50th ICAAC
F1-841

E1210, a New Broad-Spectrum Antifungal, Inhibits Glycosylphosphatidylinositol Biosynthesis in Fungi and Affects *Candida albicans* Cell Characteristics

N.-A. WATANABE*, T. HORII, M. MIYAZAKI, and K. HATA

Eisai Product Creation Systems, Eisai Co., Ltd., Tsukuba, Japan

E-mail to
n3-watanabe@hhc.eisai.co.jp

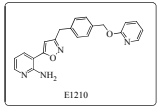
Eisai
hhc
human health care

Abstract

Background: We discovered E1210, a new broad-spectrum antifungal with a novel mechanism of action. In this study, we determined the inhibitory activity of E1210 against *Candida albicans* (Ca), *Aspergillus fumigatus* (Af), and the human Gwt1ps that catalyze the inositol acylation of glycosylphosphatidylinositol (GPI) early in the GPI biosynthesis pathway. In addition, we evaluated some effects of E1210 on *C. albicans* cells.

Methods: For Gwt1p activity, we detected PI (acyl)-GlcN produced from UDP-[^{14}C]-GlcNAc in the membrane fractions of yeasts expressing CaGWT1, AfGWT1, or PIG-W (human GWT1) instead of *Saccharomyces cerevisiae* GWT1. The Als1p level was measured by ELISA. The adherence and germ tube formation of *C. albicans* were evaluated by crystal violet staining after 1- and 4-h incubations, respectively, in MOPS-buffered RPMI 1640 medium. For biofilm formation, the amount of exopolymeric materials was measured by safranin staining after 24-h incubation in RPMI 1640 medium supplemented with 10% serum.

Results: E1210 inhibited the inositol acylation activity of CaGwt1p and AfGwt1p at IC_{50}s of 0.3 to 0.6 μM, but did not inhibit human Gwt1p activity even at 100 μM. In E1210-treated *C. albicans*, the Als1p expression on the cell surface of *C. albicans* was significantly lower compared with that of untreated cells, but the Als1p levels in the crude extract were almost the same. E1210 inhibited germ tube formation, adherence on polystyrene surfaces, and biofilm formation of *C. albicans* at concentrations above its MIC. Fluconazole inhibited neither germ tube formation nor adherence. Micafungin inhibited germ tube formation, but adversely enhanced the adherence.



Conclusion: E1210 inhibited the inositol acylation of fungus-specific GPI, which is catalyzed by Gwt1p, leading to the inhibition of GPI-anchored protein maturation. The compound not only inhibited fungal growth, but also suppressed the expression of some virulence factors of *C. albicans*.

Introduction

The incidence of serious life threatening fungal infections has increased steadily over the last two decades as a result of an increase in the susceptible immunosuppressed patient population. There is a limited choice of antifungals for the treatment of systemic infections and these are currently represented by only three classes of compounds: polyenes, azoles, and echinocandins. Resistance is becoming an increasing problem, particularly among the azole class. New antifungal drugs which show no cross-resistance to existing drugs are therefore desirable for the treatment of serious fungal infections.

Microorganisms first attach to host cell surfaces to establish infections, followed by colonization and replication on these host surfaces, and then by penetration across mucosal barriers or by systemic dissemination through the vascular route. Most fungal ligands responsible for the adhesion step are derived from glycosylphosphatidylinositol (GPI)-anchored proteins. Inhibition of GPI-anchored protein maturation is a promising therapeutic target for the treatment of fungal infections (Fig. 1).

In the course of screening compounds that inhibited cell wall assembly of GPI-anchored proteins, we discovered the GWT1 gene which encodes the inositol acyltransferase found early in the GPI biosynthesis pathway (Fig. 2) and an inhibitor, 1-(4-butylbenzyl)isoquinoline (1, 2). In our efforts to improve the efficacy of this proto-type inhibitor, we developed E1210. This compound showed potent in vitro antifungal activity against a broad range of pathogenic fungi, including *Candida* spp., *Aspergillus* spp., and other moulds such as *Fusarium* and *Scedosporium* spp. (3), and showed high therapeutic efficacy in some in vivo fungal infection models (4), in vivo post-antifungal effect (5), and good oral absorption in animals (6).

