In Vitro Antifungal Activity of (1)-N-2-Methoxybenzyl-1,10-phenanthrolinium Bromide against Candida albicans and Its Effects on Membrane Integrity

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Abstract Metal-based drugs, such as 1,10-phenanthroline, have demonstrated anticancer, antifungal and antiplasmodium activities. One of the 1,10-phenanthroline derivatives compounds (1)-N-2-methoxybenzyl-1,10-phenanthrolinium bromide (FEN), which has been demonstrated an inhibitory effect on the growth of Candida spp. This study aimed to explore the in vitro antifungal activity of FEN and its effect on the membrane integrity of Candida albicans. The minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC) of FEN against planktonic C. albicans cells were determined using the broth microdilution method according to the Clinical and Laboratory Standards Institute guidelines. Cell membrane integrity was determined with the propidium iodide assay using a flow cytometer and were visualized using scanning electron microscopy (SEM). Planktonic cells growth of C. albicans were inhibited by FEN, with an MIC of 0.39–1.56 μg/mL and a MFC that ranged from 3.125 to 100 μg/mL. When C. albicans was exposed to FEN, the uptake of propidium iodide was increased, which indicated that membrane disruption is the probable mode of action of this compound. There was cells surface changes of C. albicans when observed under SEM.

Keywords 1,10-Phenanthroline, Antifungal, Candida albicans, Cell membrane integrity

Candidiasis is one of the most common fungal infections in humans caused by the Candida species, most notably C. albicans [1, 2]. Therapy for invasive candidiasis remains a difficult medical problem. Despite the availability of extended spectrum triazoles, the incidence of invasive infections and resistance to antifungal therapy continue to increase. Resistance to antifungal drugs has become a very serious problem, especially in immunocompromised individuals because it is associated with increased incidences of opportunistic infections and systemic fungal infections [3-5]. These factors illustrate the urgent need to search for novel compounds with anticanidal activity. Metal-based drugs, such as 1,10-phenanthroline (Fig. 1A), are known to be antifungal agents that inhibit fungal metal proteases, particularly in rumen microorganisms [5-7]. The 1,10-phenanthroline is a metal chelator agent, including iron that may inhibit mitochondrial metalloproteases enzymes, required for catalytic activity [6, 8].

In this regard, we have successfully synthesized (1)-N-2-methoxybenzyl-1,10-phenanthrolinium bromide (FEN) (Fig. 1B) [9-11], which is one of the 1,10-phenanthroline derivatives. Preliminary study using broth micro dilution tests in RPMI medium have demonstrated its antifungal activity, that inhibits the growth of Candida spp. The modes of antifungal action by 1,10-phenanthroline and derivatives that have been
known was disruption of cell membrane and withdrawal of cytoplasmic membrane, drug-induced circumvention on the control of cell division (budding), damage of mitochondrial function and uncoupling of cellular respiration, chelation or sequestering essential trace metal ions inhibiting glycosylphosphatidyl inositol synthesis, ruptured internal organelles and enlarged nucleus and degradation of nuclear DNA [12, 13].

However, the mechanism of antifungal action by FEN was unknown. The cell membrane is one of essential components of *C. albicans*. The changes of the plasma membrane could affect the function of organelles so it plays an important role as antifungal targets. The aromatic ring system of FEN might present greater lipophilicity and therefore influence the penetration of the cell membrane and promote adverse intracellular interaction. This study aimed to explore the *in vitro* antifungal activity of FEN on planktonic *C. albicans* cells and the effect of this compound on the integrity of the cell membrane.

**MATERIALS AND METHODS**

**Antifungal agents.** The FEN obtained from the Faculty of Mathematics and Natural Sciences of the Gadjah Mada University, Yogyakarta, Indonesia. Fluconazole was purchased from Pharos, and a stock solution was prepared by dissolution in sterile distilled water to a concentration of 6,400 µg/mL. The stock solutions were stored frozen at −70°C until the day of the test. The final concentrations of dimethyl sulfoxide (DMSO) were 1% in all assays [14].

**Synthesis of FEN.** The FEN, which derivatives of N-alkyl and N-benzyl-1-10-phenanthroline have been synthesized by Mustofa *et al.* [10] and Hadanu *et al.* [11]. Identification of the compounds was carried out by means of infrared spectroscopy, proton nuclear magnetic resonance (1H-NMR) spectroscopy, carbon nuclear magnetic resonance (13C-NMR) spectroscopy, and mass spectroscopy. Quantitative structure-activity relationship of these 1,10-phenanthroline derivatives was also investigated. This compound was dissolved to a concentration of 1,000 µg/mL in DMSO (Sigma-Aldrich, St. Louis, MO, USA) and stored at −20°C.

