candida glabrata* were <1 µg/ml, significantly lower than fluconazole GM MICs ($P < 0.001$) and similar to those of itraconazole, posaconazole, and voriconazole (GM MIC range of 0.4 to 1.23 µg/ml). The GM MIC of compound 4 against *Candida guilliermondii* was lower than that of fluconazole (1.69 µg/ml versus 7.48 µg/ml; $P = 0.012$). MICs against *Cryptococcus neoformans* and *Cryptococcus gattii* were similar to those of fluconazole. The GM MIC of compound 4 was significantly higher for *C. neoformans* (3.83 µg/ml versus 1.81 µg/ml for *C. gattii*; $P = 0.015$). This study has identified clinically relevant *in vitro* antifungal activities of novel bisalkylpyridinium alkane compounds.

Invasive fungal disease is a significant cause of morbidity and mortality in seriously ill and immunocompromised patients (16, 26, 35). Despite the recent addition of a new class of antifungal agent (the echinocandins) (20) and more potent, broader-spectrum triazoles such as voriconazole (VRC) and posaconazole (POS) (23, 25), the number of available drugs for treatment of fungal infections remains limited. Many are fungistatic rather than fungicidal, and others are associated with substantial toxicity (4). Furthermore, clinical efficacy may be compromised by intrinsic or acquired drug resistance (29, 34). There is therefore a continuing need to develop and test novel antifungal agents with different modes of action.

Targeting of fungal virulence determinants, such as, for example, phospholipase B (PLB), is a potentially fruitful approach to new drug development. PLB is a proven virulence determinant of *Candida albicans* and *Cryptococcus neoformans* and is secreted by other pathogenic fungi, including *Aspergillus* spp. (6, 7, 13). Cryptococcal PLB (PLB1) in particular, has been well characterized (8, 13). As part of a study seeking inhibitors of cryptococcal PLB1, Ganendren et al. identified a

novel class of phospholipase inhibitors and observed that the bis(quaternary phosphonium)-alkane 1,12-bis(tributylphosphonium) dodecane dibromide not only inhibited cryptococcal PLB1 but also exhibited *in vitro* antifungal activity (18).

Properties of an “ideal” antifungal agent include ease of manufacture, potent antifungal activity, an excellent safety profile, and low cost. Bis-quaternary ammonium salts, which fulfill the above conditions, have long been recognized as potential antimicrobial agents (21, 32). Other than bisphosphonium salts (as described above) (18), we have previously determined that bisammonium-alkanes with a 12-carbon spacer between the positively charged bisammonium head groups exhibit antifungal activity with MICs of ~1 to 2.5 µg/ml against *C. neoformans* and *C. albicans* and that antifungal activity correlated with inhibition of cryptococcal PLB1 activity (27). Subsequent work on bis(aminopyridinium)alkane molecules indicated that these were also strongly antifungal, but they did not inhibit cryptococcal PLB1. This second class of compounds was significantly less toxic to human erythrocytes than the bisammonium-alkanes (28). Most recently, Obando et al. designed a third novel class of antifungal compound—the bis(alkylpyridinium)alkanes—with combined structural features of the bis(quaternary ammonium)alkanes and bis(aminopyridinium)alkanes (30). The compounds differ from previously described antimicrobial bispyridinium compounds (21, 28) as the pyridinium rings are attached to each other through the ring nitrogen atoms, with alkyl substituents appended directly to the

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TABLE 1. MICs and MFCs of 10 bis(alkylpyridinium)alkanes against 19 species of fungi

