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Research Article

Candidacidal Action of CF66I, an Antifungal Compound Produced by *Burkholderia Cepacia*

Xin Li[1], Hui-Ying Yu[1] and Chun-Shan Quan[2]*

[1]Life Science College, Yuncheng University, Yuncheng, [2]Life Science College, Dalian Nationalities University, Dalian, 116600, China.

Abstract

***Purpose*:** To investigate the candidacidal activity of a novel compound CF66I and attempt to elucidate its mode of action.
***Methods*:** Minimal inhibition concentrations (MIC) and minimal fungicidal concentrations (MFC) of CF66I for *Candida albicans* were determined using serial broth dilution method. Colony count assay, K^+ release, propidium iodide (PI) uptake and electron microscopy were used to determine the manner in which CF66I exerts its candidacidal effects.
***Results*:** MIC and MFC of CF66I were determined to be 5.0 - 7.0 µg/ml and 14.0 - 18.0 µg/ml. It showed strong candidacidal activity over a wide pH range (4.0-7.4). The compound killed *C. albicans* within 30 s. It preferentially bound to the surface of *C. albicans* via a specific interaction with β-1,6-glucan, one of fungal cell wall components, and then affected the normal structure of fungal cell wall and induced cell wall thickening, which was confirmed by transmission electron microscopy (TEM) CF66I also exerted its candidacidal activity against cell membrane, eventually induced K^+ release from *C. albicans* and PI uptake. Membrane-attacking ability was also found via electron microscopy.
***Conclusion*:** These results suggest that CF66I exerted its candidacidal activity on multiple target sites on *C. albicans*, and may have considerable potential for development of a new class of antifungal agents.

Keywords: *Burkholderia cepacia*, Candidacidal activity; CF66I; *Candida albicans*; Mode of action

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Corresponding author: E-mail: *lixin-eva@163.com;* **Tel/Fax:** *+86-(0)359-2090324*

INTRODUCTION

As an important opportunistic fungus, *Candida albicans* causes a wide range of infections ranging from surface diseases of the skin and mucosae to deep tissue infections in individuals [1,2]. Some drugs for the treatment of *Candida* infections have been developed, such as polyene and azoles, which are regarded as the most effective for controlling serious fungal infections so far [3]. However, extensive use of the currently available antifungal drugs leads to a variety of associated problems with human health due to their significant side effects, and the emergence of large amounts of drug-resistant strains worldwide [4]. Therefore, the development of new antifungal agents with increased clinical usefulness in terms of efficacy and/or safety is needed.

During the search for antagonists of plant pathogenic fungi, a novel antifungal compound named CF66I and produced by *Burkholderia cepacia* was obtained. Preliminary structural analyses confirmed that CF66I was a complicated compound having amide bonds, α-methyl fatty acid, bromine, and some structural units such as CH_2CH_2O [5]. Previous studies reported its broad-spectrum activity against plant pathogenic fungi [6]. By fluorescent staining and transmission electron microscopy, it was observed that high doses of CF66I were fungicidal with membrane-attacking ability, whereas low doses were fungistatic by interfering with the cell metabolic pathways [7, 8]. Therefore, it was considered to be a promising candidate for the development of a novel antibiotic. However, the mechanism underlying the aforementioned activities was not clearly understood. In this study, candidacidal activity of CF66I has been investigated: we also attempted to determine the manner in which CF66I exerts its candidacidal effects.

EXPERIMENTAL

Antifungal substances

The compound, CF66I, was prepared by three-step gel chromatography as previously described [6], and then held at 4 ℃ for use.

Organism and culture media

C. albicans (CGMCC 2.538) was obtained from China General Microbiological Culture Collection Center. It was subcultured twice on yeast peptone dextrose (YPD) agar plate (yeast extract 1 %, peptone 1 %, dextrose 2 % and agar 2 %) at 28 ℃ prior to testing. YPD broth used in this study contains yeast extract 1 %, peptone 1 % and dextrose 2 %.

