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In Vitro Activity of a Novel Broad-Spectrum Antifungal, E1210, Tested against *Aspergillus* spp. Determined by CLSI and EUCAST Broth Microdilution Methods▽

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E1210 is a first-in-class broad-spectrum antifungal that suppresses hyphal growth by inhibiting fungal glycophosphatidylinositol (GPI) biosynthesis. In the present study, we extend these findings by examining the activity of E1210 and comparator antifungal agents against *Aspergillus* spp. by using the methods of the Clinical and Laboratory Standards Institute (CLSI) and the European Committee for Antimicrobial Susceptibility Testing (EUCAST) to test wild-type (WT) as well as amphotericin B (AMB)-resistant (-R) and azole-R strains (as determined by CLSI methods). Seventy-eight clinical isolates of *Aspergillus* were tested including 20 isolates of *Aspergillus flavus* species complex (SC), 22 of *A. fumigatus* SC, 13 of *A. niger* SC, and 23 of *A. terreus* SC. The collection included 15 AMB-R (MIC, ≥2 μg/ml) isolates of *A. terreus* SC and 10 itraconazole-R (MIC, ≥4 μg/ml) isolates of *A. fumigatus* SC (7 isolates), *A. niger* SC (2 isolates), and *A. terreus* SC (1 isolate). Comparator antifungal agents included anidulafungin, caspofungin, amphotericin B, itraconazole, posaconzole, and voriconazole. Both CLSI and EUCAST methods were highly concordant for E1210 and all comparators. The essential agreement (EA; ±2 $\log_2$ dilution steps) was 100% for all comparisons with the exception of posaconazole versus *A. terreus* SC (EA = 91.3%). The minimum effective concentration (MEC)/MIC_{90} values (μg/ml) for E1210, anidulafungin, caspofungin, itraconazole, posaconazole, and voriconazole, respectively, were as follows for each species: for *A. flavus* SC, 0.03, ≤0.008, 0.12, 1, 1, and 1; for *A. fumigatus* SC, 0.06, 0.015, 0.12, >8, 1, and 4; for *A. niger* SC, 0.015, 0.03, 0.12, 4, 1, and 2; and for *A. terreus* SC, 0.06, 0.015, 0.12, 1, 0.5, and 1. E1210 was very active against AMB-R strains of *A. terreus* SC (MEC range, 0.015 to 0.06 μg/ml) and itraconazole-R strains of *A. fumigatus* SC (MEC range, 0.03 to 0.12 μg/ml), *A. niger* SC (MEC, 0.008 μg/ml), and *A. terreus* SC (MEC, 0.015 μg/ml). In conclusion, E1210 was a very potent and broad-spectrum antifungal agent regardless of *in vitro* method applied, with excellent activity against AMB-R and itraconazole-R strains of *Aspergillus* spp.

The introduction of mold-active antifungal agents of the triazole (itraconazole, posaconazole, voriconazole) and echinocandin (anidulafungin, caspofungin, micafungin) classes has dramatically increased the options for prophylaxis and empirical or directed therapy for the prevention and treatment of invasive aspergillosis (IA) (2, 4, 5, 11, 25, 31). Although resistance to these agents among clinical isolates of *Aspergillus* has been considered to be uncommon, both increased resistance and breakthrough infections have been reported increasingly among patients with long-term exposure to these agents (1, 3, 14, 16, 19, 20, 26, 30). These observations have prompted a call for an expanded search for new antifungal agents with novel mechanisms of action, as well as an expanded role for antifungal susceptibility testing of *Aspergillus* spp. (4, 8, 11, 12, 15, 24, 25, 30).

E1210 (Eisai Co., Tokyo, Japan) is a novel first-in-class broad-spectrum antifungal agent that inhibits the inositol acylation step in fungal glycophosphatidylinositol (GPI) biosynthesis, resulting in defects in various steps in cell wall biosynthesis with accompanying inhibition of cell wall growth, hyphal elongation, and attachment of fungal cells to biological substrates (27, 28, 32). Differences in the inositol acylation of GPI in fungi and in human cells suggest that this may prove to be a good target for drugs directed against fungi (yeasts and molds) that do not impair inositol acylation in human cells (27, 28).

