Efficacy of Oral E1210, a New Broad-Spectrum Antifungal with a Novel Mechanism of Action, in Murine Models of Candidiasis, Aspergillosis, and Fusariosis[▽]

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E1210 is a first-in-class, broad-spectrum antifungal with a novel mechanism of action—inhibition of fungal glycosylphosphatidylinositol biosynthesis. In this study, the efficacies of E1210 and reference antifungals were evaluated in murine models of oropharyngeal and disseminated candidiasis, pulmonary aspergillosis, and disseminated fusariosis. Oral E1210 demonstrated dose-dependent efficacy in infections caused by *Candida* species, *Aspergillus* spp., and *Fusarium solani*. In the treatment of oropharyngeal candidiasis, E1210 and fluconazole each caused a significantly greater reduction in the number of oral CFU than the control treatment ($P < 0.05$). In the disseminated candidiasis model, mice treated with E1210, fluconazole, caspofungin, or liposomal amphotericin B showed significantly higher survival rates than the control mice ($P < 0.05$). E1210 was also highly effective in treating disseminated candidiasis caused by azole-resistant *Candida albicans* or *Candida tropicalis*. A 24-h delay in treatment onset minimally affected the efficacy outcome of E1210 in the treatment of disseminated candidiasis. In the *Aspergillus flavus* pulmonary aspergillosis model, mice treated with E1210, voriconazole, or caspofungin showed significantly higher survival rates than the control mice ($P < 0.05$). E1210 was also effective in the treatment of *Aspergillus fumigatus* pulmonary aspergillosis. In contrast to many antifungals, E1210 was also effective against disseminated fusariosis caused by *F. solani*. In conclusion, E1210 demonstrated consistent efficacy in murine models of oropharyngeal and disseminated candidiasis, pulmonary aspergillosis, and disseminated fusariosis. These data suggest that further studies to determine E1210's potential for the treatment of disseminated fungal infections are indicated.

The expanding population of immunocompromised patients receiving immunosuppressive or anticancer therapy has resulted in an increased incidence of opportunistic mycoses. Invasive fungal infections have become increasingly common among such immunocompromised and immunosuppressed patients, including solid-organ and hematopoietic stem cell transplant recipients and individuals on immunosuppressive drug regimens (3, 17, 19, 30, 32, 45). In response, the previously established guidelines for the treatment of invasive fungal infections have been recently updated (31, 43). There is still, however, a high rate of morbidity and mortality associated with invasive fungal infections (3, 17, 20, 45), because the currently available antifungal drugs, such as polyenes, azoles, and echinocandins, are limited in terms of their antifungal spectrum, side effects, and mode of action (7). In addition, there has been an increase in resistance to commonly used antifungal compounds, especially azoles, and an epidemiological shift toward more drug-resistant strains (19, 22, 33, 34, 35, 42). Thus, there is a critical need for new antifungal compounds with a novel

mechanism of action that have a broad spectrum of activity and fewer side effects, although the development of such new antifungals is just starting to be reported (18, 23, 26).

With this in mind, we have directed our research toward the development of new promising antifungals with a novel mechanism of action. We have discovered a key compound, 1-(4-butylbenzyl) isoquinoline (BIQ) that inhibits the surface expression of glycosylphosphatidylinositol (GPI)-anchored proteins in *Saccharomyces cerevisiae*, resulting in inhibition of fungal growth, and then identified the GWT1 (GPI-anchored wall protein transfer 1) gene, the target molecule of BIQ, which encodes a new acyltransferase involved in an early step in the GPI biosynthetic pathway of fungi (39, 40). We have performed exploratory syntheses of many different compounds designed to enhance the antifungal activity of BIQ and finally discovered a candidate compound likely to be effective as a new antifungal (27, 38).

E1210, 3-(3-{4-[(pyridin-2-yloxy)methyl]benzyl}isoxazol-5-yl)pyridin-2-amine (Fig. 1), is a first-in-class, new antifungal compound that was discovered by the Tsukuba Research Laboratories of Eisai Co., Ltd. (Ibaraki, Japan). It has potent broad-spectrum antifungal activity with a novel mechanism of action, i.e., inhibition of fungal GPI biosynthesis, and favorable properties as a drug candidate (13, 24, 25, 29, 44). In the present study, the efficacies of oral E1210 and reference anti-

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FIG. 1. Chemical structure of E1210.

fungus drugs, such as fluconazole (36), voriconazole (2), caspofungin (1), and liposomal amphotericin B (8), were evaluated in murine models of candidiasis, aspergillosis, and fusariosis. In addition, the initial pharmacokinetic and *in vivo* toxicological profiles of E1210 were also demonstrated.