MIC and MFC assay

MIC and MFC were determined for CF66I against *C. albicans* using serial broth dilution method. YPD broth (100 µl) containing different concentrations of CF66I (0 to 30.0 µg/ml) was inoculated with 10 µl of fungal suspension (10^7 cfu/ml), and then the mixture was incubated in a 96-well plate at 28 ℃ for 48 h. The MIC end-point criterion was defined as the lowest compound concentration showing no visible growth after 24-h incubation. To obtain the MFC values, 10 µl of the test samples was taken from every well and spread on YPD plates, then cultivated at 28 ℃ for 48 h. MFC was defined as the lowest compound concentration at which no visible growth was observed. Triplicate determinations were performed.

Colony count assay

Aliquots (2 ml) of *C. albicans* (10^7 cfu/ml) in YPD broth was incubated with different concentrations of CF66I at 28 ℃ in a shaker incubator, and then 100-µl aliquots were removed at predetermined time intervals. Each of the aliquots directly or after dilution was spread on YPD agar plates. The resultant colonies were then counted, after incubation for 48 h at 28 ℃.

Effect of pH on candidacidal activity of CF66I

To evaluate the influence of pH on candidacidal activity of CF66I, the pH of YPD broth containing CF66I (16 µg/ml) was adjusted to 4.0, 5.0, 5.11, 6.0, 7.0 or 7.4, with 1M NaOH or 1M HCl. Each medium with different pHs was inoculated with 1% of the fungal suspensions. The cultivation was performed with shaking at 28 ºC for 1 min, while CF66I-free solutions with different pH values were used as the controls. Fungal growth was measured by counting the colonies on YPD agar plates. At each pH, CF66I activity was calculated as {1-(cfu of CF66I-treated sample/cfu of the control)} × 100 %. Triplicate determinations were performed.

Assay of K^+ efflux

The leakage of K^+ from *C. albicans* was used to monitor the cell membrane damage inflicted by CF66I. K^+ content was examined with a flame spectrometer (Hitach Z-2000). In brief, *C. albicans* cells were cultured in YPD broth at 28 ºC. After 18 h of incubation, cells were harvested by centrifugation, washed twice with distilled water, and then resuspended to 10^7 cfu/ml. One millimeter of the cell suspensions were incubated with different concentrations of CF66I at 28 ºC. At predetermined intervals, samples (0.1 ml) were taken, centrifuged to remove the cells, and the resulting filtrates were assayed for K^+ content. Total cellular K^+ was determined after the cell suspension was incubated for 30 min in a boiling water bath. Triplicate determinations were performed, and the mean values were utilized in the construction of a graph. The percent K^+ release was then calculated, according to the following equation: K^+ release (%) = ([K^+] of sample-[K^+] of CF66I-free control)/(total cellular [K^+]-[K^+] of CF66I-free control) ×100.

FACScan analysis

The integrity of cell membrane after CF66I treatment was examined by was determined by FACScan analysis, via nuclear staining with propidium iodide (PI; Sigma). *C. albicans* cells were harvested and washed twice with distilled water. The washed cells (10^7 cells) were incubated with different concentrations of CF66I at 28 ºC for 10 min, and subsequently stained with PI (100 µg/ml) for 30 min at 4 ºC. After this operation, the unbound dye was removed by washing with distilled water. Flow cytometry analysis was conducted with a flow cytometer (Becton–Dickinson).

Electron microscopy

For SEM, *C. albicans* cells were harvested by centrifugation and washed twice with PBS, then fixed with 2% glutaraldehyde in 0.1M PBS (pH 7.4) at 4 ºC for 1 h. After fixation, the samples were centrifuged at 4000 rpm and rinsed three times for 20 min each with sodium cacodylate buffer. The samples were then dehydrated through a graded ethanol series and freeze-dried for 8 h, then coated with a light coat of gold palladium. Observations were carried out with an S-3800 scanning electron microscope (Hitachi, Japan).

For TEM, cells fixed with 2% glutaraldehyde solution as described above, were postfixed with 1% OsO4 solution for 20 h at 4 ºC. Dehydration was carried out in a degraded series of ethanol before embedding in resin. Ultra-thin sections were cut with a Reichert-Nissei Ultracut N, stained with uranyl acetate, followed by lead citrate and then examined with a JEM-2000EX electron microscope (Japan) using an accelerating voltage of 120 kV.

