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In Vitro Activity of E1210 and In Vivo Activity of E1211, a Water-Soluble Prodrug of E1210, in Combination with Other Antifungals

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Abstract

Background: E1210 is a new broad-spectrum antifungal that inhibits the biosynthesis of fungal glycosylphosphatidylinositol (GPI). E1210 shows potent inhibitory effects on fungal cell growth since GPI-anchored proteins are important components of fungal cell walls. In this study, we examined the in vitro activity of E1210 and in vivo activity of E1211, a water-soluble prodrug of E1210, in combination with other antifungals.

Methods: The in vitro combination effects of E1210 plus other antifungals (fluconazole, voriconazole, micafungin, caspofungin, and amphotericin B) were assessed against *Candida* spp. and *Aspergillus* spp. using a checkerboard method. MICs were determined using a broth microdilution method. Fractional inhibitory concentration (FIC) indices were calculated. The in vivo combination effects of E1211 plus micafungin or caspofungin were further assessed in a murine model of pulmonary aspergillosis.

Results: E1210 plus fluconazole, voriconazole, or amphotericin B demonstrated synergistic in vitro activity against some strains of *Candida* spp. and E1210 plus micafungin or caspofungin showed synergistic in vitro activity against most strains of *Aspergillus* spp. tested. No antagonism was observed for the combination of E1210 and any other antifungal tested. In the murine model of *Aspergillus flavus* pulmonary aspergillosis, the efficacy of E1211 was enhanced when used in combination with micafungin or caspofungin

Conclusion: These results suggest the potential clinical usefulness of E1210 and E1211, as a novel, first-in-class antifungal drug that may effectively be used in combination with other antifungals. E1210 and E1211 demonstrated synergy in combination with other antifungals tested both in vitro and in vivo against the two most common major pathogenic fungi, *Candida* spp. and *Aspergillus* spp.

In vitro interaction activity of the combination of E1210 with other antifungals

Organism (no. of strains)	Combination compound	% of strains showing the following effects in combination with E1210[#]		
		Synergy	Indifference	Antagonism
Candida spp. (40)	Fluconazole	28	72	0
	Voriconazole	40	60	0
	Amphotericin B	10	90	0
Aspergillus spp. (22)	Micafungin	100	0	0
	Caspofungin	77	23	0

[#] Synergy, FIC index of ≤0.5; indifference, FIC index of >0.5 but ≤4; antagonism, FIC index of >4.

Introduction

Fungal infections, such as disseminated candidiasis and invasive aspergillosis, are serious threats to immunocompromised patients. Treatment outcomes could potentially be improved through the use of new antifungal agents or combination antifungal therapies. Combination therapy using antifungal agents with differing mechanisms of action might provide synergistic activity against serious fungal pathogens. E1210 is a new broad-spectrum antifungal agent (1-4) with a novel mode of action, the inhibition of glycosylphosphatidylinositol biosynthesis (5). Next we have developed E1211 (Fig. 1), a water-soluble prodrug of E1210, for IV administration since E1210 has poor solubility in water (6, 7). The differing mechanisms of action of E1210 versus other antifungal agents suggest that they might be complementary or synergistic when used in combination.

Some experimental model designs have previously been established to assess interactive combination effects. One of the best known methods for assessing interactive in vitro combination effects is the checkerboard method, in which a two-dimensional array utilizing serial concentrations of the test compounds is used as the basis for calculating a fractional inhibitory concentration (FIC) index, in order to determine whether paired combinations of the two agents can exert inhibitory effects that are greater or lesser than the sum of their individual effects (that is, that are synergistic or antagonistic, respectively). In the current study, the in vitro interactive combination effects of E1210 and other antifungal agents were assessed against *Candida* and *Aspergillus* spp. via the checkerboard method. Furthermore, we assessed the interactive in vivo combination effects by studying E1211 in combination with micafungin or caspofungin in a murine pulmonary aspergillosis model.

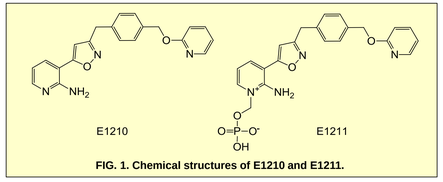

FIG. 1. Chemical structures of E1210 and E1211.