Preliminary data obtained using the Clinical and Laboratory Standards Institute (CLSI) broth microdilution (BMD) method (6) have demonstrated the excellent E1210 spectrum and potency against both yeasts and molds, including *Aspergillus* spp. (18). With regard to *Aspergillus* spp., these studies are limited by the small numbers of isolates tested, the lack of antifungal comparators, and the lack of a comparison of the E1210 potency as determined by the reference BMD methods of the CLSI and the European Committee for Antimicrobial Susceptibility Testing (EUCAST) (6, 9, 10). Currently there are two independent reference standards for BMD susceptibility testing of antifungal agents against *Aspergillus* spp. (6, 9, 10). These two methods are similar in that both use BMD, RPMI 1640 broth, incubation at 35 to 37°C for 48 h, and a complete (100%) inhibition (triazoles) or minimum effective concentration (MEC; lowest concentration of drug that produces stubby aberrant hyphal growth) MIC/MEC endpoint. They differ,

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TABLE 1. *In vitro* susceptibilities of *Aspergillus* spp. to E1210 and comparators as determined by CLSI and EUCAST broth microdilution methods

Species (no. tested)	Antifungal agent	Test method	No. of isolates at indicated MIC/MEC (μg/ml)											% EA[a]
			≤0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	≥8	
A. flavus SC[b] (20)	E1210	EUCAST		4	14	2								100.0
		CLSI	1	14	4	1								
	Anidulafungin	EUCAST	19	1										100.0
		CLSI	20											
	Caspofungin	EUCAST				4	16							100.0
		CLSI			1	15	4							
	Itraconazole	EUCAST						7	13					100.0
		CLSI							7	13				
	Posaconazole	EUCAST					2	17	1					100.0
		CLSI						1	16	3				
	Voriconazole	EUCAST							1	12	7			100.0
		CLSI							4	14	1	1		
A. fumigatus SC (22)	E1210	EUCAST		5	13	4								100.0
		CLSI	1	1	16	4								
	Anidulafungin	EUCAST	17	5										100.0
		CLSI	14	8										
	Caspofungin	EUCAST				3	15	4						100.0
		CLSI				4	17	1						
	Itraconazole	EUCAST						2	13				7	100.0
		CLSI							1	14		1	6	
	Posaconazole	EUCAST					13	1	2	5	1			100.0
		CLSI						8	7	6	1			
	Voriconazole	EUCAST						1	12	4	4	1		100.0
		CLSI						4	10	3	4		1	
A. niger SC (13)	E1210	EUCAST		5	6	2								100.0
		CLSI	8	5										
	Anidulafungin	EUCAST	13											100.0
		CLSI	12		1									
	Caspofungin	EUCAST					13							100.0
		CLSI				4	9							
	Itraconazole	EUCAST						1	3	6	3			100.0
		CLSI								6	5	2		
	Posaconazole	EUCAST					2	4	7					100.0
		CLSI						1	8	4				
	Voriconazole	EUCAST							4	5	4			100.0
		CLSI							4	5	4			
A. terreus SC (23)	E1210	EUCAST		2	17	4								100.0
		CLSI	1	11	8	3								
	Anidulafungin	EUCAST	20	3										100.0
		CLSI	15	8										
	Caspofungin	EUCAST				1	18	4						100.0
		CLSI					21	2						
	Itraconazole	EUCAST						15	7		1			100.0
		CLSI						1	15	6		1		
	Posaconazole	EUCAST				1	14	7		1				91.3
		CLSI						7	14	1	1			
	Voriconazole	EUCAST							11	11			1	100.0
		CLSI						4	13	5			1	

[a] EA, essential agreement.
[b] SC, species complex.

however, in inoculum concentration (0.4×10^4 to 5×10^4 CFU/ml [CLSI] versus 2×10^5 to 5×10^5 CFU/ml [EUCAST]), glucose content of the medium (0.2% [CLSI] versus 2.0% [EUCAST]), and rounded (CLSI) versus flat-bottom (EUCAST) microdilution wells (6, 9, 10). Although we have shown previously that both methods provide concordant results when testing *Aspergillus* spp. against itraconazole, posaconazole, and voriconazole (21), similar data are not available for E1210 or the echinocandins anidulafungin and caspofungin. Given the important role that both methods currently play in antifungal resistance surveillance and regulatory evaluations of new agents, it is important to demonstrate the comparability of their results in the preclinical development of new antifungal agents. In the present study, we have used a collection of *Aspergillus* spp. isolates selected to represent both polyene- and triazole-resistant strains to examine the activity of E1210 and comparator agents as determined by both CLSI and EUCAST reference BMD methods.

MATERIALS AND METHODS

Organisms. A total of 78 clinical isolates of *Aspergillus* spp. obtained from centers participating in the 2008-2009 ARTEMIS and SENTRY Antimicrobial Surveillance Programs (22, 23) were tested against E1210, amphotericin B, anidulafungin, caspofungin, itraconazole, posaconazole, and voriconazole. The collection included 20 isolates of *A. flavus* species complex (SC), 22 of *A. fumigatus* SC, 13 of *A. niger* SC, and of 23 *A. terreus* SC. The phenotypically resistant isolates (as determined by CLSI methods) included 15 amphotericin B-resistant (MIC, ≥2 μg/ml) isolates of *A. terreus* SC and 10 itraconazole-resistant (MIC, ≥4 μg/ml) isolates of *A. fumigatus* SC (7 isolates), *A. niger* SC (2 isolates), and *A. terreus* SC (1 isolate). The isolates were obtained from a variety of sources, including sputum, bronchoscopy, and tissue biopsy specimens, and represented individual infection episodes. The isolates were collected at individual study sites and sent to JMI Laboratories (North Liberty, IA) for identification and susceptibility testing as described previously (17, 23). All isolates were identified by standard microscopic morphology (29) and were stored as spore suspensions in sterile distilled water at room temperature until used in the study. Before testing, each isolate was subcultured at least twice on potato dextrose agar (Remel, Lenexa, KS) to ensure viability and purity.