Assay for binding of CF66I to the component of fungal cell wall

Binding property of CF66I to the surface of *C. albicans* was determined by assessing the effect of fungal cell wall components on the anti-*Candida* activity of CF66I in ultrasensitive radial diffusion assay as described previously [9]. 50 µl of 1 mg/ml

CF66I solution was added to 50 µl of laminarin (β-1,3-glucan polymer, Sigma), pustulan (β-1,6-glucan polymer, Sigma), chitin (N-acetylglucosamine polymer, Sigma) or mannan (mannose polymer, Sigma), each of which was serially diluted two-fold to concentrations ranging from 0.5 to 8.0 mg/ml, and the mixtures incubated at 30 ºC for 2 h. 50 µl samples were loaded into 6-mm diameter wells that had been pre-punched on YPD agar plates in which the washed *C. albicans* (10^7 cfu/ml) was trapped. After the plates were incubated overnight at 28ºC, the diameters of clearing zones indicating antifungal activity were plotted against polysaccharide concentrations.

Statistical analysis

The results are presented as the means of three individual experiments. Statistical significance was determined by one-way analysis of variance (ANOVA) and Student's t-test using OriginPro 8.0 software (OriginLab). $P<0.05$ was used in determining statistically significant difference.

RESULTS

MIC and MFC assay

CF66I exhibited excellent fungistatic and fungicidal activities against *C. albicans*. By the serial dilution method, an MIC of CF66I for *C. albicans* was determined to be 6.0 µg/ml, while the MFC value was 18.0 µg/ml.

Influence of pH on CF66I activity

CF66I possessed excellent candidacidal activity over a wide pH range of 4.0 to 7.4 (Fig 1). Greatest fungicidal effect against *C. albicans* was observed at pH values ranging from 6.0 to 7.4 (> 90 %), which was close to the physiological pH of human serum. While at pH lower than 6.0, CF66I activity decreased by about 75 %.

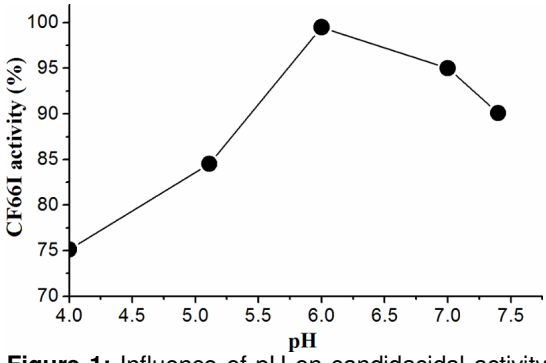

Figure 1: Influence of pH on candidacidal activity of CF66I

Effect of CF66I on the membrane integrity of *C. albicans*

Colony count assay showed that candidacidal activity of CF66I was dose-dependent. CF66I concentration of 16.0 µg/ml caused a significant reduction (approx 90 %) in cell viability. Short time course analysis (5 min) revealed that the minimal number of viable cells was achieved after 30 s of incubation with CF66I, and then reached a plateau thereafter (Fig 2a), thereby illustrating the marked rapidity with which CF66I killed the *C. albicans* cells; its candidacidal activity was completed within 30 s.

Fig 2b showed the release of K^+ from *C. albicans* treated with CF66I for the predetermined time. About 95.2 % of the total intracellular K^+ was released after 5-min incubation with 16.0 µg/ml of CF66I, and the percentage of K^+ efflux remained constant during further incubation. The time course associated with K^+ release from the *C. albicans* exhibited a pattern consistent with the results obtained from the colony count assay for the candidacidal activity of CF66I. Therefore, we concluded that CF66I induced an immediate and massive efflux of K^+ from *C. albicans*. Fig 2c shows the results of the intracellular PI measurements. The *C. albicans* cells were labeled fluorescently after 5-min of incubation with CF66I, thereby indicating that CF66I induced the uptake of PI into the cells.