Strain	MIC/MFC ($\mu\text{g/ml}$) of test compound ^a :									
	1	2	3	4	5	6	7	8	9	10
<i>Candida albicans</i> ATCC 90028	1.48	1.48	2.96/5.9	1.48/2.96	0.79	3.2	1.74	1.58	1.86	1.86
<i>C. albicans</i> ATCC 10231	1.48 ^b	1.48	2.96	1.48/5.9	0.79	3.2	1.74	1.58	1.86 ^b	1.86
<i>Candida krusei</i> ATCC 6258	5.9 ^b /11.8	23.7	23.7	5.9/11.8	3.2	25.3	13.9	6.3	1.86 ^b	3.7
<i>C. krusei</i> ATCC 9258	5.9/11.8	23.7	11.8/23.7	11.8	ND/ND ^c	ND/ND	ND/ND	ND/ND	1.86/3.7	ND/ND
<i>C. krusei</i> 04-202328	11.8	23.7	23.7/47.3	11.8	3.2	25.3	13.9/27.9	6.3	1.86	3.7
<i>Candida parapsilosis</i> ATCC 22019	0.74 ^b /1.48	0.74/1.48	1.48/2.96	1.48/2.96	0.79	1.58/3.2	0.87/1.74	0.79	1.86 ^b	1.86
<i>C. parapsilosis</i> 04-202398	47.3	47.3	>47.3	47.3	25.3/50.7	50.7	55.8/>55.8	25.3/50.7	7.5/14.9	14.9/29.8
<i>Candida glabrata</i> 04-202345	1.48	0.37	1.48/5.9	1.5/3.0	0.79	0.79/1.58	0.87	0.79/3.2	0.93	1.86
<i>Candida tropicalis</i> 04-202361	0.74	0.74	2.96	1.48/5.9	0.79	1.58	0.87	0.79/1.58	1.86	1.86
<i>Cryptococcus neoformans</i> ATCC 90112	1.48 ^b	5.9	2.96	11.8	0.79	1.58	1.74	0.79	1.86 ^b	1.86/3.7
<i>C. neoformans</i> 05-202523	47.3	47.3	11.8/47.3	11.8/23.7	6.3/25.3	25.3/50.7	27.9	12.7/25.3	7.5	3.7/7.5
<i>Cryptococcus gattii</i> 05-200122	0.74	1.48	1.48	1.48	0.79	1.58	1.74	0.79	1.86	1.86
<i>Aspergillus fumigatus</i> ATCC 204305	47.3 ^b	>47.3	>47.3	>47.3	12.7/50.7	>50.7	27.9	25.3/50.7	7.5 ^b /14.9	14.9/29.8
<i>A. fumigatus</i> 05-203114	47.3	>47.3	>47.3	>47.3	12.7/50.7	>50.7	13.9/55.8	25.3	7.5/14.9	14.9/59.7
<i>Aspergillus flavus</i> ATCC 204304	23.7 ^b /47.3	>47.3	>47.3	>47.3	12.7/25.3	>50.7	55.8	12.7/25.3	7.5 ^b /14.9	14.9/59.7
<i>A. flavus</i> 05-203758	47.3	>47.3	>47.3	>47.3	25.3	>50.7	>55.8	25.3/50.7	14.9/29.8	59.7
<i>Aspergillus terreus</i> 05-202844	5.9 ^b /11.8	0.74/1.48	2.96/5.9	1.48	0.79	50.7	1.74	0.7	0.93 ^b /1.86	3.7/14.9
<i>Scedosporium prolificans</i> 05-203060	1.48 ^b /2.96	1.48/5.9	2.96	1.48	25.3	1.58/12.7	>55.8	1.58/6.83	1.86 ^b /3.7	3.7
<i>Scedosporium apiospermum</i> 05-203100	2.96 ^b	1.48	11.8	11.8	50.7	6.3/12.7	>55.8	6.3	3.7 ^b	1.86
<i>Cunninghamella bertholiae</i> 99-12187	47.3	47.3	>47.3	>47.3	12.7/25.3	>50.7	27.9	ND/ND	7.5	14.9/29.8
<i>Absidia corymbifera</i> 04-200170	>47.3	>47.3	>47.3	>47.3	50.7	>50.7	>55.8	50.7	14.9/27.8	29.8/59.7
<i>Exophiala jeanselmei</i> 03-200268	1.48/2.96	0.74/2.96	2.96	5.9	0.79	1.58/3.2	1.74	1.58	1.86/3.7	3.7
<i>Exophiala spinifera</i> 03-200803	0.37	0.37/0.74	1.48	0.74	1.58	0.79	1.74	0.79/1.58	1.86	1.86
<i>Fusarium solani</i> 04-203102	47.3	23.7	47.3	47.3	12.7	>50.7	27.9	12.7	14.9	29.8/59.7
<i>Paecilomyces lilacinus</i> 05-200601	>47.3	>47.3	>47.3	>47.3	50.7	>50.7	>55.8	>50.7	14.9/29.8	59.7
<i>Rhizopus oryzae</i> 05-203111	1.48 ^b /5.9	2.96/5.9	5.9/23.7	5.9/23.7	0.79	6.3/25.3	0.87/1.74	1.58/6.3	1.86 ^b	3.7

^a Minimal fungicidal concentrations (MFC) are given only where they differ from the MIC. The MFCs in many cases were the same as the MIC.

^b As previously tested in the study by Obando et al. (30).

^c ND, not done.

pyridinium rings at the 2-, 3-, or 4-positions; preliminary testing of two of these compounds (compounds 1 and 9 in the present study) against 11 unique fungal strains indicated that they may have useful antifungal activities (30).

Given the promising antifungal activity of this class of compounds as observed by Obando et al. (30), we evaluated the *in vitro* antifungal activities of 10 novel bisalkylpyridinium compounds, including compounds designated in the present study as 1 and 9 (described above); the *in vitro* hemolytic and cytotoxic activities of these compounds have previously been determined (30). Initially, the 10 compounds were screened for antifungal activity against a panel of key fungal pathogens. The MICs and minimum fungicidal concentrations (MFCs) of four of the most active compounds and MICs of marketed triazoles, amphotericin B (AMB), and caspofungin (CAS) were then determined against a large number of *Candida* (representing eight species) and cryptococcal isolates.

MATERIALS AND METHODS

Isolates. A total of 126 fungal isolates (112 yeasts and 14 filamentous fungi) were studied. These comprised eight American Type Culture Collection (ATCC; Rockville, MD) strains and 118 clinical isolates obtained from the Centre for Infectious Diseases and Microbiology, Westmead Hospital, Sydney, Australia, and the Mycology Unit, South Australia Pathology, Adelaide, Australia (Tables 1 and 2). All isolates were identified using standard phenotypic methods (15, 22) and stored in sterile distilled water at 25°C. When required, they were subcultured onto Sabouraud's dextrose agar (SDA; Difco Laboratories, Detroit, MI) (for yeasts) or potato dextrose agar (PDA; for molds) to ensure adequate growth (22). *Candida parapsilosis* ATCC 22019, *Candida krusei* ATCC 6258, *Aspergillus fumigatus* ATCC 204305, and *Aspergillus flavus* ATCC 204304 were the quality control strains (10, 11).

Medium. RPMI 1640 broth (Sigma-Aldrich, Castle Hill, Australia) with L-glutamine and without sodium bicarbonate was used for susceptibility testing.

The medium was buffered to pH 7.0 at 25°C with 0.165 M 3-(N-morpholino)-propanesulfonic acid (MOPS; Sigma-Aldrich). Sterility was confirmed prior to use.

Investigational bis(alkylpyridinium) compounds. Initially, MICs and MFCs of 10 investigational bis(alkylpyridinium) alkanes (compounds 1 to 10) (Fig. 1) were determined against 26 fungal isolates representing 19 species (Table 1). Synthesis of the compounds has been described previously (30). Compounds 1, 4, 5, and 8 were tested further against 80 isolates of *Candida* spp. and 10 isolates each of *C. neoformans* and *C. gattii* (Table 2). These compounds were selected for testing against the expanded set of isolates as they were the most active *in vitro* (see Table 1 and Results) and were shown previously to exhibit the least cytotoxicity and hemolytic activity (30). The compounds were dissolved in water to achieve a stock concentration of 373 to 474 $\mu\text{g/ml}$ (i.e., 700 μM for all compounds) and diluted in RPMI medium according to Clinical Laboratory Standards Institute (CLSI) methodology (10, 11) to provide a final test concentration range of 0.175 to 87.5 μM for all compounds; the concentration range corresponds to the lowest concentrations of 0.09 to 0.12 $\mu\text{g/ml}$ and the highest concentrations of 47 to 59 $\mu\text{g/ml}$, depending on the compound.