**Fungal strains.** This study used 23 strains of *C. albicans*. These strains were comprised of one standard strain of *C. albicans*, i.e., ATCC 10231, that was acquired from the Regional Laboratory of Yogyakarta, Indonesia and 22 clinical isolates of *C. albicans* that were obtained from the Department of Microbiology of the Faculty of Medicine, Gadjah Mada University, Yogyakarta, Indonesia. The stock cultures were stored at −80°C, subcultured on Sabouraud dextrose agar (SDA) and stored at 4°C.

**Inoculum preparation.** Fungal inocula were prepared by inoculating fungal colonies from the SDA (Sigma-Aldrich) medium into 25 mL of yeast peptone dextrose (Sigma-Aldrich) medium and then incubated in a shaking incubator overnight at 35°C. After overnight culture, the samples were centrifuged at 3,000 rpm for 5–10 min. The cells were washed twice with sterile phosphate buffered saline (PBS; Sigma-Aldrich), and the pellets were resuspended with 20 mL of RPMI 1640 (Sigma-Aldrich) buffered to pH 7.0 with MOPS (Sigma-Aldrich) at 0.165 mol/L. The final concentration of the cell suspension was adjusted at 1 × 10⁶ colony-forming unit (CFU)/mL according to McFarland standard 0.5 [15, 16].

**Antifungal activity of FEN.** The minimum inhibitory concentrations (MICs) were determined using the microdilution method described in the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [15, 16]. Briefly, serial two-fold dilutions were prepared precisely as described in the CLSI document. Yeast inoculum (0.1 mL) was added to each well of the microdilution plates. Drug-free and yeast-free controls were included. The plates were incubated at 35°C, and the MIC end points were read after 48 hr. The MIC was defined the lowest concentration of drug that resulted in complete inhibition of growth of the fungi and determined by visual observation [17]. The experiments were performed three times with three replicate wells for each experiment. After the MICs had been measured, the samples from the corresponding wells were withdrawn and plated onto SDA plates. The inoculated plates were incubated at 35°C and the percentages of viable cells were calculated at 48 hr and used to determine the minimum fungicidal concentrations (MFCs) of the tested compound. MFC was defined as the lowest drug concentration that resulted in 100% inhibition of growth of the fungi. An antifungal agent was considered as fungicidal if the ratio of the MFC to MIC did not exceed a value of 4 and fungistatic if the ratio higher than 4 [17].

**Membrane integrity analysis.** The membrane integrities of the *C. albicans* cells exposed to FEN were examined by the method as described by Ali *et al.* [18] with modification using propidium iodide (PI; Sigma-Aldrich). *C. albicans* cells (1 × 10⁶ CFU/mL) were incubated with two to eight times the MIC (3.125 to 12.5 µg/mL) of FEN at 35°C for 4 hr. Fluconazole at eight times the MIC (4.0 µg/mL) was used.
as the positive control and the cells without FEN served as the negative control treated in similar fashion. The suspensions were centrifuged, washed, and resuspended in PBS. PI solution in PBS (5 µL) was added to the cell suspensions to obtain a final concentration of 1 µg/mL PI. Furthermore, the suspension was also supplemented with 300 mL of 25 mM sodium deoxycholate (Sigma-Aldrich). The samples were then incubated at 35°C for 50 min in the dark. The unstained cells were always included as auto-fluorescence controls. After incubation, 75 mL aliquots were transferred into fluorescence-activated cell sorting (FACS) tubes at a rate of 10 mL/min. Each tube was analyzed using a FACScan flow cytometer (Becton-Dickinson) [18, 19].

**Scanning electron microscopy (SEM).** For SEM observations, *C. albicans* were incubated on sterile polyvinyl chloride coverslips (with a thickness of 0.13–17 mm and a diameter of 22 mm) in 12-well microtiter plates (Corning Costar; Sigma-Aldrich) with FEN (MIC value), fluconazole (MIC value) and untreated cells for 24 hr at 37°C. Thereafter, the coverslip was coated twice with platinum vanadium (Becton-Dickinson Biosciences, Mansfield, MA, USA) with a blue argon laser at 488 nm and 15 mW, and the results were analyzed using CellQuest Pro Software (Becton-Dickinson) [18, 19].