Methods

Antifungals. E1210 and E1211 were synthesized by Eisai Co. (Tokyo, Japan). Fluconazole and voriconazole were extracted from fluconazole tablets and voriconazole tablets (Pfizer Japan Inc., Tokyo, Japan), respectively. Micafungin sodium (Astellas Pharma Inc., Tokyo, Japan) and caspofungin acetate (Merck & Co., Whitehouse Station, NJ) were obtained as lyophilized powder in vials. Amphotericin B was purchased from Sigma-Aldrich Co. (St. Louis, MO). For in vitro studies, all compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted with MOPS-buffered RPMI 1640 (pH 7.0) to yield a DMSO concentration of 1%. For in vivo studies, E1211 was dissolved in 30 mM NaOH at a concentration of 10 mg/ml as E1210 and then diluted with 5% glucose to concentrations of 0.25, 0.5, and 1 mg/ml. Micafungin and caspofungin solutions were prepared according to each manufacturer's instructions.

Fungal Strains. The *C. albicans* (20 strains), *C. tropicalis* (10 strains), *C. glabrata* (10 strains), *A. fumigatus* (18 strains), and *A. flavus* (four strains) used in this study were clinical isolates provided from the Medical Mycology Research Center, Chiba University (Chiba, Japan) and the Life Science Research Center, Gifu University (Gifu, Japan).

Animals. Specific-pathogen-free female DBA/2N mice (age, 7 weeks; weight, approximately 19 g; Charles River Japan Inc., Kanagawa, Japan) were used. They were housed in cages of 5 to 10 animals per group and had access to food and water ad libitum. All procedures were performed in an animal facility accredited by the Center for Accreditation of Laboratory Animal Care and Use by the Japan Health Sciences Foundation. All protocols were approved by the Institutional Animal Care and Use Committee and carried out according to Eisai animal experimentation regulations.

MIC Determinations. For the checkerboard assay, the broth microdilution method was conducted in accordance with the guidelines presented in the Clinical and Laboratory Standards Institute (CLSI) documents M27-A3 (8) and M38-A2 (9). The compounds were diluted serially twofold. The *Candida* strains were subcultured in Sabouraud dextrose broth (Becton, Dickinson and Company, Sparks, MD, USA) at 35°C for 2 days. The *Aspergillus* strains were subcultured on potato dextrose agar (PDA; Eiken Chemical Co., Tokyo, Japan) and incubated at 35°C for 1 week. The resultant conidia were scraped from the PDA surfaces and suspended in sterile physiological saline containing 0.05% Tween 80. The cell suspensions were then diluted with MOPS-buffered RPMI 1640 medium (pH 7.0). The final inoculum size was 1×10^3 cells/ml for *Candida* strains and 1×10^4 cells/ml for *Aspergillus* strains. The plates were incubated at 35°C for 46–48 h.

The MICs in this study were defined as the lowest concentration of the compound tested alone or in combination at which a prominent reduction in growth was observed after incubation, as the MIC definitions of the compounds tested vary in terms of the incubation times and the extent of growth inhibition (as specified in the CLSI guidelines. For the *Candida* spp., the MICs were defined as the lowest concentration at which a 50% reduction in turbidity relative to the compound-free control's growth was observed. For the *Aspergillus* spp., the growth reduction was graded visually using a numerical score, ranging from 0 to 4, according to the following scale: 0 = optically clear; 1 = slightly hazy (approximately 25% of the growth of the compound-free control); 2 = a prominent reduction in turbidity (approximately 50% of the growth of the compound-free control or the presence of small, rounded and compact hyphal forms as compared to the hyphal growth seen in the growth of the compound-free control); 3 = a slight reduction in turbidity (approximately 75% of the growth of the compound-free control); and 4 = no reduction in turbidity. The MICs were then defined as the lowest concentration at which a score of 2 was observed.

To evaluate the interaction effects of the antifungal agent combinations, the FIC was calculated for each compound in each combination. The following formulae were used to calculate the FIC index (10):

FIC of A = MIC of A in the combination/MIC of A alone

FIC of B = MIC of B in the combination/MIC of B alone

FIC index = FIC of A + FIC of B

For calculation purpose, off-scale MICs were converted to the next higher dilution when MICs were above the maximum concentration prepared and they were converted to the minimum concentration prepared when MICs were the minimum concentration prepared or lower. The compound interaction effects were classified as synergy, indifference, and antagonism on the basis of the FIC index. Synergy was defined as an FIC index of ≤0.5. Indifference was defined as an FIC index of >0.5 but ≤4. Antagonism was defined as an FIC index of >4.