Antifungal agents. The following antifungal drugs were obtained as pure powders: amphotericin B (AMB; Bristol-Myers Squibb, Princeton, NJ), fluconazole (FLC; Sigma-Aldrich), itraconazole (ITC; Janssen Research Foundation, Beerse Belgium), voriconazole (VRC; Pfizer, North Ryde, Australia), posaconazole (POS; Schering-Plough, Kenilworth, NJ), and caspofungin (CAS; Merck and Co., Inc., Whitehouse Station, NJ). AMB, ITC, VRC, and POS were dissolved in 100% dimethyl sulfoxide (DMSO), and FLC and CAS were dissolved in 100% sterile distilled water. Stock concentrations and drug dilutions were made according to Clinical Laboratory Standards Institute (CLSI) methodology to yield the recommended final concentration ranges for the fungi tested (10, 11).

Susceptibility testing. MICs were determined in round-bottom 96-well microtiter plates using standard broth microdilution protocols described in the CLSI M27-A3 (for yeasts) or M38-A2 (for filamentous fungi) documents (10, 11). The final inoculum was verified by plating a small volume (100 μl) of the adjusted inoculum onto SDA. Plates were incubated at 35°C and examined daily for fungal colonies for up to 3 days for yeasts and up to 7 days for molds. Two drug-free controls were included in each test run: one contained medium alone (sterile control), and the other contained medium plus the inoculum (growth

TABLE 2. MICs of four bis(alkylpyridinium) compounds and five standard antifungal drugs against clinical isolates of yeasts

Species and antifungal agent or compound ^a	MIC (µg/ml) ^b				MFC (µg/ml) ^c		
	Range	GM	50%	90%	Range	50%	90%
<i>Candida albicans</i>							
AMB	0.06–0.5	0.20	0.25	0.5			
FLC	0.26–64	1.15	0.50	16			
ITC	0.02–0.18	0.07	0.04	0.09			
VRC	0.002–0.17	0.04	0.01	0.17			
POS	0.01–0.18	0.04	0.02	0.18			
CAS	0.07–0.27	0.12	0.14	0.27			
Compound 1	0.75–1.48	0.21	1.48	1.48	0.75–1.48	1.48	1.48
Compound 4	0.75–1.47	1.28	1.47	1.47	0.75–2.94	1.47	1.47
Compound 5	1.56	1.56	1.56	1.56	1.56–3.11	1.56	1.56
Compound 8	0.79–1.56	1.36	1.56	1.56	0.79–1.56	1.56	1.56
<i>Candida tropicalis</i>							
AMB	0.06–0.5	0.22	0.25	0.5			
FLC	0.076–2.45	1.41	1	4			
ITC	0.03–0.25	0.11	0.125	0.25			
VRC	0.008–0.25	0.07	0.125	0.25			
POS	0.016–0.25	0.061	0.06	0.25			
CAS	0.06–0.25	0.14	0.125	0.25			
Compound 1	0.38–0.75	0.702	0.75	0.75	0.38–0.75	0.75	0.75
Compound 4	0.37–1.47	0.746	0.75	1.47	0.37–1.47	0.75	1.47
Compound 5	0.39–1.56	1.183	1.56	1.56	0.39–1.56	1.56	1.56
Compound 8	0.39–1.56	0.790	0.79	0.79	0.39–1.56	0.79	1.56
<i>Candida glabrata</i>							
AMB	0.06–0.5	0.18	0.125	0.25			
FLC	16–256	32	16	256			
ITC	0.5–16	1.15	0.5	16			
VRC	0.125–4	0.41	0.25	4			
POS	0.25–8	1.23	1	8			
CAS	0.125–0.5	0.19	0.125	0.25			
Compound 1	0.38–1.48	0.701	0.75	0.75	0.38–1.48	1.48	1.48
Compound 4	0.37–1.47	0.975	1.47	1.47	0.75–5.87	1.47	5.87
Compound 5	0.39–0.79	0.739	0.79	0.79	0.39–1.56	1.56	1.56
Compound 8	0.39–1.56	0.642	0.79	0.79	0.39–1.56	0.79	1.56
<i>Candida parapsilosis</i>							
AMB	0.03–0.5	0.28	0.25	0.5			
FLC	1–8	2.14	2	4			
ITC	0.02–0.125	0.07	0.06	0.125			
VRC	0.02–0.125	0.043	0.06	0.06			
POS	0.02–0.125	0.049	0.06	0.06			
CAS	0.125–2	0.66	0.5	1			
Compound 1	>47	>47	>47	>47	>47	>47	>47
Compound 4	23.5	23.5	23.5	23.5	23.5	23.5	23.5
Compound 5	24.9–49.5	28.56	24.9	49.5	24.9–49.5	49.5	49.5
Compound 8	24.9–49.5	30.58	24.9	49.5	49.5	49.5	49.5
<i>Candida krusei</i>							
AMB	0.06–0.5	0.22	0.25	0.5			
FLC	64–128	84.45	64	128			
ITC	0.125–1	0.33	0.25	0.5			
VRC	0.125–1	0.41	0.25	1			
POS	0.06–0.5	0.18	0.125	0.25			
CAS	0.06–1	0.31	0.25	0.5			
Compound 1	0.38–11.8	2.253	2.96	5.91	0.38–11.8	2.96	5.91
Compound 4	0.75–11.74	3.383	2.94	5.87	0.75–11.74	5.87	11.74
Compound 5	0.79–3.11	1.362	1.56	1.56	0.79–3.11	1.56	3.11
Compound 8	0.79–12.45	3.122	3.11	6.22	0.79–12.45	6.22	6.22
<i>Candida dubliniensis</i>							
AMB	0.016–0.25	0.10	0.125	0.125			
FLC	0.125–32	0.43	0.25	0.5			
ITC	0.008–0.06	0.028	0.03	0.06			
VRC	0.008–0.06	0.011	0.008	0.016			
POS	0.008–0.06	0.014	0.008	0.03			