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MATERIALS AND METHODS

Antifungals. E1210 was synthesized at Eisai Co. Ltd., Tokyo, Japan. The negative logarithm of the dissociation constant (pK_a) of E1210 was determined by capillary electrophoresis. The pK_a values for the conjugate acid of E1210 were 3 and 5.1 (I [ionic strength] = 0.05). The hydrophobicity (LC18; apparent log P determined by high-performance liquid chromatography [HPLC]) of E1210 at neutral pH was 3.48. Fluconazole and voriconazole were extracted at Eisai Co. Ltd. from commercial products obtained from Pfizer, Inc. (Tokyo, Japan). Caspofungin, amphotericin B, and liposomal amphotericin B were commercially obtained from Merck & Co., Inc. (Whitehouse Station, NJ), Bristol-Myers KK (Tokyo, Japan), and Dainippon Sumitomo Pharma Co., Ltd. (Osaka, Japan), respectively. All drugs were dissolved individually in dimethyl sulfoxide (DMSO) and then diluted with culture medium at required concentrations for *in vitro* studies. For *in vivo* efficacy studies in mice, E1210 was dissolved in 250 mmol/liter HCl at a concentration of 25 mg/ml and then diluted with vehicle to the required concentrations. For *in vivo* toxicological studies in rats, E1210 was dissolved in 400 mmol/liter HCl at a concentration of 100 mg/ml and then diluted with 400 mmol/liter HCl to the required concentrations. Voriconazole was dissolved in 1 mol/liter HCl to a concentration of 20 mg/ml and then diluted with vehicle to the required concentrations. The dosing formulations of E1210 and voriconazole were prepared and stored in a -40°C freezer until use. Other drugs were prepared on the day of use according to the manufacturer's specifications.

Organisms. In total, the following six fungal strains were used for these studies: *Candida albicans* IFM49971, *Candida albicans* IFM49738, *Candida tropicalis* E83037, *Aspergillus flavus* IFM50915, *Aspergillus fumigatus* IFM51126, and *Fusarium solani* IFM50956. These strains were provided by Chiba University (Chiba, Japan) and Gifu University (Gifu, Japan) and were stored as glycerin stock at -80°C .

Animals. Specific-pathogen-free female ICR mice (age, 5 weeks; weight, approximately 25 g; Charles River Japan Inc., Kanagawa, Japan), specific-pathogen-free female DBA/2N mice (age, 8 weeks; weight, approximately 18 g; Charles River Japan Inc., Kanagawa, Japan), or specific-pathogen-free male and female Sprague-Dawley rats (age, 8 weeks; weight, approximately 190 to 270 g; Charles River Japan Inc., Kanagawa, Japan) were used for these experiments. 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.

***In vitro* susceptibility testing.** The MICs of E1210 and the reference compounds were determined using the broth microdilution method detailed by the Clinical and Laboratory Standards Institute (CLSI) in documents M27-A3 (6) and M38-A2 (5). RPMI 1640 medium buffered to pH 7.0 with 0.165 M 3-(*N*-morpholino)-propanesulfonic acid (MOPS) was used. The results were expressed as the median MIC of each compound derived from three independent experiments.

The *Candida* spp. were subcultured in Sabouraud dextrose broth (SDB) at

35°C for 1 to 2 days. The *Aspergillus* spp. were subcultured onto potato dextrose agar (PDA) and then incubated at 35°C for 1 to 2 weeks. *F. solani* was subcultured onto PDA and incubated at 35°C for 2 to 3 days and then at 25°C for 4 to 5 days. The conidia were scraped from the PDA surface and suspended in sterile normal saline containing 0.05% Tween 80. The cell counts from the yeast cultures or conidial suspensions were determined with a hemocytometer by a modification of the CLSI method, and the cell suspensions were diluted with RPMI 1640 medium buffered to pH 7.0 with 0.165 M MOPS to obtain an inoculum size of 1.5×10^3 cells/ml for *Candida* spp. or 1.2×10^4 cells/ml for the filamentous fungi. The test organisms were cultured in medium containing E1210 (0.001 to 32 $\mu\text{g/ml}$), fluconazole (0.001 to 32 $\mu\text{g/ml}$), voriconazole (0.001 to 32 $\mu\text{g/ml}$), caspofungin (0.0005 to 16 $\mu\text{g/ml}$), amphotericin B (0.016 to 8 $\mu\text{g/ml}$), or 0.5% DMSO, and the growth inhibition induced by the test compounds was evaluated. For all the test compounds except caspofungin, the plates were incubated under the following conditions: at 35°C for 22 to 26 h for *C. albicans* and *C. tropicalis* and at 35°C for 46 to 50 h for the filamentous fungi. For caspofungin, the plates were incubated at 35°C for 22 to 26 h for all test strains.

For *Candida* spp., the reduction in growth was determined based on the changes in optical density of the medium at 660 nm (using a MTP-450 microplate reader; Corona Electric Co., Ltd., Ibaraki, Japan). The MICs of E1210, fluconazole, voriconazole, and caspofungin were defined as the lowest concentrations resulting in a prominent decrease in turbidity (that is, a 50% reduction in growth determined spectrophotometrically) relative to that in a control well by a modification of the CLSI method. The MICs of amphotericin B were defined as the lowest concentration resulting in complete growth inhibition determined visually.

For the filamentous fungi, the growth reductions were graded visually and expressed as a numerical score, ranging from 0 to 4, in accordance with the CLSI document for filamentous fungi (5). The MICs of amphotericin B and voriconazole were defined as the lowest concentration at which a score of 0 was observed, while those of E1210, fluconazole, and caspofungin were defined as the lowest concentration at which a score of 2 was observed. The MICs of caspofungin against the filamentous fungi corresponded to the minimal effective concentrations (MECs) defined in the CLSI guidelines (5).