Pulmonary aspergillosis model. DBA/2N mice were immunosuppressed with subcutaneously administered 200 mg/kg of 5-fluorouracil given 5 days prior to infection. The mice were also administered 0.1 mg/ml ciprofloxacin orally via their drinking water, from 3 days prior to infection until 7 days after infection. *A. flavus* IFM50915 was cultured on a PDA plate and incubated at 35°C for 1 week. The conidia from the surface of the agar plate were suspended in sterile normal saline containing 0.05% Tween 80, and the cell number was counted using a hemocytometer. The final inoculum was adjusted to the required concentration with sterile normal saline containing 0.05% Tween 80. The mice were anesthetized with 0.1 ml ketamine hydrochloride (4.17 mg/ml) intravenously. Infection was induced in the neutropenic mice by the intranasal inoculation of 0.05 ml of an *A. flavus* conidia suspension ($2\text{-}3\times10^4$ conidia/mouse). Antifungal therapy was initiated two hours after infection and was continued for four consecutive days (days 0-3). E1211 was administered intraperitoneally twice daily, and micafungin or caspofungin was administered intraperitoneally once daily. The survival rate and survival duartion were determined over 14 days.

Results

In Vitro Combination Study

***Aspergillus* spp.**

Against *A. fumigatus* (18 strains) and *A. flavus* (four strains), combinations of E1210 with echinocandins demonstrated a high incidence of synergy (Table 1). No antagonism was observed for the combination of E1210 and any of the other antifungal agents.

TABLE 1. In vitro interactions between combinations of E1210 and other antifungals against *Aspergillus* spp.

Organism (no. of strains)	Combination compound	% of strains showing the following effects in combination with E1210		
		Synergy	Indifference	Antagonism
A. fumigatus (18)	Voriconazole	6	94	0
	Micafungin	100	0	0
	Caspofungin	78	22	0
	Amphotericin B	0	100	0
A. flavus (4)	Voriconazole	0	100	0
	Micafungin	100	0	0
	Caspofungin	75	25	0
	Amphotericin B	0	100	0

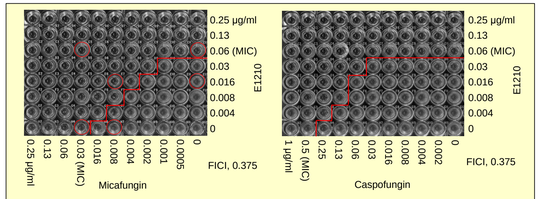

FIG. 2. Microtiter plates inoculated with *A. fumigatus* IFM51747 in the presence of E1210 , micafungin (left panel) or caspofungin (right panel) alone or in combination.

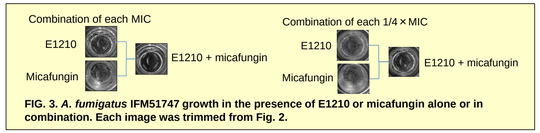

FIG. 3. *A. fumigatus* IFM51747 growth in the presence of E1210 or micafungin alone or in combination. Each image was trimmed from Fig. 2.

In Vivo Combination Study

Since the in vitro combination of E1210 plus micafungin or caspofungin were synergistic against most strains of *Aspergillus* spp., we assessed the in vivo combination of E1211 plus micafungin or caspofungin in a murine pulmonary aspergillosis model. When E1211 was administered with micafungin or caspofungin, survival rates of infected mice increased compared with those treated with each compound alone (Figs. 4 and 5).

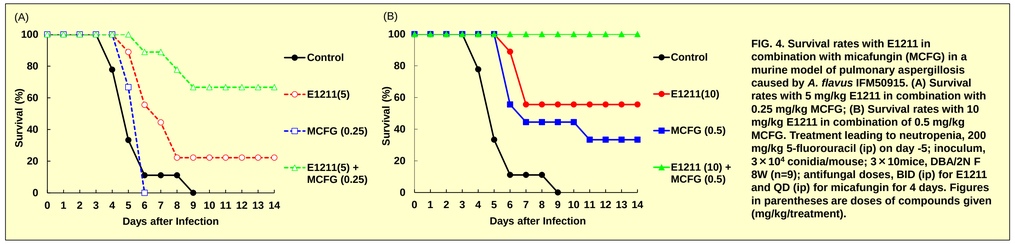

FIG. 4. Survival rates with E1211 in combination with micafungin (MCFG) in a murine model of pulmonary aspergillosis caused by *A. flavus* IFM50915. (A) Survival rates with 5 mg/kg E1211 in combination with 0.25 mg/kg MCFG; (B) Survival rates with 10 mg/kg E1211 in combination of 0.5 mg/kg MCFG. Treatment leading to neutropenia, 200 mg/kg 5-fluorouracil (ip) on day -5; inoculum, 3×10^4 conidia/mouse; 3×10mice, DBA/2N F 8W (n=9); antifungal doses, BID (ip) for E1211 and QD (ip) for micafungin for 4 days. Figures in parentheses are doses of compounds given (mg/kg/treatment).