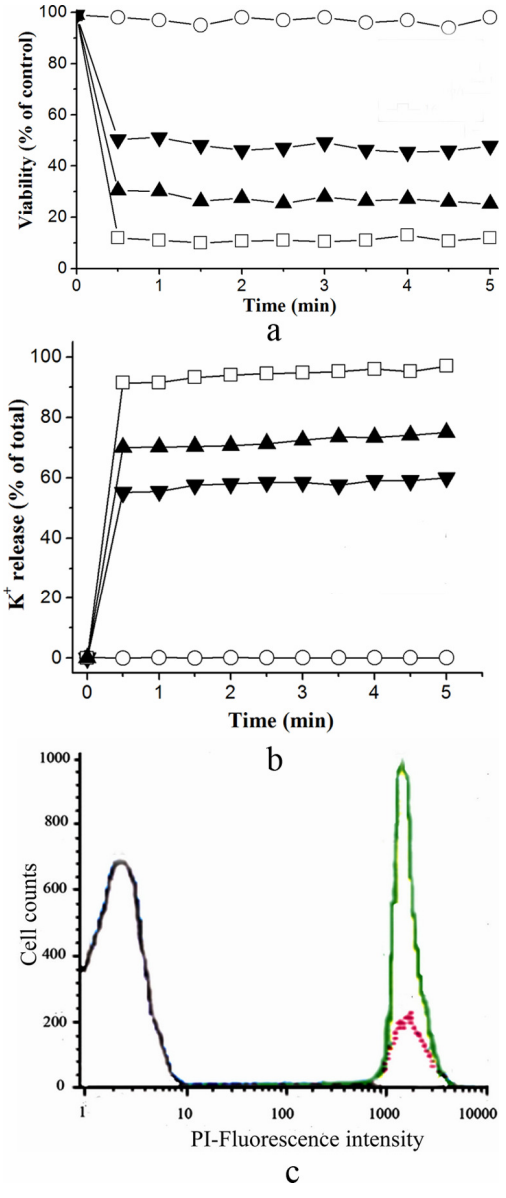

Figure 2: Effect of CF66I on the cell membrane of *C. albicans* (a) Colony count assay for the candidacidal activity of CF66I (b) CF66I-induced K^+ release from *C. albicans*. (c) Flow cytometric analysis. *C. albicans* cells were treated without (dark line) and with CF66I (green line, 16 µg/ml; red line, 8 µg/ml), and then stained with PI. Cellular fluorescence was examined by FACScan flow cytometry. The increase in fluorescence intensity represents the PI uptake to the cells (○= control; ▼= 8 µg/ml; ▲= 12 µg/ml; □= 16 µg/ml)

Electron microscopic studies

Morphological changes of *C. albicans* induced by CF66I were shown in Figs 3 and 4. Control cells exhibited an ovoid shape with smooth surfaces. The average diameters were 1 to 3 µm. Normal buds and bud scars were observed (Fig 3a). TEM images revealed that the control cells presented a typical multilayered cell wall with an outer electron-dense layer, and the thickness was about 100 nm. Typical features of organelles, i.e., nucleus, mitochondria and vacuoles are also visible (Fig 4a).

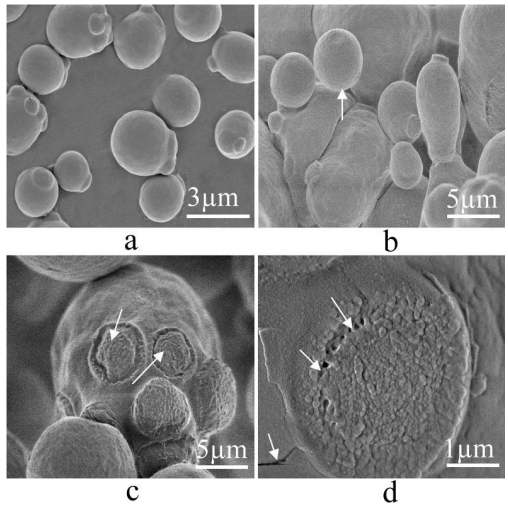

Figure 3: Scanning electron microscopy. (a) Control cells. All cells lie apart, showing smooth surfaces and normal bud scars; (b, c, d) Cells exposure to CF66I (16 µg/ml) for 30 min. Note the obvious increased volume of CF66I-treated cells and the formation of interconnected cells. Cell surfaces were wrinkled, and some pores and cracks (d, arrows) were observed on the cell wall. In addition, randomly distributed bud scars were visible (c, arrows).