Continued on following page

TABLE 2—Continued

Species and antifungal agent or compound ^a	MIC (μg/ml) ^b				MFC (μg/ml) ^c		
	Range	GM	50%	90%	Range	50%	90%
CAS	0.03–0.125	0.070	0.06	0.125			
Compound 1	0.75–1.48	0.805	0.75	0.75	0.75–1.48	0.75	1.48
Compound 4	1.47–5.87	2.224	1.47	2.93	1.47–5.87	2.93	2.93
Compound 5	0.39–0.79	0.739	0.79	0.79	0.79–1.48	0.79	0.79
Compound 8	0.79–3.11	0.908	0.79	0.79	0.79–3.11	0.79	1.56
<i>Candida guilliermondii</i>							
AMB	0.06–0.5	0.15	0.125	0.25			
FLC	1–32	7.46	4	16			
ITC	0.125–1	0.5	0.5	1			
VRC	0.03–0.25	0.124	0.125	0.25			
POS	0.03–0.25	0.14	0.125	0.25			
CAS	0.5–2	1.15	1	2			
Compound 1	0.38–1.48	0.569	0.38	0.75	0.38–1.48	0.75	0.75
Compound 4	0.75–5.87	1.692	1.47	1.47	0.75–5.87	1.47	5.87
Compound 5	0.39–3.11	0.789	0.79	1.56	0.39–3.11	0.79	1.56
Compound 8	0.39–3.11	0.903	0.79	1.56	0.39–3.11	0.79	1.56
<i>Candida lusitanae</i>							
AMB	0.06–0.25	0.12	0.125	0.25			
FLC	0.5–32	2.64	2	4			
ITC	0.03–0.25	0.09	0.06	0.25			
VRC	0.008–0.25	0.02	0.03	0.16			
POS	0.008–0.06	0.015	0.016	0.03			
CAS	0.125–4	0.54	0.5	1			
Compound 1	0.75–1.48	0.86	0.75	1.48	0.75–1.48	1.48	1.48
Compound 4	1.47–5.87	2.74	2.93	2.93	1.47–5.87	2.93	5.87
Compound 5	0.79–1.56	1.04	0.79	1.56	0.79–12.44	0.79	6.22
Compound 8	1.56–6.22	2.71	3.11	3.11	1.56–12.44	3.11	6.22
<i>Cryptococcus neoformans</i>							
AMB	0.06–0.5	0.19	0.25	0.5			
FLC	1–32	4.59	4	16			
ITC	0.03–0.125	0.06	0.06	0.125			
VRC	0.008–0.125	0.04	0.03	0.125			
POS	0.03–0.25	0.07	0.06	0.125			
CAS	8	8	8	8			
Compound 1	0.75–23.66	2.41	1.48	5.91	1.48–47.05	1.48	47
Compound 4	1.47–11.74	3.83	2.94	5.87	2.94–11.74	5.87	11.74
Compound 5	0.79–6.22	1.19	0.79	3.11	0.79–12.45	0.79	12.45
Compound 8	0.79–6.22	1.28	0.79	3.11	0.79–12.45	0.79	12.45
<i>Cryptococcus gattii</i>							
AMB	0.06–0.5	0.19	0.25	0.25			
FLC	2–8	4	4	8			
ITC	0.03–0.125	0.07	0.06	0.125			
VRC	0.03–0.06	0.05	0.06	0.06			
POS	0.03–0.125	0.04	0.03	0.06			
CAS	8	8	8	8			
Compound 1	0.75–1.48	1.06	0.75	1.48	0.75–1.48	1.48	1.48
Compound 4	1.47–2.94	1.81	1.48	2.94	2.94	2.94	2.94
Compound 5	0.79	0.79	0.79	0.79	0.79–1.56	0.79	0.79
Compound 8	0.79	0.79	0.79	0.79	0.79	0.79	0.79

^a There were 10 isolates tested for each species.^b 50% and 90%, concentrations at which 50% and 90% of the isolates, respectively, were inhibited.^c 50% and 90%, concentrations at which 50% and 90% of the isolates, respectively, were killed.

control). Plates were incubated at 35°C according to established protocols (10, 11). Each isolate was tested in duplicate in two independent experiments.

Determination of MIC endpoints and MFCs. MICs were read visually after 24 to 72 h of incubation, depending on the test organism (10, 11). For the investigational compounds and AMB, the MIC was defined as the lowest concentration corresponding to 100% inhibition compared with the growth in the control well, and for FLC, ITC, VRC, and POS, the MIC was defined as the lowest drug concentration resulting in either 50% (MIC₅₀) or 100% inhibition, depending on

the test organism. MICs to CAS were determined according to published methodology (10, 11).

MFCs were determined for the 10 investigational compounds only. After the MIC was read, 30-μl aliquots of suspension from each well showing total inhibition of visible growth were plated onto SDA and incubated at 35°C. MFCs were read after 48 to 72 h (for yeasts) or for up to 7 days (for molds). The MFC was defined as the lowest drug concentration at which no colonies were observed.

FIG. 1. Chemical structures of compounds 1 through 10.

Analysis of results. Where 10 or more isolates of a species were tested, data were grouped by MIC range, MIC₅₀, MIC₉₀, MFC₅₀, and MFC₉₀. Geometric mean (GM) MICs were compared using the Mann-Whitney U test or Wilcoxon signed-rank test, as appropriate with SPSS version 14.0 software (SPSS, Inc., Chicago, IL). *P* values of <0.05 were considered statistically significant.