Oropharyngeal candidiasis model. *C. albicans* was used to infect mice that were immunosuppressed with cortisone, and the number of *C. albicans* cells in the oral cavity of each mouse was measured following drug treatment (16, 37). ICR mice were immunosuppressed using 4 mg of subcutaneously administered cortisone acetate given 1 day before and 3 days after infection. The mice were also given 1 mg/ml tetracycline hydrochloride via their drinking water, starting on the day of cortisone administration and continuing throughout the experiment, in order to prevent bacterial infection. *C. albicans* IFM49971 was grown on Sabouraud dextrose agar (SDA) at 35°C for 2 days. The cells were suspended in sterile normal saline. The cells were counted with a hemocytometer and adjusted to the required density with sterile normal saline. The mice were then anesthetized with chlorpromazine hydrochloride (0.5 mg/mouse given subcutaneously). By use of a micropipette, aliquots (10 μl) of *C. albicans* IFM49971 suspension were inoculated into the oral cavities of the anesthetized mice. Then the challenge dose of 4×10^5 CFU of *C. albicans* (CFU)/mouse was given. This was followed with either E1210 orally administered twice daily (BID) or fluconazole orally administered once daily (QD) for three consecutive days starting 3 days after infection. The control group was given the equivalent volume of 5% glucose BID. The mice were anesthetized with chlorpromazine hydrochloride (0.5 mg/mouse subcutaneously) the day after the final dose of the study drug. Efficacy was assessed by determination of the number of *C. albicans* cells in the oral cavity of each mouse after study drug treatment. The oral cavity (that is, the cheek, tongue, and soft palate) was thoroughly swabbed using a fine-tipped cotton swab. After swabbing, the cotton end was placed into a test tube containing 1 ml sterile normal saline. The cells recovered were suspended in sterile normal saline by mixing them on a vortex mixer before being cultured, after serial 10-fold dilutions, on SDA plates supplemented with ampicillin (0.1 mg/ml). The SDA plates were incubated at 35°C overnight, and then the viable cells were counted as the number of CFU. The cell number was expressed in units of $\log_{10}$ CFU/swab. The lowest detectable number of cells in the oral cavity was 10 CFU ($1 \log_{10}$ CFU). The viable cell counts were performed in duplicate.

Disseminated candidiasis model. ICR mice were immunosuppressed utilizing 5-fluorouracil (5-FU) at 200 mg/kg of body weight subcutaneously administered 6 days prior to infection. These mice were also administered 0.1 mg/ml ciprofloxacin orally via their drinking water, from 2 to 3 days prior to infection to 5 to 7 days after infection, in order to prevent endogenous bacterial infections. *C. albicans* IFM49971, *C. albicans* IFM49738, and *C. tropicalis* E83037 were each cultured on an SDA plate at 35°C for 2 days. The cells from the surface of the agar plate were suspended in sterile normal saline, and the cells were counted with a hemocytometer. The final inoculum was adjusted to the required density

TABLE 1. Comparative *in vitro* antifungal susceptibilities of experimental infection strains to E1210 and reference compounds^a

Organism	Strain	MIC ($\mu\text{g/ml}$)				
		E1210	Fluconazole	Voriconazole	Caspofungin	Amphotericin B
<i>Candida albicans</i>	IFM49971	0.004	0.13	0.002	0.06	0.25
<i>Candida albicans</i>	IFM49738	0.008	>32	>32	0.06	0.5
<i>Candida tropicalis</i>	E83037	0.016	>32	0.5	0.25	0.5
<i>Aspergillus flavus</i>	IFM50915	0.03	>32	0.5	0.13	1
<i>Aspergillus fumigatus</i>	IFM51126	0.03	>32	0.25	0.13	0.25
<i>Fusarium solani</i>	IFM50956	0.06	>32	4	>16	1

^a MICs were determined with the broth microdilution method developed by the CLSI and described in documents M27-A3 (6) and M38-A2 (5). The results are the median MIC of each compound derived from three independent experiments.

using sterile normal saline. Infection was induced in the neutropenic mice by the intravenous administration of 0.2 ml of a *C. albicans* cell suspension (0.8 to 1.4×10^4 CFU/mouse or 5.3×10^4 CFU/mouse for IFM49971) or of a *C. tropicalis* cell suspension (3.0×10^5 CFU/mouse) injected into the lateral tail vein. Antifungal therapy was initiated 1 h or 24 h after infection and was continued for three consecutive days (days 0 to 2 or 1 to 3). E1210 or voriconazole was each orally administered two or three times daily, fluconazole was orally administered once daily, and caspofungin or liposomal amphotericin B was intravenously administered once daily. The control group received an equivalent volume of vehicle (5% glucose, 10 ml/kg) orally two or three times daily. In our preliminary studies, the survival curve of control mice receiving vehicle orally was similar to that of mice receiving vehicle intravenously. Therefore, we did not set up the control group to receive vehicle intravenously. The survival rate and survival period were determined over 14 days.