**Statistical analysis.** The inhibitions of the growth of planktonic *C. albicans* cells are expressed as the MICs and MFCs. The cell membrane integrities of *C. albicans* were indicated by the ability of cells to uptake PI. These values were then analyzed with normality and homogeneity tests. Because of the data distributions abnormal, we transformed it to normalize distributions. The data were analyzed using one-way ANOVA, followed by post-hoc Bonferroni multiple comparison tests with a significance level of *p* < 0.05.

**RESULTS AND DISCUSSION**

**Antifungal activity of FEN against planktonic *C. albicans* cells.** Despite the introduction of improved antifungal drugs for treatment and prophylaxis, invasive fungal infections remain a significant clinical problem. Infections caused by eukaryotic organisms, such as yeasts generally present more difficult therapeutic problems than do bacterial infections. There are relatively few antifungal agents that can identify unique targets not shared with human hosts [22]. The antifungal activities of FEN against the tested *C. albicans* strains are illustrated in Table 1. All of *C. albicans* strains used in this study were susceptible to FEN with MIC values in the range of 0.39 to 1.56 µg/mL. Importantly, the MICs of FEN against the 22 clinical strains *C. albicans* ranged from 0.39 to 1.56 µg/mL, while *C. albicans* ATCC 10231 demonstrated a susceptibility similar to those of most of the clinical strains with an MIC value of 1.56 µg/mL. Additionally, the MICs of fluconazole against these clinical strains *C. albicans* were higher with values in the ranged of 0.5 to 8 µg/mL.

In this study, the MFC values of between the positive control and clinical strains tested exposure to FEN obtained ranged from 3.125 to 100 µg/mL, resulted in substantial losses of viabilities indicated by the reductions in fungal colony counts compared with the negative control (Table 1). These finding suggest that FEN is capable of anticanidal activity against *C. albicans*. This compound exhibited antifungal potency that included both fungistatic and fungicidal activities because the ratio of MFC to MIC ranged 2 to 64. To our knowledge, these data represent the first report of the antifungal potency of FEN against clinical strains of *C. albicans*. The mechanism of action of FEN against *C. albicans* may be partially due the induction of cellular oxidative stress, damaged mitochondrial function, induced

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**Table 1.** MIC, MFC, and MFC/MIC values of FEN against 23 isolates of *Candida albicans*

| Clinical isolate | MIC (mg/mL) | MFC (mg/mL) | MFC/MIC |
|------------------|-------------|-------------|---------|
| 2                | 1.56        | 3.13        | 2       |
| 20               | 1.56        | 25.00       | 16      |
| 531              | 1.56        | 6.25        | 4       |
| 610              | 1.56        | 6.25        | 4       |
| 521              | 1.56        | 6.25        | 4       |
| 420              | 1.56        | 25.00       | 16      |
| 476              | 1.56        | 25.00       | 16      |
| 493              | 1.56        | 6.25        | 4       |
| 604              | 0.39        | 6.25        | 16      |
| 654              | 1.56        | 25.00       | 16      |
| 559              | 1.56        | 6.25        | 4       |
| 508              | 1.56        | 3.13        | 2       |
| 760              | 1.56        | 50.00       | 32      |
| 507              | 1.56        | 25.00       | 16      |
| 510              | 1.56        | 6.25        | 4       |
| 519              | 1.56        | 12.5        | 8       |
| 576              | 1.56        | 3.13        | 2       |
| 518              | 1.56        | 12.50       | 8       |
| 302              | 1.56        | 3.13        | 2       |
| 263              | 1.56        | 12.50       | 8       |
| 769              | 1.56        | 6.25        | 8       |
| 537              | 1.56        | 100.00      | 64      |
| ATCC 10231       | 1.56        | 6.25        | 4       |

MIC, minimum inhibitory concentration; MFC, minimum fungicidal concentration; FEN, (1)-N-(2-methoxybenzyl)-1,10-phenanthroline bromide.
changes in the internal structures of the yeast cells, such as cytoplasm retraction and splitting of the cell nuclei, the induction of apoptosis in yeast cells [13, 23, 24] and an effect on cell respiration [15].