RESULTS

Antifungal activity of investigational bis(alkylpyridinium) compounds. The MICs and MFCs of the 10 investigational bis(alkylpyridinium) salts (compounds 1 to 10) for 26 unique isolates of fungi (19 species) are shown in Table 1.

In general, the test compounds had good activity and were more potent against *Candida* and *Cryptococcus* spp. than against molds (Table 1). Two clinical isolates, *C. neoformans* 05-202398 and *C. parapsilosis* 04-202398, were uniformly less susceptible than the other yeasts tested (Table 1). Among *Candida* spp., MICs against *C. krusei* were 4 to 16 times greater than those observed for the other species (Table 1). Of the molds tested, *Exophiala* spp. were the most susceptible and the zygomycetes, *Aspergillus*, *Fusarium*, and *Paecilomyces* spp. were

the least susceptible (Table 1). However, many test compounds had good activity against single isolates of *Aspergillus terreus*, *Scedosporium prolificans*, *Scedosporium apiospermum*, and *Rhizopus oryzae* (Table 1).

MFCs of the 10 bis(alkylpyridinium) compounds were in general the same as or 1 to 2 dilutions higher than the corresponding MICs. The MFC/MIC ratio was highest (i.e., 4) for compound 8 against the *C. glabrata*, *A. terreus*, and *R. oryzae* isolates and one of two *C. neoformans* strains (Table 1).

Overall, compounds 1, 4, 5, 8, 9, and 10 were the most active. Although most potent against *Candida* and *Cryptococcus* spp., MICs were also relatively low for *Exophiala* and *Scedosporium* spp., *R. oryzae*, and *A. terreus* (range, 0.37 to 6.9 µg/ml). Compound 2 likewise had good activity against yeasts, but MICs were higher against *C. krusei* than those of compounds 1, 4, 5, and 8 to 10 (23.7 µg/ml versus 1.48 to 6.3 µg/ml (Table 1). Compound 7 was the least potent compound.

Antifungal susceptibility of *Candida* and *Cryptococcus* spp. to compounds 1, 4, 5, and 8. To confirm the promising activities of compounds 1, 4, 5, and 8 against pathogenic yeasts, the susceptibilities of 80 *Candida* isolates (eight species), 10 *C. neoformans* isolates, and 10 *C. gattii* isolates were determined. These four compounds were chosen, since they also demonstrated favorable toxicity profiles, with (50% inhibitory concentrations [IC₅₀s]) against two mammalian cell lines of 48 to >350 µM and HC₅₀s of 129 to >350 µM against human erythrocytes (30). Although compounds 9 and 10 also had good antifungal activity (Table 1), they were previously shown to have significantly higher toxicity (IC₅₀s of 10 to 70 µM and hemolytic concentrations [HC₅₀s, defined as the concentration at which there was 50% lysis determined by plotting percent lysis against concentration of drug] of 17.5 to 31 µM) (30) and were therefore not investigated further (30). MIC results from compounds 1, 4, 5, and 8 and those from AMB, FLC, ITC, VRC, POS, and CAS are shown in Table 2.

With the exception of *C. parapsilosis*, for which MICs ranged from 44 to >87.5 µg/ml, the four bis(alkylpyridinium)alkanes demonstrated good activity against *Candida* and *Cryptococcus* spp. (Table 2). In general, GM MICs were similar to those of AMB and/or CAS, either substantially lower than or comparable to those of FLC and 10- to 100-fold higher than those of ITC, VRC, and POS. MIC₉₀ values were 4- to 20-fold lower than those of FLC against most *Candida* spp. and 2- to 8-fold lower against *C. neoformans* and *C. gattii* (Table 2). The MICs of all compounds were comparable, although for some species, compound 4 was the least potent and compounds 5 and 1 were the most potent (Table 2).

Of the eight *Candida* species studied, the least susceptible, other than *C. parapsilosis* (see above), was *C. krusei*. The GM MICs of the compounds were 5 to 10 times greater than those for marketed antifungal drugs other than FLC, although all MIC₉₀s were <6 µg/ml (Table 2). In particular, the MIC₉₀ of compound 5 was 1.56 µg/ml, substantially lower than that of the MIC₉₀ for FLC (128 µg/ml) (Table 2). The compounds also demonstrated good activity against *C. glabrata*: GM MICs and MIC₉₀s of all four compounds were <1 µg/ml and were significantly lower than those for FLC (*P* < 0.001; data not shown) (Table 2). The GM MIC of compound 4 (0.98 µg/ml) was comparable to those of ITC (1.15 µg/ml), POS (1.23 µg/ml), and VRC (0.40 µg/ml) (all *P* values not significant). The

compounds were also active against *C. guilliermondii*, *C. lusitaniae*, and *C. dubliniensis* (all MIC₉₀s ≤ 3.11 $\mu\text{g/ml}$). The GM MIC of the least active test agent, compound 4, against *C. guilliermondii* was 1.69 $\mu\text{g/ml}$, significantly lower than that of FLC (7.476 $\mu\text{g/ml}$; $z = -2.50$, $P = 0.012$ [Wilcoxon's signed-rank test]).

Among the members of the *Cryptococcus* complex, GM MICs of the four compounds against *C. neoformans* were ≈ 2 -fold higher and MIC₉₀s were ≈ 4 - to 5-fold higher than the corresponding MICs against *C. gattii*, with the GM MIC of compound 4 being significantly higher (3.83 $\mu\text{g/ml}$ versus 1.81 $\mu\text{g/ml}$; Mann-Whitney test $U = 14$, $P = 0.005$). MICs of one or more of the compounds against three strains of *C. neoformans* were high (11 to >44 $\mu\text{g/ml}$), whereas the MICs of all 10 *C. gattii* strains were ≤ 5.5 $\mu\text{g/ml}$ (data not shown). Overall, GM MICs of the test compounds were similar to that of FLC (Table 2) but ≈ 100 -fold higher than those of ITC, VRC, and POS.