Antifungal agents. Reference powders of E1210, amphotericin B, anidulafungin, caspofungin, itraconazole, posaconazole, and voriconazole were obtained from their respective manufacturers. Stock solutions were prepared in dimethyl sulfoxide, and serial doubling dilutions in RPMI 1640 medium (Sigma, St. Louis, MO) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) buffer (Sigma) were made.

Antifungal susceptibility testing. All isolates were tested for *in vitro* susceptibility to E1210 and comparators using the CLSI and EUCAST BMD methods (6, 9, 10). CLSI BMD testing was performed as outlined in document M38-A2 (6) by using RPMI 1640 medium with 0.2% glucose, round-bottom microdilution trays, inocula of 0.4×10^4 to 5×10^4 CFU/ml, and incubation at 35°C for 48 h. MIC values for the triazoles were determined visually as the lowest concentration of drug that caused complete inhibition of growth (first clear well) relative to that of the growth control. MEC values for E1210, anidulafungin, and caspofungin were determined as described previously (18, 22) and by the CLSI (6). The MEC endpoint was chosen for E1210 due to the fact that, similar to the echinocandins, E1210 inhibits cell wall synthesis and hyphal extension of *Aspergillus* spp., resulting in aberrant hyphal growth (short, stubby, highly branched hyphae) but not complete growth inhibition. Preliminary data generated by the sponsor and in experiments at JMI Laboratories (unpublished) found that as with the echinocandins, when an MIC for E1210 is determined (complete growth inhibition), an endpoint is not achieved for *Aspergillus* spp.

EUCAST BMD testing was performed exactly as detailed by EUCAST (10) by using RPMI 1640 medium with 2.0% glucose, flat-bottom microdilution trays, inocula of 2×10^5 to 5×10^5 CFU/ml, and incubation at 35°C. MICs (triazoles) and MECs (E1210, echinocandins) were determined after 48 h as described above and by both CLSI (6) and EUCAST (10). Quality control was assured by testing the following strains recommended by CLSI and by EUCAST (7): *Candida parapsilosis* ATCC 22019, *C. krusei* ATCC 6258, and *A. flavus* ATCC 204304 (6, 9).

Analysis. The MIC/MEC results for each antifungal agent obtained with the EUCAST method (9) were compared to those of the CLSI BMD method (6). High off-scale MIC/MEC results were converted to the next highest concentration and low off-scale MIC/MEC results were left unchanged. Discrepancies of more than ±2 $\log_2$ dilution steps among MIC results were used to calculate the level of essential agreement (EA) between the two methods with a target of ≥95% EA at ≤2 doubling dilutions.

RESULTS AND DISCUSSION

Table 1 summarizes the *in vitro* susceptibilities of tested *Aspergillus* spp. to E1210 and five comparators as determined by the CLSI and EUCAST BMD methods (6, 9, 10). All *Aspergillus* spp. were inhibited by ≤0.06 μg/ml of E1210 as determined by both methods. The echinocandins (anidulafungin and caspofungin) displayed the lowest MEC_{50}/MIC_{50} values among comparator agents (MEC_{50}, ≤0.008 μg/ml for anidulafungin for all species; MEC_{50} range from 0.06 to 0.12 μg/ml for caspofungin) followed by posaconazole (MIC_{50}s of 0.5 μg/ml for all *Aspergillus* spp.).

The EA between the two reference methods was 100.0% for E1210 across all four *Aspergillus* spp. (Table 1). The MEC values generated by the EUCAST method (9, 10) tended to be 1 $\log_2$ dilution higher than those obtained by the CLSI method (6). Likewise, the overall EA between EUCAST and CLSI results was 100.0% for anidulafungin, caspofungin, itraconazole, and voriconazole but was 97.4% (76 of 78 results) for posaconazole. These results confirm the high level of concordance between the MIC results produced by both methods for the triazoles when testing *Aspergillus* spp. (21) and demonstrates for the first time that both reference methods generate comparable MEC values for E1210, anidulafungin, and caspofungin.