Pulmonary aspergillosis model. DBA/2N mice were immunosuppressed with subcutaneously administered 5-FU at 200 mg/kg, 5 to 6 days prior to infection. The mice were also administered 0.1 mg/ml ciprofloxacin orally via their drinking water, from 3 to 4 days prior to infection until 7 days after infection, in order to prevent endogenous bacterial infections. *A. flavus* IFM50915 and *A. fumigatus* IFM51126 were cultured on a PDA plate at 35°C for 7 days. The conidia from the surface of the agar plate were suspended in sterile normal saline containing 0.05% Tween 80, and the cells were counted with a hemocytometer. The final inoculum was adjusted to the required density 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 these neutropenic mice by the intranasal inoculation of 0.05 ml of an *A. flavus* conidial suspension (3.0×10^4 conidia/mouse) or 0.05 ml of an *A. fumigatus* conidial suspension (6.0×10^4 conidia/mouse). Antifungal therapy was initiated 1 h after infection and was continued for four or seven consecutive days (days 0 to 3 or 0 to 7). E1210 or voriconazole was administered orally twice daily, and caspofungin or liposomal amphotericin B was administered intraperitoneally once daily. The control group received an equivalent volume of vehicle (5% glucose, 10 ml/kg) orally twice daily. In our preliminary studies, the survival curve of control mice receiving vehicle orally was similar to that of mice receiving vehicle intraperitoneally. Therefore, we did not set up the control group to receive vehicle intraperitoneally. The survival rate and survival period were determined over 14 days.

Disseminated fusariosis model. DBA/2N mice were immunosuppressed with 200 mg/kg of subcutaneously administered 5-FU, 6 days prior to infection. The mice were also administered 0.1 mg/ml ciprofloxacin orally in their drinking water, from 3 days prior to infection until 7 days after infection, to prevent bacterial infections. *F. solani* IFM50956 was cultured on a PDA plate at 30°C for 7 days. The cells from the surface of the agar plate were suspended in sterile normal saline containing 0.05% Tween 80, and the cells were counted using a hemocytometer. The final inoculum was adjusted to the required density using sterile normal saline containing 0.05% Tween 80. Infection was induced in the neutropenic mice by the intravenous inoculation of a 0.2-ml *F. solani* cell suspension (5.0×10^5 cells/mouse) into the lateral tail vein. Antifungal therapy was initiated 1 h after infection and was continued for five consecutive days (days 0 to 4). E1210 was orally administered three times a day (TID). The control group received an equivalent volume of 5% glucose orally TID. The survival rate and survival period were determined over 14 days.

Pharmacokinetic study. E1210 was intravenously or orally administered to male ICR mice. After administration of E1210, blood samples were drawn from the vena cava of each mouse at designated time points (0.08, 0.25, 0.5, 1, 2, 4, 6, 8 h). Plasma samples were obtained by centrifuging blood. After deproteinization with methanol, the extracted sample was analyzed by liquid chromatography-tandem mass spectrometry (LC/MS/MS). The concentrations of E1210 in plasma

were determined by an internal standard method using MassLynx (Waters, Milford, MA). The pharmacokinetic parameters of E1210 were calculated by model independent analysis.

Toxicology study. E1210 was administered orally by gavage once a day for 7 days to male and female Sprague-Dawley rats (3 animals/group/gender) at doses of 100, 300, or 1,000 mg/kg. A control group received an equivalent volume (10 ml/kg) of vehicle (0.4 mol/liter hydrochloric acid). All rats found dead or moribund were necropsied as soon as they were discovered, and all surviving animals were necropsied after 7 days of administration. The following were evaluated: mortality, clinical signs, body weight, food consumption, hematology, blood chemistry, toxicokinetics, hepatic drug-metabolizing enzymes, and macroscopic and microscopic pathologies.

Statistical analysis. In the oropharyngeal candidiasis model, data are expressed as the mean $\pm$ standard error of the mean (SEM). The differences in the viable cell counts between the control group and the E1210-treated groups or the fluconazole-treated groups were evaluated using one-way analysis of variance (ANOVA), followed by the Dunnett multiple-comparison test. The dose responsiveness of E1210 or fluconazole was determined using regression analysis.

The differences between the survival curves of the vehicle-treated (control) and antifungal-treated groups over 14 days postinfection were analyzed by the log rank test with the Bonferroni adjustment. Additionally, based on the survival rate at day 14 after infection, the 50% effective dose (ED_{50}) and 95% confidence interval (CI) of each antifungal were estimated with a probit method. For the antifungals for which the 95% CIs were not calculated by a probit method, the 95% CIs of the ED_{50} s were calculated based on the exact 95% confidence limits of the survival rate at each dose. Statistical analyses were performed with SAS version 8.2 software package (SAS Institute Japan Ltd., Tokyo, Japan). A probability (*P*) value of <0.05 (two-sided) was considered statistically significant.