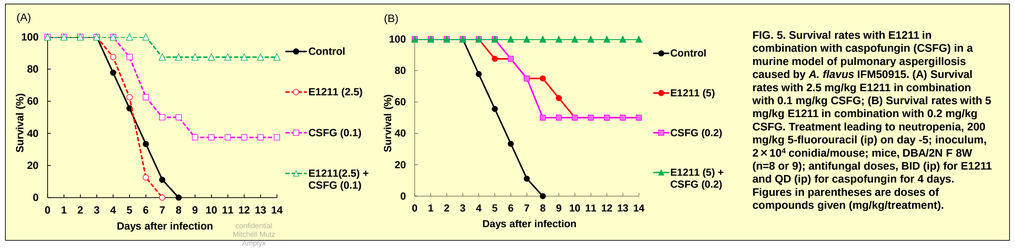

FIG. 5. Survival rates with E1211 in combination with caspofungin (CSFG) in a murine model of pulmonary aspergillosis caused by *A. flavus* IFM50915. (A) Survival rates with 2.5 mg/kg E1211 in combination with 0.1 mg/kg CSFG; (B) Survival rates with 5 mg/kg E1211 in combination with 0.2 mg/kg CSFG. Treatment leading to neutropenia, 200 mg/kg 5-fluorouracil (ip) on day -5; inoculum, 2×10^4 conidia/mouse; mice, DBA/2N F 8W (n=8 or 9); antifungal doses, BID (ip) for E1211 and QD (ip) for caspofungin for 4 days. Figures in parentheses are doses of compounds given (mg/kg/treatment).

***Candida* spp.**

The in vitro combinations of E1210 with the azoles, fluconazole and voriconazole, were synergistic against *C. tropicalis* (10 strains) (Table 2). No antagonism was observed with the combination of E1210 and any other antifungal agent tested against *C. albicans* (20 strains), *C. tropicalis*, and *C. glabrata* (10 strains).

TABLE 2. In vitro interaction effects for combinations of E1210 and other antifungals against *Candida* spp.

Organism (no. of strains)	Combination compound	% of strains showing the following effects in combination with E1210		
		Synergy	Indifference	Antagonism
C. albicans (20)	Fluconazole	10	90	0
	Voriconazole	15	85	0
	Micafungin	0	100	0
	Caspofungin	0	100	0
	Amphotericin B	5	95	0
C. tropicalis (10)	Fluconazole	90	10	0
	Voriconazole	100	0	0
	Micafungin	0	100	0
	Caspofungin	0	100	0
	Amphotericin B	0	100	0
C. glabrata (10)	Fluconazole	0	100	0
	Voriconazole	30	70	0
	Micafungin	0	100	0
	Caspofungin	0	100	0
	Amphotericin B	30	70	0

Conclusions

- No antagonism was observed in vitro with any combination of E1210 with fluconazole, voriconazole, micafungin, caspofungin or amphotericin B tested against *Candida* and *Aspergillus* spp.
- The in vitro combinations of E1210 with the azoles, fluconazole and voriconazole, were synergistic against *C. tropicalis*.
- Against *A. fumigatus* and *A. flavus*, the in vitro combinations of E1210 with the echinocandins, micafungin and caspofungin, demonstrated a high incidence of synergy. Co-administration of E1210 with echinocandins enhanced survival rates of mice in a pulmonary aspergillosis model.
- E1210 and E1211 demonstrated synergy in combination with virtually all other antifungals tested both *in vitro* and *in vivo* against the two most common major pathogenic fungi , *Candida* spp. and *Aspergillus* spp. These results suggest the potential clinical usefulness of E1210 and E1211, as a novel, first-in-class antifungal drugs that may effectively be used in combination with other antifungal drugs. Such combination therapy may also significantly reduce the risk of drug-related adverse events caused by these other antifungal agents while maintaining a high level of efficacy.
- The synergistic effects between E1210 and other antifungals seem to occur when E1210 is combined with other antifungals that show fungistatic rather than fungicidal activity, e.g., echinocandins against *Aspergillus* spp. or azoles against *Candida* spp. E1210 when combined with each of these antifungals showed greater suppression of fungal growth than either E1210 or any of the other antifungal compounds alone. However, its precise mechanism for this combined activity remains unknown.

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