In this study, we investigated the effects of E1210 on the inositol acylation of GPI, on the expression of Als1 protein, a GPI-anchored protein, on the cell surface of *Candida albicans*, and on some virulence factors: adherence, germ tube formation, and biofilm formation, of *C. albicans*.

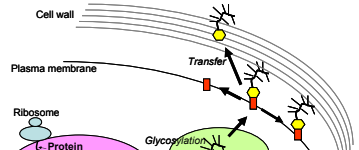


FIG. 1. Schematic maturation process of GPI-anchored proteins.

Methods

Fungal strains

The *Saccharomyces cerevisiae* GWT1 disruptant expressing CaGWT1, AfGWT1 or PIG-W (human GWT1) protein was genetically engineered in Eisai and was used as enzyme sources for early GPI biosynthesis. *C. albicans* IFM49971 was obtained from the Medical Mycology Research Center, Chiba University (Chiba, Japan).

In Vitro Assays for Inositol Acylation in GPI Biosynthesis

Yeast cells grown in SD (−Ura) medium were ruptured by glass beads in 50 mM Tris-HCl buffer (pH 7.5) containing 2 mM $MgCl_2$ (TM buffer). The membrane fractions were obtained as pellets by differential centrifugation. Membrane fractions (70-μg protein) were incubated in TM buffer containing 2 mM $MnCl_2$, 10 μM nikkomycin Z, 21 μg/ml tunicamycin, 0.5 mM dithiothreitol, and 0.925 kBq of UDP-[^{14}C]-GlcNAc for 20 min at 30°C. Then, an excess of cold UDP-GlcNAc and compounds was added and further incubated for 20 min. Palmitoyl-CoA (0.1 mM) was added as an acyl donor and incubation was continued for 2 h (2). The reaction was stopped by adding $CHCl_3$/CH_3OH (1:1), and the supernatant was then separated. The pellet was re-extracted by $CHCl_3$/CH_3OH/H_2O (10:10:3). The lipid extracts were pooled, desalted by n-butanol extraction, and then dried. The lipids were separated on TLC plates with $CHCl_3$/CH_3OH/H_2O (65:25:4). Labeled products were detected by autoradiography and analyzed with a BAS2500 imaging analyzer.

Als1 Protein Levels on the Cell Surface of *C. albicans*

To easily estimate the amount of Als1 protein expressed on the cell surface, the compound-treated cells were retained on the filters, and the relative Als1 protein levels on the surface of intact cells were measured with an ELISA. *C. albicans* IFM49971 was grown in Sabouraud dextrose broth for 3 days at 25°C without shaking, to obtain a saturated culture containing synchronized cells (7). Cells were then resuspended in MOPS-buffered RPMI 1640 medium (5×10^6 cells/ml). Since Fu *et al.* (8) showed that the Als1 protein expression on the surface of *C. albicans* cells was induced after 1 h of incubation in RPMI 1640 medium, *C. albicans* cells were incubated for 1 h at 35°C in RPMI 1640 medium on a flat-plate (MultiScreen HTS 96-well filtration plate; Millipore Corp.). After incubation, the medium was removed by using a vacuum manifold. The remaining *C. albicans* cells on the filter were washed with PBS. For the ELISA, anti-Als1 rabbit antibody was used as the primary antibody and anti-rabbit IgG and HRP-linked antibody was used as the secondary antibody. The amount of Als1 protein was estimated based on the peroxidase activity, which was assayed on the filter plate by adding 0.1 ml SureBlue Reserve TMB microwell peroxidase substrate. The reaction mixture was filtered and the absorbance of filtrates at 450 nm was measured.