The effects of FEN on the cell membrane integrity of \textit{C. albicans}. FEN affected the integrity of the cell membranes of \textit{C. albicans} as indicated by the percentages fungal cells that were positive for PI. PI is a marker nucleic acid that is unable to penetrate healthy cell membranes. When cells undergo apoptosis or necrosis, the permeabilities of the plasma membrane and nucleolus change such that PI can enter cells and cause a red fluorescence. The fluorescence due to PI in the cells indicates a defect in the cell plasma membrane [12]. The sodium deoxycholate used in this study enhanced the susceptibility of membranes of \textit{C. albicans}, so PI could easier diffuses into the cells to result a red fluorescence and it is not toxic to \textit{C. albicans} cells [19, 25]. The percentage of PI uptake is presented in Fig. 2.

The increasing concentrations of the test compound that were used to treat the fungal cells resulted in higher uptake of PI. The percentage of PI uptake in the \textit{Candida} cells that were exposed to FEN for 4 hr at a concentration of 8 times the MIC was 18% higher than the fluconazole uptake at the same concentration and period of incubation, which were 30% and 12%, respectively (Fig. 2).

A one-way ANOVA test followed post-hoc Bonferroni tests revealed that there were significant differences of PI uptake between the negative control group and the groups treated with FEN at concentrations of 4 MIC and, 8 MIC and the group treated with fluconazole at 8 MIC. Exposing the cell suspensions of \textit{C. albicans} ATCC 10231 to two to eight times (3.12–12.5 µg/mL) the MIC of FEN for 4 hr increased the cell permeability to the fluorescent nucleic acid.

Fig. 2. Percentages of propidium iodide uptake by \textit{Candida albicans} ATCC 10231. The exposure of the fungal cells by (1)-N-2-methoxybenzyl-1,10-phenanthroline bromide (FEN) at concentrations of 2 minimum inhibitory concentration (MIC), 4 MIC, and 8 MIC. Fluconazole at 8 MIC was used as the positive control and fungal cells without treatment with the test compound were used as the negative control. The data were analyzed by one-way ANOVA followed by post-hoc Bonferroni tests. *\(p < 0.05\) (compared to the negative control).

Fig. 3. Scanning electron microscopy visualization of \textit{Candida albicans} ATCC 10231 cultured for 24 hr at 37°C. A, Control (untreated) cells showed smooth surface (×10,000); B, Treatment with fluconazol at minimum inhibitory concentration (MIC) (0.5 µg/mL) appeared shrunked cells (×10,000); C, Cells after treatment with (1)-N-2-methoxybenzyl-1,10-phenanthroline bromide (FEN) at MIC (1.56 µg/mL), the cell surface appeared rough and ruptured (×5,000); D, Cracked cells after treatment with FEN at MIC value (×10,000).
acid stain, PI due to the disruption of membrane integrity. This increase in fluorescence was proportional to the increase in the FEN concentration.

In this study, we also used SEM to investigate yeast cells surface in the absence or presence of FEN. Untreated cells of C. albicans 10231 showed smooth cell surface (Fig. 3A), whereas fluconazole treated cells appeared oval shaped and shrunk (Fig. 3B). Treatment with FEN resulted ruptured and cracked of the fungal cell (Fig. 3C and 3D). Some studies have suggested that the 1,10-phenanthroline complex targets cells by disrupting the mitochondria, inducing oxidative stress, and interfering with cell respiration, which results in changes to the structures of the cells, which eventually undergo apoptosis [15, 24]. This mechanisms associated with the activity of 1,10-phenanthroline as a metal chelator that can bind bivalent cations. The cell membranes are permeable, which are used for the catalytic activities of numerous enzymes that require metal atoms including matrix metalloprotease-processing peptidase enzyme. 1,10-Phenanthroline is a non-competitive inhibitor of this enzyme [8]. Unfortunately, this study did not used original compound, 1,10-phenanthroline to compared it. As positive controls we just used fluconazole which is regarded as commonly used and standard antifungal. Coyle et al. [13] and McCann et al. [12] had been studied about the effect of 1,10-phenanthroline to fungal cells and find that the compounds had the potential to induce apoptosis.

So far, the precise antifungal mechanism of FEN is still not clear yet. However, this study represented initial research provided data concerning antifungal effect of FEN on cell membrane integrity of C. albicans.

The FEN tested in this study exhibited antifungal effects on standard and clinical strains of C. albicans; thus, this compound has the potential to be developed as a new antifungal agent. The establishment of this and similar compounds as antifungal agents requires further research, especially regarding the mechanisms of action and safety of these compounds.

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