RESULTS

***In vitro* antifungal activity.** Table 1 shows the MICs of E1210 and reference comparator antifungal compounds against *Candida* spp., *Aspergillus* spp., and *F. solani* used in the *in vivo* efficacy studies of E1210. E1210 showed potent antifungal activity against *C. albicans* IFM49971 (MIC = 0.004 $\mu\text{g/ml}$). E1210 was more active than fluconazole, caspofungin, and amphotericin B and showed activity similar to that of voriconazole against *C. albicans* IFM49971. E1210 also had potent activity against both azole-resistant *Candida* strains, *C. albicans* IFM49738 (MIC = 0.008 $\mu\text{g/ml}$) and *C. tropicalis* E83037 (MIC = 0.016 $\mu\text{g/ml}$); E1210 was more active than all of the reference antifungals tested. E1210 showed potent antifungal activity against *A. flavus* IFM50915 (MIC = 0.03 $\mu\text{g/ml}$), *A. fumigatus* IFM51126 (MIC = 0.03 $\mu\text{g/ml}$), and *F. solani* IFM50956 (MIC = 0.06 $\mu\text{g/ml}$); E1210 was the most active compound tested against these strains of filamentous fungi.

Efficacy in the oropharyngeal candidiasis model. The efficacy of orally administered E1210 compared to that of fluconazole for the treatment of *C. albicans*-induced oral candidiasis is shown in Fig. 2. In this model, the control group showed a viable oral cavity cell count of $5.62 \pm 0.11 \log_{10}$ CFU. The oral

FIG. 2. Comparative efficacies of E1210 and fluconazole in a murine oropharyngeal candidiasis model. Mice, immunosuppressed with cortisone acetate administered subcutaneously 1 day before and 3 days after infection, were orally infected with 4×10^5 CFU of *Candida albicans*. E1210 was orally administered twice daily and fluconazole was orally administered once daily for three consecutive days starting 3 days after infection. The oral cavity of each mouse was thoroughly swabbed, and the recovered cells were cultured on SDA plates. After incubation at 35°C overnight, the viable cells were counted and expressed as the number of CFU. The viable cell counts were performed in duplicate. *, $P < 0.05$ versus control (one-way ANOVA with the Dunnett multiple-comparison test).

cavity cell counts in mice treated with E1210 (BID) doses of 2.5, 5 and 10 mg/kg were 4.97 ± 0.16 , 4.24 ± 0.31 , and 3.08 ± 0.27 log₁₀ CFU, respectively. The oral cavity cell counts in mice treated with doses of fluconazole (once daily [QD]) of 2.5, 5, and 10 mg/kg were 5.28 ± 0.18 , 3.88 ± 0.23 , and 2.75 ± 0.24 log₁₀ CFU, respectively. Mice treated with E1210 or fluconazole at doses of 5 and 10 mg/kg showed significantly better resolution of oral candidiasis than control mice. E1210 or fluconazole reduced the number of viable *C. albicans* cells in the oral cavity in a dose-dependent manner.

Efficacy in the disseminated candidiasis model. The efficacies of E1210, fluconazole, caspofungin, and liposomal amphotericin B on *C. albicans*-induced mortality in mice are shown in Fig. 3. In this model, all control mice died within 5 days. The mice treated with E1210 (BID) at 5 and 12.5 mg/kg, with fluconazole (QD) at 2 and 5 mg/kg, with caspofungin (QD) at 0.064 and 0.16 mg/kg, and with liposomal amphotericin B (QD) at 0.4 and 1 mg/kg (as amphotericin B) showed significantly higher survival rates than control mice. E1210 at doses of 0.8, 2, 5, and 12.5 mg/kg protected 0%, 11%, 56%, and 89% of the mice at day 14, respectively, and its ED₅₀ was 4.8 mg/kg (95% CI, 3.0 to 7.9 mg/kg). Fluconazole at doses of 0.32, 0.8, 2, and 5 mg/kg protected 0%, 0%, 67%, and 100% of the mice at day 14, respectively, and its ED₅₀ was 1.9 mg/kg (95% CI, 0.80 to 5.0 mg/kg). Caspofungin at doses of 0.01, 0.026, 0.064, and 0.16 mg/kg protected 0%, 11%, 100%, and 100% of the mice at day 14, respectively, and its ED₅₀ was 0.030 mg/kg (95% CI, 0.026 to 0.064 mg/kg). Liposomal amphotericin B at doses of 0.064, 0.16, 0.4, and 1 mg/kg protected 0%, 22%, 56%, and 100% of the mice at day 14, respectively, and its ED₅₀ was 0.31 mg/kg (95% CI, 0.20 to 0.49 mg/kg). The ED₅₀s of all com-

pounds tested in the disseminated candidiasis model are summarized in Table 2.

The efficacies of E1210 and voriconazole on azole-resistant *C. albicans*-induced mortality in mice are shown in Fig. 4. In this model, all of the control mice died within 5 days. The mice treated with E1210 (TID) at 2.5 mg/kg showed a significantly higher survival rate than the control mice. E1210 at doses of 0.313, 0.625, 1.25, 2.5, and 5 mg/kg protected 0%, 13%, 38%, 88%, and 100% of the mice at day 14, respectively, and its ED₅₀ was 1.3 mg/kg (95% CI, 0.93 to 2.0 mg/kg). Voriconazole (TID) at a dose of 5 mg/kg protected 25% of the mice at day 14.