Adherence and Germ Tube Formation in *C. albicans*

C. albicans cells were diluted to concentrations of 5×10^6 and 1×10^6 cells/ml for the adherence assay and the germ tube formation assay, respectively. Cells were incubated at 35°C in RPMI 1640 medium that contained antifungals. The amounts of *C. albicans* cells adhered to the wells of 96-well polystyrene plates were determined with the crystal violet staining assay (7). The plates were incubated for 1 h and 4 h for the adherence assay and the germ tube formation assay, respectively. As the incubation for >1 h in RPMI 1640 medium induces the germ tube formation in *C. albicans* cells, it is difficult to compare the inhibitory effects of compounds on *C. albicans* adherence with and without the potential to inhibit germ tube formation in *C. albicans* cells for >1-h incubation. Thus, to evaluate the inhibitory effects of compounds on the adherence of *C. albicans* cells, they were incubated for 1 h at which time point no distinct cell elongation was apparent (Figure 6, B). After incubation, the plates were processed by the procedures of Brayman and Wilks (7). The *C. albicans* cells attached to the wells were stained for 20 min at room temperature with 0.1 ml of 0.02% crystal violet. They were washed three times with water, once with 0.25% SDS and twice again with water. After the plates were dried, 0.2 ml isopropanol containing 0.04 N HCl and 0.05 ml of 0.25% SDS were added to the wells. The absorbance at 590 nm was measured.

Biofilm Formation in *C. albicans*

The 96-well plates were pre-treated with 0.1 ml FBS at 35°C for 24 h, and washed twice with PBS. Then, *C. albicans* cell suspension (1×10^6 cells/ml) in RPMI 1640 medium supplemented with 10% FBS was added. The plates were incubated at 35°C for 1 h. Planktonic cells were removed, and then RMPI 1640 medium that contained the antifungals was added. After incubation at 35°C for 24 h, the amount of exopolymeric materials and the metabolic activity of the cultures were measured by safranin staining (9) and the XTT-reduction assay (10), respectively. Safranin is capable of staining the exopolymeric material structure; therefore, biofilm formation was evaluated by determining the biofilm density using safranin staining. The absorbance at 492 nm was measured for both assays.

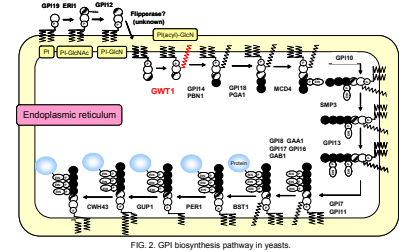


FIG. 2. GPI biosynthesis pathway in yeasts.

Results

In Vitro Inositol Acylation of GPI

E1210 inhibited inositol acylation in a concentration-dependent manner at an early step in the GPI biosynthesis pathway of *C. albicans* and *A. fumigatus* (IC_{50}s, 0.27 and 0.60 μM, respectively) but not of humans (IC_{50}, >100 μM) (Fig. 3). Fluconazole, amphotericin B, and micafungin had little or no effect (IC_{50}s, >100 μM).

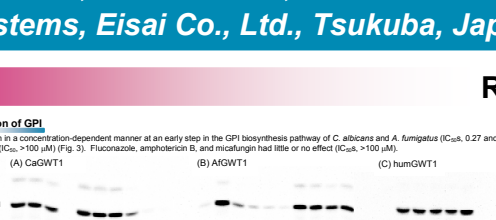


FIG. 3. Autoradiograph of TLC separation of GPI intermediates produced from UDP-[^{14}C]-GlcNAc in the presence of E1210.
Abbreviations: FLCZ, fluconazole; AMPH, amphotericin B; MCFG, micafungin.

Effect of E1210 on *C. albicans* Adherence and Germ Tube Formation

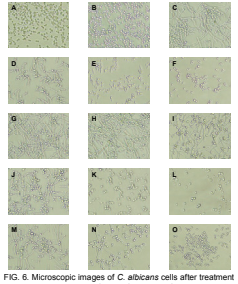


FIG. 6. Microscopic images of *C. albicans* cells after treatment with E1210 and other antifungals for 4 h. (A) Control at 0 h. (B) Control after 1 h incubation. (C) Control after 4 h incubation. (D–F) E1210 treatment at 0.004, 0.008, and 0.5 μg/ml, respectively. (G–I) Fluconazole treatment at 0.06, 0.13, and 8 μg/ml, respectively. (J–L) Micafungin treatment at 0.008, 0.016, and 0.5 μg/ml, respectively. (M–O) Amphotericin B treatment at 0.13, 0.25, and 8 μg/ml, respectively.