MFCs of the test compounds were either the same as or 1 to 2 dilutions higher than the MICs for the majority of isolates. The MFC/MIC ratio varied with the compound-organism combination (Table 2).

DISCUSSION

Given the increase in invasive fungal disease due to previously uncommon or new fungal pathogens, many of which are resistant or less susceptible to currently available antifungal drugs, testing the antifungal activity of novel and existing antimycotic agents is critical to the development of new therapeutic strategies. The results of this study indicate that bis(alkylpyridinium)alkane compounds are fungicidal at relatively low concentrations against a broad range of clinically important fungi, suggesting that this class of compound can be exploited for antifungal drug development. Among yeasts, the important exception was *C. parapsilosis*, for which the MIC₉₀s of the four most active compounds against clinical strains were more than 50-fold higher than those against other yeasts.

The potential of a series of bis(alkylpyridinium)alkanes as candidate antifungal drugs was affirmed first by their low MICs and fungicidal activity against *Candida* spp. and cryptococci (Table 1). In the extended study of yeast susceptibility to the most promising (and least toxic) (30) of the 10 compounds tested (compounds 1, 4, 5, and 8), it is noteworthy that overall the antifungal activity was similar to that of FLC and AMB, and for *C. guilliermondii* and *C. lusitaniae*, also that of CAS (9, 10, 24). *C. parapsilosis* was the notable exception, with MICs of all clinical isolates greatly exceeding those of the other *Candida* species. The compounds were generally 10- to 100-fold less potent than ITC, VRC, and POS (23, 25). The reasons for the apparent resistance of *C. parapsilosis* are not apparent. Curiously, unlike clinical strains of *C. parapsilosis*, *C. parapsilosis* ATCC 22019 tested "susceptible" to all 10 compounds examined in the first part of the study (Table 1), indicative of the importance of testing of clinical strains and of large numbers of strains for susceptibility. Against *C. krusei*, although MICs were in general higher than those for other *Candida* spp. (except for *C. parapsilosis*), GM MICs were <3.5 $\mu\text{g/ml}$, indicating potentially useful antifungal activity against this species.

Not only was the GM MIC of one or more of compounds 1, 4, 5, and 8 similar to, or significantly lower than, that of FLC against most *Candida* species, but the MIC₉₀s were also 2 to 20 times lower, reflecting the relatively narrow range of MICs and lack of cross-resistance with FLC (the differences in FLC MIC₉₀ were most apparent for the azole-resistant or potentially azole-resistant species *C. krusei* and *C. glabrata*). Consistent with this observation, for *C. glabrata*, the GM MICs of the test compounds were similar to those of POS and ITC, with MIC₉₀s substantially lower than those of all four azoles. The compounds were also active against the more uncommon, but potentially drug-resistant, species *C. guilliermondii* and *C. lusitaniae* (34), with MICs similar to or lower than those of FLC. Importantly, the compounds were generally fungicidal, with an MFC/MIC ratio of 1 to 2, except for compound 4, for which for *C. glabrata*, *C. krusei*, *C. guilliermondii*, and *C. lusitaniae* the ratio ranged from 2 to 4 (Table 2). Given that azole antifungals are fungistatic (23), the role of bis(alkylpyridinium)alkanes compounds as an alternative to FLC for the treatment of infections caused by potentially FLC-resistant *Candida* species is worthy of further study. The anticytotoxic activities of the compounds against *C. neoformans* and *C. gattii*, with MICs comparable to or lower than those of fluconazole, suggest they may also be potentially useful in the treatment of cryptococcosis (33). MICs against both cryptococcal species were also broadly comparable. All four compounds 1, 4, 5, and 8 were fungicidal for *C. gattii*, but tolerance (MFC/MIC ratio of >4) was demonstrated for *C. neoformans*. AMB is frequently used during the induction phase of treatment of cryptococcosis because of its fungicidal activity, especially in combination with the agent flucytosine. Examination of larger numbers of both cryptococcal species for fungicidal activity will be required to determine if bis(alkylpyridinium)alkanes exhibit killing kinetics similar to those of AMB (5).

Another finding of the study was that the MICs of most of the bis(alkylpyridinium)alkanes tested were similar to those of AMB against single strains of relatively, or highly, resistant filamentous fungi, namely, *R. oryzae*, *S. prolificans*, *S. apiospermum*, and *A. terreus*, and the dematiaceous fungi (1, 3, 14, 17, 19). Furthermore, these compounds were fungicidal for these pathogens. MICs of the compounds were also substantially lower than those of CAS, which has no useful activity against non-*Aspergillus* molds (20). Further study of the activity of these compounds against molds resistant or nonsusceptible to current antifungal drugs is warranted. These include *Rhizopus* spp., especially *R. oryzae*, which are major zygomycete pathogens causing $\geq 50\%$ of zygomycoses (2). Clinical responses to AMB, the cornerstone of drug therapy for zygomycoses, are often suboptimal despite prolonged treatment with high doses. *Scedosporium* spp. are also emergent pathogens with high associated mortality (12). Other than compounds 7 and 5, MICs of this class of compound against *S. prolificans* in particular were within or similar to MICs, not only of AMB, but also of ITC, POS, and VRC (VRC MIC range for *S. prolificans* of 1 to 8 $\mu\text{g/ml}$, versus a range of 1.48 to 11.8 $\mu\text{g/ml}$ for the test compounds) (12, 19). The susceptibility of the single *A. terreus* strain to the compounds was similar to that reported for AMB (AMB MIC range of 2 to 4 $\mu\text{g/ml}$) (3).