However, profound alterations in cell morphology of *C. albicans* were observed after exposure to CF66I, including surface roughening and disruption (Fig 3b, 3c and 3d). In addition, this compound caused the clusters of interconnected cells with buds and bud scars irregularly located on the cell surface. Abnormal cell division observed in

morphologically changed cells demonstrated inhibition of normal development of buds or daughter cells and their separation from parent cells. Meanwhile, SEM images showed swollen and aberrantly deformed cells of *C. albicans*, which were at least three times larger than control (Fig 3b and 3c).

Ultrastructural modifications of CF66I-treated *C. albicans* were observed via TEM. 16.0 µg/ml of CF66I induced the partial solubilization of cytoplasmic membrane and the prominent enlargement of nucleus (Fig 4b); however, the cell volume did not change at this time. After incubation with CF66I for 30 min, cells swollen obviously, and the dramatic disruptions of cell membrane were observed. Whereas some CF66I-treated cells remain intact like the control, the cell interior was completely necrotic, and only several fat deposits remained (Fig 4c and 4d).

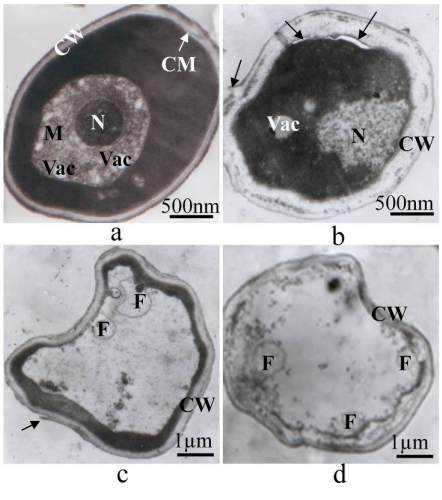

Figure 4: Ultrastuctural modifications of *C. albicans* examined by TEM after exposure to CF66I. (a) Control cell. Cell wall (CW), cell membrane (CM), mitochondria (M), nucleus (N) and vacuoles (Vac) were clearly seen (×12000). (b) Cells exposure to 16 µg/ml of CF66I for 10 min. Note the loosening and thickening of cell walls, damage of the cell membrane (arrows) and the enlargement of nucleus. (c, d) Cells exposure to 16 µg/ml of CF66I for 30 min. The cells swelled, whereas the cell remained intact, cell interior was completely necrotic and became empty. Only some fat deposits (F) were observed.

The cytoplasm leakage probably related with the cracks and pores on the cell wall (Fig 3d). However, of particular interest were the observations of fungal cell wall thickening with its thickness increasing to about 500 nm (Fig 4b, 4c and 4d).

Specific binding of CF66I to the component of fungal cell wall

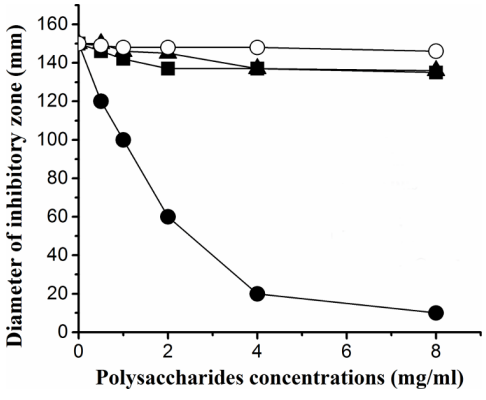

Figure 5: Specific binding of CF66I to pustulan. Radial diffusion assay was performed by mixing various amounts of laminarin (■), pustulan (●), chitin (○) or mannan (▲) with 25 µg of CF66I in a final volume of 50 µl. The mixtures of CF66I with each polysaccharide were introduced into the wells of YPD agar plates pre-seeded with *C. albicans* suspensions (10^7 cfu/ml). The anti-*Candida* activities of CF66I in the mixture were graphed against concentrations of polysaccharides.

In order to investigate if CF66I specifically binds to fungal cell surface, the effects of main cell wall polysaccharides on the anti-*Candida* activity of this compound were examined (Fig 5). The constant amount of CF66I was incubated with different concentrations of laminarin, mannan, chitin or pustulan, and then the mixture was tested for anti-*Candida* activity in agar diffusion assay. As a result, its activity was significantly reduced as the amount of pustulan increased in the mixture. By contrast, the other polysaccharides affected the anti-*Candida* activity of CF66I. These results indicated that pustulan prevented the compound from interacting with the cell surface of *C.*

albicans, resulting in the abrogation the CF66I-induced antifungal activity in a dose-dependent manner. Therefore, it was concluded that CF66I bound to *C. albicans* cells via a specific interaction with β-1,6-glucan, thereby exerted its antifungal effects.