E1210, micafungin, and amphotericin B showed dose-dependent inhibition of the yeast-to-hyphal transition, and showed marked inhibition of germ tube formation at each MIC (Fig. 6; E, K and N, respectively), whereas germ tube formation was observed even at a fluconazole concentration of 8 μg/ml (Fig. 6; I).

C. albicans cells not incubated in RPMI 1640 medium (0-h incubation) showed little adherence to the polystyrene surface (2.9% of control cells grown in RPMI 1640 medium for 1 h). E1210 inhibited the adherence of *C. albicans* cells to the polystyrene surface with an IC_{50} value of 0.0039 μg/ml, which was lower than its MIC (Fig. 7). Amphotericin B also inhibited *C. albicans* adherence, but its IC_{50} was 1.2 μg/ml, which was about fivefold higher than its MIC. Fluconazole showed little inhibition of *C. albicans* adherence within the range of concentrations tested. By contrast, micafungin enhanced *C. albicans* adherence about twofold at concentrations above its MIC. E1210, micafungin, and amphotericin B inhibited germ tube formation by *C. albicans* with IC_{50} values of 0.0071, 0.015 and 0.24 μg/ml, respectively, which were close to their MICs (Fig. 8). No inhibition of germ tube formation was seen with fluconazole at concentrations of ≤8 μg/ml.

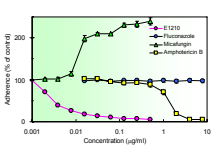


FIG. 7. Effects of E1210 and other antifungals on the adherence of *C. albicans* cells to a polystyrene surface.

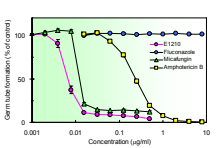


FIG. 8. Effects of E1210 and other antifungals on germ tube formation in *C. albicans* cells.

Als1p Expression Levels in *C. albicans*

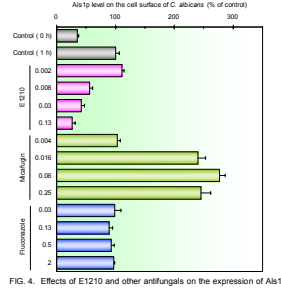


FIG. 4. Effects of E1210 and other antifungals on the expression of Als1 protein on the surface of *C. albicans* cells.

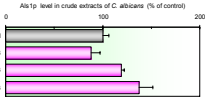


FIG. 5. Effects of E1210 on the Als1 protein level in the crude extracts of *C. albicans* cells.

Als1 protein expression on the surface of *C. albicans* cells grown for 1 h in RPMI 1640 medium was induced as described by Fu *et al.* (8). E1210 suppressed this induction in a concentration-dependent manner (Figure 5). E1210 suppressed Als1 protein expression on the cell surface by about 50%, 60%, and 75% at concentrations of 0.008, 0.03 and 0.13 μg/ml, respectively. By contrast, micafungin further enhanced the Als1 protein expression two- to three-fold at concentrations above its MIC. Fluconazole showed no effects on Als1 protein expression.

Als1 protein levels in the crude extracts of *C. albicans* cells grown in RPMI 1640 medium for 1 h were also investigated. E1210 showed an increase trend in the Als1 protein levels in a dose-dependent manner, but these increases were not significant compared to the control cells (Fig. 6).

Effect of E1210 on *C. albicans* Biofilm Formation

Biofilm formation by *C. albicans* was assessed by a biofilm-density assay using safranin staining of the extracellular polymeric materials that the biofilm cells produced. E1210 inhibited biofilm formation with an IC_{50} value of 0.0044 μg/ml and almost entirely inhibited biofilm formation at a concentration of 0.008 μg/ml (Fig. 9; A). Micafungin and amphotericin B also inhibited *C. albicans* biofilm formation, and their IC_{50} values were 0.014 μg/ml and 0.085 μg/ml, respectively. Fluconazole showed some inhibition of biofilm formation with an IC_{50} value of 0.86 μg/ml, but did not completely inhibit biofilm formation even at the highest concentration tested.

