World J Microbiol Biotechnol (2009) 25:151–154
DOI 10.1007/s11274-008-9861-9

SHORT COMMUNICATION

Assessment of antifungal effects of a novel compound from *Burkholderia cepacia* against *Fusarium solani* by fluorescent staining

Xin Li · Chun-Shan Quan · Hui-Ying Yu · Jian-Hua Wang · Sheng-Di Fan

Received: 17 July 2008 / Accepted: 12 September 2008 / Published online: 7 October 2008

Abstract A novel compound CF66I produced by *Burkholeria cepacia* was investigated for its antifungal effects against *Fusarium solani* by three different fluorescent dyes. Dual staining with propidium iodide (PI) and fluorescein diacetate (FDA) demonstrated high doses of CF66I (120.0 μg ml^{-1}) killed the fungi by acting primarily on the cell membrane. However, at fungistatic concentration (20.0 μg ml^{-1}) of this compound, microscopic observations revealed swelling hyphae with abnormal chitin deposition, as determined by Calcofluor white (CFW) staining, which was indicative of the alterations in cell wall structure. In addition, inhibition of intracellular esterases activity was observed. These results led us to conclude that low doses of CF66I probably inhibited the fungal growth by interfering with the cell metabolic pathways.

Keywords *Burkholeria cepacia* · *Fusarium solani* · CF66I · Fluorescent staining · Fungicidal · Fungistatic

X. Li · J.-H. Wang
Dalian Institute of Chemical Physics, Chinese Academy of Sciences, 457 Zhong-Shan Road, Dalian 116023, China

X. Li · J.-H. Wang
Graduate University of the Chinese Academy of Sciences, Beijing 100049, China

C.-S. Quan (✉) · S.-D. Fan
Life Science College, Dalian Nationalities University, Economical and Technological Development Zone, Dalian 116600, China
e-mail: mikyeken@dlnu.edu.cn

H.-Y. Yu
Tieling Forestry Bureau, Tieling 112000, China

Introduction

As well-known plant pathogens and food contaminants, the fungi belonging to the genus *Fusarium* are also causative agents of superficial and systemic infections in humans, such as onychomycosis and keratomycosis (Nelson et al. 1994). Among the *Fusarium* species, *F. solani*, the most pathogenic species of the genus, accounts for nearly half of the cases of invasive infections (Guarro and Gene 1995; Ponton et al. 2000; Torres and Kontoyiannis 2003). Many studies have been conducted to investigate the activities of currently used antifungal agents against *Fusarium* spp. both in vitro and in animal models (Pujol et al. 1997; Guarro et al. 1999). However, few data are available, and as a result, the treatment of choice for *Fusarium* infections remains to be determined. Therefore, the introduction of new and more effective antifungal substances with therapeutic activity is urgently required.

The novel compound CF66I produced by *Burkholderia cepacia*, is an antifungal agent that is active against a broad spectrum of pathogenic fungi (Quan et al. 2006; Li et al. 2007). Therefore, CF66I was considered to be an appropriate candidate for the development of a novel antibiotic. Previous studies reported its strong inhibitory effects against *Fusarium* species; however, it was not clear whether the compound could kill the fungi, and meanwhile, the mechanisms underlying the aforementioned activities was little known. In the present study, by using specific fluorescent dyes, we attempted to elucidate the antifungal effects of CF66I against *F. solani*.

Materials and methods

The antifungal compound CF66I was obtained by three-step gel chromatography as previously described (Quan

et al. 2006). *F. solani* (Mart.) Sacc (MIC of CF66I, 20.0 μg ml^{-1}) was subcultured twice on potato dextrose agar (PDA; Oxiod, Basingstoke, UK) at 28°C prior to testing.

Antifungal susceptibility tests were carried out in potato dextrose broth (PDB; Oxiod, Basingstoke, UK). Briefly, precultures of hyphal suspensions (10^7 cfu ml^{-1}) of *F. solani* were incubated with 20.0 μg ml^{-1} and 120.0 μg ml^{-1} of CF66I at 28°C for 12 h, respectively. Hyphae were harvested by centrifugation, and stained with 100 μg ml^{-1} of fluorescein diacetate (FDA; Sigma-Aldrich) and 0.1% (wt/vol) propidium iodide (PI, Sigma-Aldrich), then incubated in the dark for 10 min. To examine the effect of CF66I on the localization of skeletal polysaccharides constituting the cell wall of *F. solani*, Calcofluor white (CFW, Fluorescent Brightener 28; Sigma-Aldrich) staining was performed on the same samples by adding CFW to a final concentration of 50 μg ml^{-1}, and then incubated in the dark for 30 min. Each sample was washed three times with distilled water, and then examined under an Olympus light microscopy equipped with differential interference contrast (Nomarski) and fluorescence capabilities (IX-71, Olympus). Micrographs that exhibited typical morphology of *F. solani* were shown for each condition.

After incubation with CF66I for 12 h, Intracellular K^+ released from the fungi (extracelluar K^+) was determined by using a K^+-specific electrode as previously described (Sud and Feingold 1982). Total cellular K^+ was measured after a cell suspension was incubated for 30 min in a boiling water bath. The K^+ released was calculated in terms of percentage of total cellular K^+ by using the formula: K^+ release (%) = (extracelluar K^+ in sample − extracelluar K^+ in control)/(total cellular K^+) × 100.