DISCUSSION

Despite the existence of many currently available antifungal drugs, the increasing prevalence of life-threatening fungal diseases means that it is necessary to develop new, safer and more effective antifungal agents. In this paper, the compound CF66I exhibited excellent candidacidal activity. MIC and MFC values indicated that it could be regarded as a most promising candidate for the development of novel therapeutic agents for *Candida* infections. Moreover, it kept a good biological control performance over a wide pH range of 4.0 to 7.4. Especially at pH values near to the human physiological environment (6.0-7.4), the compound could exert its optimal candidacidal effect.

In order to elucidate the mechanisms underlying candidacidal activity, we attempted to determine the manner in which CF66I carried out the destruction of *C. albicans*. Colony count assay indicated CF66I could kill the *C. albicans* cells within 30 s. Many reports have been published about the rapid killing process of antifungals, for instance, some natural peptides could exert their antibacterial or fungicidal effects within a few minutes, and this effect was most plausible attributed to the action of these compounds on the cytoplasmic membranes [9, 10]. Accordingly, the results of our study led us to hypothesize that CF66I might act on the cell membrane of *C. albicans*, thus inducing cell lysis. Consistent with our hypothesis, CF66I treatment induced the release of intracellular K^+ (Fig 2b) and an obvious uptake of PI into the cells (Fig 2c). Consequently, the rapid candidacidal manner of CF66I was considered to act primarily on the cell membrane of *C. albicans*.

Cell wall is a unique organelle to fungi, not found in mammal cells, which has been regarded as an ideal target for the search of novel antifungal agents [11]. SEM and TEM images revealed the prominent alterations on the cell wall of *C. albicans* induced by CF66I (Fig. 3 and Fig. 4). Thickening of the fungal cell wall and an obvious increase in the thickness was observed (more than three times than control). However, no obvious alterations were observed in the composition of the fungal cell wall polysaccharides (data not shown). Thus, the ultrastructural changes of cell wall was not similar to some inhibitors of glucan synthesis, such as cilofungin (LY121019) and papulacandin B, which were reported to have secondary effects on other components of intact cells including an increase in the chitin content of the cell wall, and this could result in thickened fungal cell walls [3]. Recent evidences indicated that another mechanism similar to benanomicins and pradimicins may be used to explain the action mode of CF66I against the fungal cell wall. These two drugs killed the fungal cells by damaging the fungal cell wall structure by forming an insoluble complex with polysaccharide (mannan) [12,13]. However, when CF66I was mixed with cell wall polysaccharides, no insoluble complex was observed, but specific binding of CF66I to β-1,6-glucan was observed and its antifungal activity changed, which maybe the reason of CF66I-inducing changes on fungal cell wall.

Electron microscopic observations also clearly confirmed the candidacidal action exerted by CF66I. Profound morphological alterations were observed, such as cells swollen and distortion, which were most probably due to a change in cell permeability. These results were in agreement with earlier ultrastructural observations, indicating that the first changes are localized at the cell membrane or cell wall before any alteration can be detected in the cell interior [14]. Meanwhile, an obvious increase in buds and bud scars at several regions of the cell surface (Fig 3c) implied that the compound CF66I probably affected the normal division

process of *C. albicans*; however, that such attempts did not result in a great number of viable cells was evidenced by colony count assay. Moreover, the inability of cells to separate, resulting in the formation of numerous clusters of interconnected cells, points to the antifungal activity of CF66I.

CONCLUSION

Results from the present study indicated that the novel compound CF66I possesses excellent and rapid candidacidal activity, and its action mode can be interpreted in multiple ways. During the interaction of CF66I with *C. albicans*, it preferentially bound to β-1,6-glucan, and then exerted its antifungal activity to the cell membrane, eventually inducing the profound morphological changes and cell death. Studies on this compound maybe useful for us to develop a new class of antibiotic for *Candida* infections.

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