The metabolic activity of the *C. albicans* cultures was assessed by the XTT-reduction assay (Fig. 9; B). E1210 did not completely inhibit the metabolic activity of cells even at a concentration of 0.25 μg/ml, at which biofilm formation was completely inhibited. In addition, enhancement of metabolic activity was observed at concentrations below its MIC. Micafungin and amphotericin B greatly reduced metabolic activity at concentrations above their MICs. Micafungin enhanced the metabolic activity of the cells by about twofold at concentrations below its MIC. By contrast, fluconazole enhanced metabolic activity at concentrations above its MIC.

TABLE 1. IC_{50} values of E1210 and other antifungals for growth, adherence, germ tube formation, and biofilm formation of *C. albicans*

Assay	Supplement	Incubation (h)	IC_{50} (μg/ml) E1210	FLCZ	MCFG	AMPH
Growth (MIC)	None	48	0.008	0.13	0.016	0.25
Adherence		1	0.0039	>8	>0.5	1.2
Germ tube formation		4	0.0071	>8	0.015	0.24
Growth (MIC)	10% FBS	48	0.016	0.25	0.06	0.25
Biofilm formation		24	0.0044	0.86	0.014	0.085
Biofilm metabolic activity		24	0.18	>8	0.061	0.25

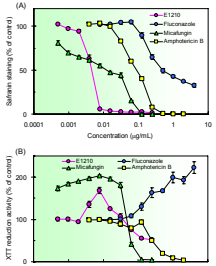


FIG. 9. Effects of E1210 and other antifungals on the density of *C. albicans* biofilms (A) and the metabolic activity of cultures (B) after incubation for 24 h.

Conclusion

E1210 inhibited inositol acylation at an early step in the GPI biosynthesis pathway of *C. albicans* and *A. fumigatus*, but not of humans. Indeed, E1210 suppressed Als1 protein expression on the *C. albicans* cell surface at concentrations above its MIC, while it showed no effects on the Als1 protein levels in the crude extracts of *C. albicans* cells. It was suggested that E1210 reduced Als1 protein levels on the cell surface, attributable to the maturation inhibition of Als1 protein based on GPI biosynthesis inhibition by E1210 but not by protein synthesis inhibition.

E1210 potently inhibited both the adherence and germ tube formation of *C. albicans* cells at concentrations above its MIC, and E1210 inhibited *C. albicans* biofilm formation at concentrations below its MIC, although the compound did not completely suppress the metabolic activity of *C. albicans* cells even at concentrations above its MIC.

Thus, GPI biosynthesis inhibition by E1210 leads to impairment in the maturation or translocation of GPI-anchored proteins, resulting in growth defects and in the reduced expression of some virulence factors, such as the adherence, germ tube formation, and biofilm formation, of *C. albicans*. The antifungal characteristics of E1210 are fascinating and further studies leading to its clinical development are warranted.

References

1. Tsukahara, K. *et al.* Mol. Microbiol. 48:1029–1042 (2003); 2. Umemura, M. *et al.* J. Biol. Chem. 278:23639–23647 (2003); 3. Miyazaki, M. *et al.* 50th ICAAC abstr. F1-840 (2010); 4. Hata, K. *et al.* 50th ICAAC, abstr. F1-842 (2010); 5. Horii, T. *et al.* 50th ICAAC abstr. F1-843 (2010); 6. Okubo, M. *et al.* 50th ICAAC abstr. F1-844 (2010); 7. Brayman, T. G., and J. W. Wilks. Antimicrob. Agents Chemother. 47:3305–3310 (2003); 8. Fu, Y. *et al.* Mol. Microbiol. 44:61–72 (2002); 9. Seidler, M. *et al.* Int. J. Antimicrob. Agents 28:568–573 (2006); 10. Ramage, G. *et al.* Antimicrob. Agents Chemother. 45:2475–2479 (2001)