The primary molecular target(s) of the bis(alkylpyridinium)alkane compounds and the mechanism(s) of their antifungal

effect have not been elucidated. While inhibition of PLB1 may contribute to the antifungal activity of this class of compounds in cryptococci, it is unlikely to be the major mode of action (30). Based on evidence from older bisquaternary ammonium salts and their well-established antibacterial and antifungal effects (21), we postulate that the compounds examined in the present study exert their antifungal effect by interfering with fungal cell wall or cell membrane biochemistry. We observed a correlation between the substituted head groups of the designed compounds and antifungal activity: compounds with straight chain head groups or branching at the carbon atom directly attached to the pyridinium ring—that is, those with 4-pentyl, 4-pentenyl, 4-hexyl, (3-methyl, 4-pentyl), 3,4-dipentyl, and [3-methyl 4-(5'-nonyl)] head group substitutions (as represented by compounds 1, 4, 5, 8, 9, and 10, respectively)—were the most active. Conversely, compounds 3, 6, and 7, in which the head group substituents bear branched or aromatic groups three to four atoms distal from the pyridinium ring, were the least potent, indicating that the introduction of steric bulk at this position decreases activity. This may reflect differences in the mode of interaction of these compounds with membrane phospholipids (31).

Although achievable serum drug levels of the compounds *in vivo* and other pharmacokinetic data are not known, on a molar basis (formula weights of compounds 1, 4, 5, and 8 range from 535 to 566, in comparison with those of fluconazole [306], itraconazole [705], voriconazole [349], amphotericin B [924], and caspofungin [1,093]), the compounds overall were at least 10-fold more active than fluconazole (except for *C. dublinensis* [MIC_{90} of 1.4 μM versus 1.6 μM for fluconazole]). In general, the compounds were about 5 times less active than amphotericin B. Studies correlating MICs with pharmacokinetic parameters and with clinical outcome are indicated.

In summary, the bis(alkylpyridinium)alkanes are fungicidal compounds with potentially useful *in vitro* activities against common as well as more resistant emerging fungi. Two common pathogens, *C. parapsilosis* and *A. fumigatus*, are the notable exceptions. Because of their ease of synthesis, these compounds may also lend themselves to development as topical agents. Further synthesis and evaluation of additional members of this class of compounds as novel antifungal agents are in progress.

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REFERENCES

- Alastruey-Izquierdo, A., M. V. Castelli, I. Cuesta, A. Monzon, M. Cuenca-estrella, and J. L. Rodríguez-Tudela. 2009. Activity of posaconazole and other antifungal agents against the Mucorales strains identified by sequencing of internal transcribed spacers. *J. Clin. Microbiol.* **53**:1686–1689.
- Alvarez, E., D. A. Sutton, J. Cano, A. W. Fothergill, A. Stchigel, M. G. Rinaldi, and J. Guarro. 2009. Spectrum of zygomycete species identified in clinically significant specimens in the United States. *J. Clin. Microbiol.* **47**:1650–1656.
- Baddley, J. W., K. A. Marr, D. R. Andes, T. J. Walsh, C. A. Kauffman, D. P. Kontoyiannis, J. I. Ito, S. A. Balajee, P. G. Pappas, and S. A. Moser. 2009. Patterns of susceptibility of *Aspergillus* isolates recovered from patients enrolled in the Transplant-Associated Infection Surveillance Network. *J. Clin. Microbiol.* **47**:3271–3275.
- Barrett, D. 2002. From natural products to clinically useful antifungals. *Biochim. Biophys. Acta* **1587**:224–233.
- Bicanic, T., R. Wood, G. Meintjes, K. Rebe, A. Brouwer, A. Loyse, L. G. Bekker, S. Jaffar, and T. Harrison. 2008. High-dose amphotericin B with flucytosine for the treatment of cryptococcal meningitis in HIV-infected patients: a randomized trial. *Clin. Infect. Dis.* **47**:123–130.
- Birch, M., D. W. Denning, and G. D. Robson. 2004. Comparison of extracellular phospholipase activities in clinical and environmental *Aspergillus fumigatus* isolates. *Med. Mycol.* **42**:81–86.
- Calderone, R. A., and W. A. Fonzi. 2001. Virulence factors of *Candida albicans*. *Trends Microbiol.* **9**:327–335.
- Chen, S. C. A., M. Muller, J. Z. Zhou, L. C. Wright, and T. C. Sorrell. 1997. Phospholipase activity of *Cryptococcus neoformans*: a new virulence factor? *J. Infect. Dis.* **175**:414–420.
- Chen, S. C. A., D. Marriott, E. G. Playford, Q. Nguyen, D. Ellis, W. Meyer, T. C. Sorrell, M. Slavin, and the Australian Candidemia Study. 2009. Candidemia with uncommon *Candida* species: predisposing factors, outcome, antifungal susceptibility and implications for management. *Clin. Microbiol. Infect.* **15**:662–669.
- Clinical and Laboratory Standards Institute. 2007. Reference method for broth dilution antifungal susceptibility testing of yeasts: approved standard, 3rd ed. CLSI document M27-A3. CLSI, Wayne, PA.
- Clinical and Laboratory Standards Institute. 2008. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi: approved standard, 2nd ed. CLSI document M38-A2. CLSI, Wayne, PA.
- Cortez, K., E. Roilides, F. Quiroz-Telles, J. Meletiadis, C. Antachopoulos, T. Knudsen, W. Buchanan, J. Milanovich, D. A. Sutton, A. Fothergill, M. G. Rinaldi, Y. R. Shea, T. Zaoutis, S. Kottitil, and T. J. Walsh (ed.). 2008. Infections caused by *Scedosporium* spp. *Clin. Microbiol. Rev.* **21**:157–197.
- Cox, G. M., H. C. McDade, S. C. A. Chen, S. Tucker, M. Gottfredson, L. C. Wright, T. C. Sorrell, S. D. Leidich, A. Casadevall, M. Ghannoum, and J. Perfect. 2001. Extracellular phospholipase activities are a virulence factor for *Cryptococcus neoformans*. *Mol. Microbiol.* **39**:166–175.
- Dannaoui, E., J. Meletiadis, J. W. Mouton, J. F. G. M. Meis, P. E. Verweij, and the Eurofung Network. 2003. *In vitro* susceptibilities of zygomycetes to conventional and new antifungals. *J. Antimicrob. Chemother.* **51**:45–52.
- de Hoog, G. S., J. Guarro, J. Gene, and M. J. Figueras (ed.). 2008. Atlas of clinical fungi, 2nd ed. Centraalbureau voor Schimmelcultures/Universitat Rovira i Virgili, Utrecht, Netherlands.
- Eggimann, P., J. Garbino, and D. Pittet. 2003. Epidemiology of *Candida* species infections in critically ill non-immunosuppressed patients. *Lancet Infect. Dis.* **3**:685–702.
- Fothergill, A. W., M. G. Rinaldi, and D. A. Sutton. 2009. Antifungal susceptibility testing of *Exophiala* spp.: a head-to-head comparison of amphotericin B, itraconazole, posaconazole and voriconazole. *Med. Mycol.* **47**:41–43.
- Ganendren, R., F. Widmer, V. Singhal, C. Wilson, T. Sorrell, and L. Wright. 2004. *In vitro* antifungal activities of inhibitors of phospholipases from the fungal pathogen *Cryptococcus neoformans*. *Antimicrob. Agents Chemother.* **48**:1561–1569.
- Heath, C., M. Slavin, T. C. Sorrell, R. Handke, A. Harun, M. Phillips, Q. Nguyen, L. Delhaes, D. Ellis, W. Meyer, and S. C. A. Chen, on behalf of the Australian *Scedosporium* Study Group. 2009. Population-based surveillance for scedosporiosis in Australia: epidemiology, disease manifestations and emergence of *Scedosporium aurantiacum* infection. *Clin. Microbiol. Infect.* **15**:689–693.
- Kauffman, C. A., and P. L. Carver. 2008. Update on echinocandin antifungals. *Semin. Respir. Crit. Care. Med.* **29**:211–220.
- Kourai, H., T. Yabuhara, A. Shirai, T. Maeda, and H. Nagamune. 2006. Syntheses and antimicrobial activities of a series of new bis-quaternary ammonium compounds. *Eur. J. Med. Chem.* **41**:437–444.
- Larone, D. H. 2002. Medically important fungi: a guide to identification, 4th ed. ASM Press, Washington, DC.
- Maschmeyer, G., and A. Haas. 2006. Voriconazole: a broad spectrum triazole for the treatment of serious and invasive fungal infections. *Future Microbiol.* **1**:365–385.
- Messer, S. A., G. Moet, J. T. Kirby, and R. N. Jones. 2009. Activity of contemporary antifungal agents, including the novel echinocandin anidulafungin, tested against *Candida* spp., *Cryptococcus* spp., and *Aspergillus* spp.: report from the SENTRY antimicrobial surveillance program (2006–2007). *J. Clin. Microbiol.* **47**:1942–1946.
- Nagappan, V., and S. Deresinski. 2007. Posaconazole: a broad-spectrum triazole antifungal agent. *Clin. Infect. Dis.* **45**:1610–1617.
- Neofytos, D., D. Horn, E. Anaissie, et al. 2009. Epidemiology and outcome of invasive fungal infection in adult hematopoietic stem cell transplant recipients: analysis of multicenter Prospective Antifungal Therapy (PATH) Alliance registry. *Clin. Infect. Dis.* **48**:265–273.
- Ng, C. K. L., D. Obando, F. Widmer, L. C. Wright, T. C. Sorrell, and K. A. Jolliffe. 2006. Correlation of antifungal activity with fungal phospholipase inhibition using a series of bisquaternary ammonium salts. *J. Med. Chem.* **49**:811–816.