In addition, E1210 was effective in the model of disseminated candidiasis caused by *C. tropicalis* (data not shown). In this model, all of the control mice died within 4 days. The mice treated with E1210 (TID) at 2.5 mg/kg showed a significantly higher survival rate than the control mice. E1210 at doses of 1.25 and 2.5 mg/kg protected 20% and 100% of the mice at day 14, respectively.

The impact of E1210 administered orally for 3 consecutive days starting 1 h or 24 h after infection on *C. albicans*-induced mortality in mice is shown in Fig. 5. In this model, all of the control mice died within 5 days. The mice treated with E1210 (TID) at 2.5 mg/kg showed a significantly higher survival rate than the control mice regardless of the time that treatment was initiated. When therapy was started 1 h after infection, E1210 at doses of 0.625, 1.25, and 2.5 mg/kg protected 0%, 50%, and 100% of the mice at day 14, respectively, and its ED₅₀ was 1.3 mg/kg (95% CI, 0.63 to 2.5 mg/kg). When therapy was started 24 h after infection, E1210 at doses of 0.625, 1.25, and 2.5 mg/kg protected 0%, 38%, and 75% of the mice at day 14, respectively, and its ED₅₀ was 1.7 mg/kg (95% CI, 1.1 to 2.8 mg/kg). The efficacy of E1210 for the treatment of disseminated candidiasis was minimally affected by a treatment delay of 24 h.

Efficacy in the pulmonary aspergillosis model. The efficacies of E1210, voriconazole, caspofungin, and liposomal amphotericin B on *A. flavus*-induced mortality in mice are shown in Fig. 6. In this model, all of the control mice died within 6 days. The mice treated with E1210 (BID) at 10 and 25 mg/kg, voriconazole (BID) at 4 and 10 mg/kg, and caspofungin (QD) at 0.4 and 1 mg/kg showed significantly higher survival rates than the control mice. E1210 at doses of 1.6, 4, 10, and 25 mg/kg protected 0%, 11%, 33%, and 100% of the mice at day 14, respectively, and its ED₅₀ was 10 mg/kg (95% CI, 6.8 to 16 mg/kg). Voriconazole at doses of 0.64, 1.6, 4, and 10 mg/kg protected 0%, 11%, 44%, and 100% of the mice at day 14, respectively, and its ED₅₀ was 3.7 mg/kg (95% CI, 2.5 to 5.9 mg/kg). Caspofungin at doses of 0.064, 0.16, 0.4, and 1 mg/kg protected 11%, 22%, 44%, and 78% of the mice at day 14, respectively, and its ED₅₀ was 0.41 mg/kg (95% CI, 0.21 to 1.3 mg/kg). Liposomal amphotericin B (QD) at a dose of 10 mg/kg did not protect the mice against mortality. The ED₅₀s of all compounds tested in the pulmonary aspergillosis model are summarized in Table 2.

E1210 was also effective in treating pulmonary aspergillosis caused by *A. fumigatus* (data not shown). In this model, all of the control mice died within 6 days. The mice treated with E1210 (TID) at 10 and 20 mg/kg showed significantly higher survival rates than the control mice. E1210 at doses of 2.5, 5,

FIG. 3. Efficacy of E1210 compared to that of reference antifungals in a murine model of disseminated candidiasis caused by *C. albicans* IFM49971. Mice ($n = 9$) were immunosuppressed with 5-FU administered subcutaneously 6 days prior to infection and then intravenously infected with 0.8×10^4 CFU of *Candida albicans*. E1210 was administered orally twice daily, fluconazole was administered orally once daily, and liposomal amphotericin B or caspofungin was administered intravenously once daily for three consecutive days starting 1 h after infection. The survival rate and survival period were determined over 14 days. (A) E1210; (B) fluconazole; (C) caspofungin; (D) liposomal amphotericin B. *, $P < 0.05$ versus control group (log rank test with Bonferroni's adjustment).

10, and 20 mg/kg protected 0%, 14%, 43%, and 100% of the mice at day 14, respectively, and its ED_{50} was 9.3 mg/kg (95% CI, 6.4 to 15 mg/kg).

Efficacy in the disseminated fusariosis model. The efficacy of oral E1210 on *F. solani*-induced mortality in mice is shown in Fig. 7. In this model, 80% of the control mice died within 6 days and 20% of the control mice survived at day 14. E1210 at doses of 5, 10, and 20 mg/kg protected 25%, 63%, and 100% of the mice at day 14, respectively. The mice treated with E1210 (TID) at 20 mg/kg showed significantly higher survival rates than the control mice.

Pharmacokinetic profile. Mean plasma concentrations of E1210 after oral and intravenous administrations to mice are shown in Fig. 8. In mice, after intravenous administration, E1210 exhibited moderate clearance and volume of distribution and the elimination half-life was 2.2 h. E1210 dosed as an

oral solution was rapidly absorbed and achieved a maximum concentration at 0.5 h after dosing. Oral bioavailability was calculated at 57.5% in mice.

Toxicological profile. At an E1210 dose of 1,000 mg/kg, mortality (1 male) and morbidity (1 male) were observed, which were caused by anorexia and gastrointestinal lesion. Adaptive hepatocellular hypertrophy resulting from liver enzyme induction was observed at 300 mg/kg and higher. No E1210-related change was observed at 100 mg/kg. E1210 was generally well tolerated when given orally to rats for 1 week at doses up to 300 mg/kg.