TABLE 2. *In vitro* activities of E1210 and comparators against amphotericin B-resistant *A. terreus* SC as determined by CLSI broth microdilution methods[a]

Isolate no.	MIC/MEC (μg/ml) for[b,c]:						
	AMB	ITR	PSC	VRC	ANF	CSF	E1210
446	4	0.5	0.5	0.5	0.008	0.12	0.015
618	4	0.5	0.25	0.25	0.008	0.12	0.03
8691	2	1	0.5	1	0.015	0.25	0.06
8693	2	1	0.5	0.5	0.015	0.25	0.06
8694	2	0.5	0.25	0.5	0.008	0.12	0.03
8695	2	1	0.5	1	0.008	0.12	0.06
8696	2	0.25	0.25	0.25	0.008	0.12	0.015
8722	2	0.5	0.5	1	0.015	0.12	0.03
8723	2	0.5	0.25	0.5	0.008	0.12	0.03
8727	2	0.5	0.5	1	0.008	0.12	0.03
8728	2	0.5	0.5	0.5	0.008	0.12	0.03
8729	2	1	0.5	1	0.008	0.12	0.03
8730	2	0.5	0.5	1	0.008	0.12	0.03
8731	2	4	2	>8	0.008	0.12	0.015
8733	2	0.5	0.5	1	0.015	0.12	0.015

[a] The MICs and minimum effective concentrations (MECs) were determined after 48 h of incubation.
[b] The MICs for AMB (amphotericin B), ITR (itraconazole), PSC (posaconazole), and VRC (voriconazole) were read at complete (100%) inhibition.
[c] The MECs for ANF (anidulafungin), CSF (caspofungin), and E1210 were read as described in CLSI document M38-A2 (6).

E1210 and the echinocandins were the most active agents against amphotericin B-resistant strains of *A. terreus* SC as determined by the CLSI method (Table 2). Anidulafungin was the most potent agent (MEC_{90}, 0.015 μg/ml) followed by E1210 (MEC_{90}, 0.06 μg/ml), caspofungin (MEC_{90}, 0.25 μg/ml), posaconazole (MIC_{90}, 0.5 μg/ml), itraconazole and voriconazole (MIC_{90}, 1 μg/ml), and amphotericin B (4 μg/ml).

A similar rank order of activity was determined with the itraconazole-resistant isolates: anidulafungin (MEC_{90}, 0.015 μg/ml) > E1210 (MEC_{90}, 0.06 μg/ml) > caspofungin (MEC_{90}, 0.12 μg/ml) > amphotericin B (MIC_{90}, 1 μg/ml) > posaconazole (MIC_{90}, 2 μg/ml) > voriconazole (MIC_{90}, 8 μg/ml) > itraconazole (MIC_{90}, >8 μg/ml) (Table 3). Whereas cross-resistance was apparent between itraconazole, posaconazole, and voriconazole, the distinctly different mechanisms of action represented by E1210, amphotericin B, and the echinocandins result in activity of these antifungal agents against this subset of *Aspergillus* species isolates that are of great clinical concern worldwide (3, 12, 13, 15, 30).

There were several important findings derived from this investigation. First, we have documented the excellent *in vitro* potency for E1210 tested against contemporary isolates of *Aspergillus* spp., including polyene- and triazole-resistant

TABLE 3. *In vitro* activities of E1210 and comparators against itraconazole-resistant *Aspergillus* spp. as determined by CLSI broth microdilution methods

Species	Isolate no.	MIC/MEC (μg/ml) for[a]:						
		ITR	PSC	VRC	AMB	ANF	CSF	E1210
A. fumigatus SC	8686	>8	1	2	1	0.015	0.12	0.12
	8687	>8	1	2	1	0.015	0.12	0.06
	8688	>8	1	1	1	0.008	0.12	0.03
	8689	>8	1	2	1	0.015	0.12	0.06
	8690	>8	2	8	1	0.008	0.12	0.06
	8737	4	1	2	1	0.015	0.12	0.03
	8737	>8	1	0.25	1	0.008	0.12	0.03
A. niger SC	8698	4	1	2	1	0.008	0.12	0.008
	301	4	1	2	0.5	0.008	0.12	0.008
A. terreus SC	8731	4	2	>8	2	0.008	0.12	0.015

[a] Definitions for MIC and MEC and all abbreviations are as described in Table 2. Resistance to itraconazole is defined as an MIC of ≥4 μg/ml (8, 29, 30).

strains. Second, we have confirmed previous observations (21) regarding the comparability of the CLSI and EUCAST BMD methods (6, 9, 10) when testing the triazoles against *Aspergillus* spp. Finally, we document the high level of agreement between the CLSI and EUCAST methods for testing E1210 and the echinocandins against *Aspergillus* spp. In conclusion, E1210 is a potent, novel antifungal agent with impressive activity against both wild-type and antifungal-resistant strains of *Aspergillus* species. Further development of this new antifungal agent appears warranted.

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