28. Ng, C. K. L., V. Singhai, F. Widmer, L. C. Wright, T. C. Sorrell, and K. A. Jolliffe. 2007. Synthesis, antifungal and hemolytic activity of a series of bis(pyridinium)alkanes. *Bioorg. Med. Chem.* **15**:3422–3429.
29. Norrby, S. R., C. E. Nord, and R. Finch. 2005. Lack of development of new antimicrobial drugs: a potential serious threat to public health. *Lancet Infect. Dis.* **5**:115–119.
30. Obando, D., N. Pantarat, R. Handke, Y. Koda, F. Widmer, J. T. Djordjevic, D. H. Ellis, T. C. Sorrell, and K. A. Jolliffe. 2009. Synthesis, antifungal, haemolytic and cytotoxic activities of a series of bis(alkylpyridinium)alkanes. *Bioorg. Med. Chem.* **17**:6329–6339.
31. Pashynskaya, V. A., M. V. Kosevich, A. Gomory, O. V. Vashchenko, and L. N. Lisetski. 2002. Mechanistic investigation of the interaction between bisquaternary antimicrobial agents and phospholipids by liquid secondary ion mass spectrometry and differential scanning calorimetry. *Rapid Commun. Mass. Spectrom.* **16**:1706–1713.
32. Pavlikova-Moricka, M., I. Lacko, F. Devinsky, L. Masarova, and D. Mily-narcik. 1994. Quantitative relationships between structure and antimicrobial activity of new “soft” bisquaternary ammonium salts. *Folia Microbiol. (Praha)* **39**:176–180.
33. Perfect, J. R., W. E. Dismukes, F. Dromer, D. L. Goldman, J. R. Graybill, R. J. Hamill, T. S. Harrison, R. A. Larsen, O. Lortholary, M. H. Nguyen, P. G. Pappas, W. G. Powderly, N. Singh, J. D. Sobel, and T. C. Sorrell. 2010. Clinical practice guidelines for the management of cryptococcal disease: 2010 update by the Infectious Diseases Society of America. *Clin. Infect. Dis.* **50**:291–322.
34. Pfaller, M. A., and D. J. Diekma. 2007. Epidemiology of invasive candidiasis: a persistent public health problem. *Clin. Microbiol. Rev.* **20**:133–163.
35. Singh, N., and D. L. Paterson. 2005. *Aspergillus* infections in transplant recipients. *Clin. Microbiol. Rev.* **18**:44–69.