DISCUSSION

E1210 is a first-in-class, broad-spectrum antifungal with a novel mechanism of action—inhibition of fungal GPI biosyn-

TABLE 2. ED₅₀s of E1210 and reference compounds based on day 14 survival rates^a

Strain	Compound	Route	Frequency of doses	ED ₅₀ (mg/kg/dose)	95% CI (mg/kg/dose)
<i>Candida albicans</i> IFM47991	E1210	p.o.	BID	4.8	3.0–7.9
	Fluconazole	p.o.	QD	1.9	0.80–5.0
	Caspofungin	i.v.	QD	0.03	0.026–0.064
	Liposomal amphotericin B	i.v.	QD	0.31	0.20–0.49
<i>Aspergillus flavus</i> IFM50915	E1210	p.o.	BID	10	6.8–16
	Voriconazole	p.o.	BID	3.7	2.5–5.9
	Caspofungin	i.p.	QD	0.41	0.21–1.3
	Liposomal amphotericin B	i.p.	QD	>10	

^a The 50% effective dose (ED₅₀) and 95% confidence interval (CI) were estimated with a probit method. For the antifungals for which the 95% CIs were not calculated by a probit method, the 95% CIs of the ED₅₀s were calculated based on the exact 95% confidence limits of the survival rate at each dose. p.o., per os; i.v., intravenous; i.p., intraperitoneal.

thesis. E1210 has a broad spectrum of potent activity against major pathogenic fungi, such as *Candida* spp., *Aspergillus* spp., and other filamentous fungi which are resistant to existing antifungals (24, 25). Invasive fungal infections, including disseminated candidiasis, pulmonary aspergillosis, and disseminated fusariosis, are serious, life-threatening infections recognized increasingly more frequently in immunocompromised patients (17, 19, 28, 30, 45). Oropharyngeal candidiasis, the most commonly encountered opportunistic infection in human immunodeficiency virus-infected patients (10), is becoming increasingly more resistant to fluconazole and, soon, other azoles. We therefore developed experimental models of these fungal infections in immunosuppressed mice to evaluate the therapeutic efficacy of E1210. In our previous studies to eval-

uate the efficacy of ravuconazole, we showed that 5-fluorouracil treatment elicited a considerable reduction in the number of circulating leukocytes, especially neutrophils, in mice (41), and disseminated and pulmonary infections caused by *Candida* spp. or *Aspergillus* spp. were then readily induced in these mice (11, 12). The neutropenic disseminated or pulmonary infection model appears to more closely mimic the immunocompromised patient situation in clinical settings. Furthermore, a disseminated fusariosis model was also able to be established in these neutropenic mice, as part of this study. Oropharyngeal infection caused by *C. albicans* was able to be induced in cortisone acetate-treated mice.

In order to better evaluate the comparative efficacies of E1210 and reference antifungals, the experimental infection models were studied using various numbers of doses per day for each antifungal compound. It has been reported that the plasma half-lives of voriconazole (2), fluconazole (14), caspofungin (9), and liposomal amphotericin B (8) were 0.7 to 2.9 h,

FIG. 4. Efficacy of E1210 compared to that of voriconazole in a murine model of disseminated candidiasis caused by azole-resistant *C. albicans* IFM49738. Mice ($n = 8$) were immunosuppressed with 5-FU administered subcutaneously 6 days prior to infection and then intravenously infected with 5.3×10^4 CFU of *Candida albicans*. E1210 or voriconazole was administered orally three times daily for three consecutive days starting 1 h after infection. The survival rate and survival period were determined over 14 days. *, $P < 0.05$ versus control group (log rank test with Bonferroni's adjustment).

FIG. 5. Effect of treatment delay on the efficacy of E1210 in a murine model of disseminated candidiasis. Mice ($n = 8$) were immunosuppressed with 5-FU administered subcutaneously 6 days prior to infection and then intravenously infected with 1.4×10^4 CFU of *C. albicans* IFM49971. E1210 was administered orally three times daily for three consecutive days starting 1 h or 24 h after infection. The survival rate and survival period were determined over 14 days.

FIG. 6. Efficacies of E1210 and reference antifungals in a murine model of pulmonary aspergillosis caused by *A. flavus* IFM50915. Mice ($n = 9$) were immunosuppressed with 5-FU administered subcutaneously 5 days prior to infection and then intranasally infected with 3.0×10^4 of *Aspergillus flavus* conidia. E1210 or voriconazole was administered orally twice daily and caspofungin or liposomal amphotericin B was administered intraperitoneally once daily for four consecutive days starting 1 h after infection. The survival rate and survival period were determined over 14 days. (A) E1210; (B) voriconazole; (C) caspofungin; (D) liposomal amphotericin B. *, $P < 0.05$ versus control group (log rank test with Bonferroni's adjustment).

5.1 h, 7.6 h, and 10.1 to 12.5 h, respectively, in mice. Therefore, the plasma half-life of E1210 (2.2 h) was noted to be similar to that of voriconazole and about two to five times shorter than those of the reference antifungals other than voriconazole. From these data, we determined the optimal frequency of dosing for E1210 and voriconazole to be BID or TID and those for other drugs to be QD for these murine models of infection. Thus, E1210 had a relatively shorter plasma half-life than those of the reference drugs, except for voriconazole, in mice. Also, the addition of serum (90%) greatly affected the MICs of E1210 for the strains tested; the MICs of E1210 increased 64-fold (24, 25). This result indicates that E1210 may have a high plasma protein binding ratio. For all these reasons, we expect that the higher doses of E1210 were needed for effective treatment in mouse models of infection. We are currently conducting further pharmacokinetic and metabolic studies for

the future clinical development of E1210 in rats, dogs, and monkeys.

Orally administered E1210 demonstrated clear dose-dependent therapeutic responses in the various experimental infection models in mice. First, E1210 showed efficacy in treating oropharyngeal candidiasis in mice. Treatment with E1210 significantly reduced the number of *Candida* CFU in the oral cavity in comparison to that of the control treatment ($P < 0.05$), with the extent of eradication comparable to that of fluconazole. Second, E1210 increased the survival time in a dose-dependent manner in mice infected with *Candida* spp. or *Aspergillus* spp. E1210 was consistently effective in treating disseminated candidiasis caused by azole-susceptible *C. albicans*. Furthermore, E1210 had the additional benefit of an efficacy against azole-resistant *C. albicans* infections in mice comparable to that against azole-susceptible *C. albicans* infec-

FIG. 7. Efficacy of E1210 in a murine model of disseminated fusariosis caused by *F. solani* IFM50956. Mice ($n = 8-10$) were immunosuppressed with 5-FU administered subcutaneously 6 days prior to infection and then intravenously infected with 5.0×10^3 of *F. solani* conidia. E1210 was administered orally three times daily for five consecutive days starting 1 h after infection. The survival rate and survival period were determined over 14 days. *, $P < 0.05$ versus control group (log rank test with Bonferroni's adjustment).

tions. The frequency of clinical reports of azole-resistant *C. albicans* and azole-resistant *Candida* spp. other than *C. albicans* is increasing (22, 33, 35). E1210 proved to be effective against disseminated candidiasis caused by azole-resistant strains of *C. albicans* and *C. tropicalis* in mice. E1210 is further characterized by its efficacy against invasive pulmonary aspergillosis caused by *A. flavus* and *A. fumigatus* in mice. Invasive aspergillosis currently constitutes the most common cause of infectious pneumonic mortality in patients undergoing hematopoietic stem cell transplantation (HSCT) and is an important cause of opportunistic infection in other immunocompromised patients (3, 4, 17, 19, 30, 32). For primary treatment of invasive pulmonary aspergillosis, intravenous or oral voriconazole is recommended for most patients based on the Infectious Diseases Society of America's guideline published in 2008 (43). Interestingly, E1210 at doses of >20 mg/kg showed a maximum therapeutic efficacy (100% survival) that was comparable to that of voriconazole at 10 mg/kg in murine invasive pulmonary aspergillosis models. In addition, we confirmed that E1210 was effective against disseminated aspergillosis caused by *A. fumigatus* in mice (data not shown). In the future, it will be very important to evaluate the antifungal activity of E1210 against azole-resistant *Aspergillus* strains, which are increasingly being recognized in the clinic (34, 42).

From the viewpoint of antifungal spectrum of activity, one of the important characteristics of E1210 is its activity against non-*Aspergillus* filamentous fungi, such as *F. solani* and *Scedosporium prolificans*, which are intrinsically resistant to all currently approved or available antifungals (15, 21, 28). We therefore conclude that it will be very important to evaluate the *in vivo* efficacy of E1210 in animal models of infections attributable to these pathogens. We showed the efficacy of E1210 in a disseminated fusariosis model in this study and have

FIG. 8. Time-plasma concentration profiles of E1210 after oral and intravenous administrations of a single dose of 1 mg/kg to mice. After administration of E1210, blood samples were drawn from the vena cava of mice at designated time points. After deproteinization with methanol, the extracted sample was analyzed by LC/MS/MS. Each value represents the mean of the results for two animals.

already started to investigate the efficacy of E1210 in the same model for *S. prolificans* pulmonary infections.

Furthermore, E1210 proved to be effective in treating disseminated candidiasis, even if treatment was started 24 h after infection (Fig. 5). We also confirmed that E1210 was effective against disseminated aspergillosis caused by *A. fumigatus* even if treatment was started 24 h after infection in mice (data not shown). These results strongly suggest that E1210 has the potential for being effective if given during acute or subacute infections.

As a preliminary toxicological study, we have conducted a 7-day oral dose range toxicity study in rats. E1210 was generally well tolerated when given orally to rats for 1 week at doses up to 300 mg/kg. Further toxicological studies are needed to confirm the safety of E1210 in rats and other animals, such as dogs and monkeys.

In conclusion, these results suggest that E1210 has the potential for efficacy as a single-agent therapy in the treatment of oropharyngeal candidiasis, disseminated candidiasis, pulmonary aspergillosis, and disseminated fusariosis. E1210 is thus a very promising drug for the treatment of fungal infections, and therefore, further studies on its pharmacokinetics/pharmacodynamics and toxicological characteristics are warranted.

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