Results and discussion

Control hyphae of *F. solani* were well extended, as shown in Figs. 1a and 2a. FDA is a fluorescent dye that can permeate into the cytoplasm through cell membrane and is hydrolyzed to the fluorescent carboxy fluorescein in live cells by intracellular esterases, which was used to detect cell metabolic activity (Breeuwer et al. 1995). PI is used for rapid viability assessment for various cells. The membrane-impermeable fluorochrome can only pass through the membrane of injured or dead cells, which has already been used widely as a good marker for cell death associated with membrane alterations (Pore 1990). Microscopic observations indicated that all hyphae from CF66I-free cultures were well stained with FDA (Fig. 1b) and scarcely stained with PI (Fig. 1c). The dye CFW, a compound which was specific for chitin binding (Pringle 1991; Watanabe et al. 2005), was used on the same samples and showed a normal

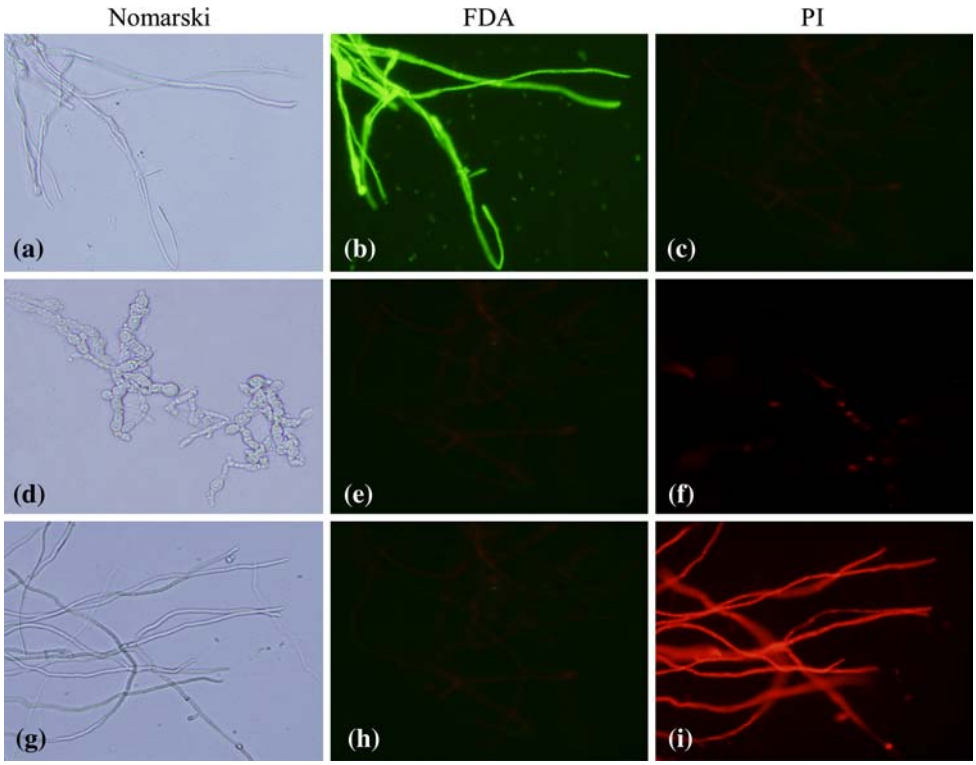

Fig. 1 Fluorescent photomicrographs of untreated and CF66I-treated *F. solani*. Hyphal cells (10^7 cfu ml^{-1}) from an untreated culture (**a**) or from cultures treated with CF66I at the concentrations of 20 μg ml^{-1} (**d**) and 120 μg ml^{-1} (**g**) were harvested after 12 h, and stained with FDA (**b**, **e**, **h**) and PI (**c**, **f**, **i**)

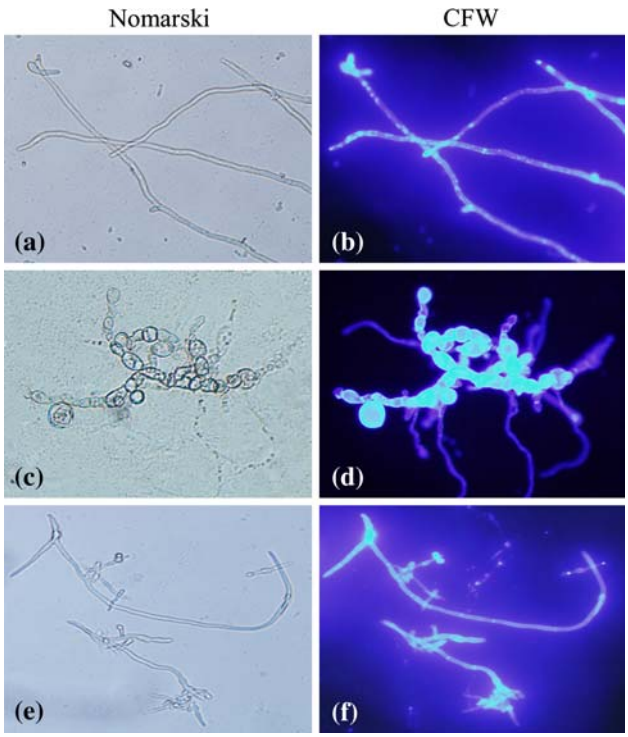

Fig. 2 CFW staining of untreated and CF66I-treated *F. solani*. Hyphal cells (10^7 cfu ml^{-1}) were incubated at 28°C without (**a**) or with CF66I at the concentrations of 20 μg ml^{-1} (**c**) and 120 μg ml^{-1} (**e**). After incubation for 12 h, samples were stained with CFW. Corresponding fluorescent images were shown in the left panels (**b**, **d**, **f**)

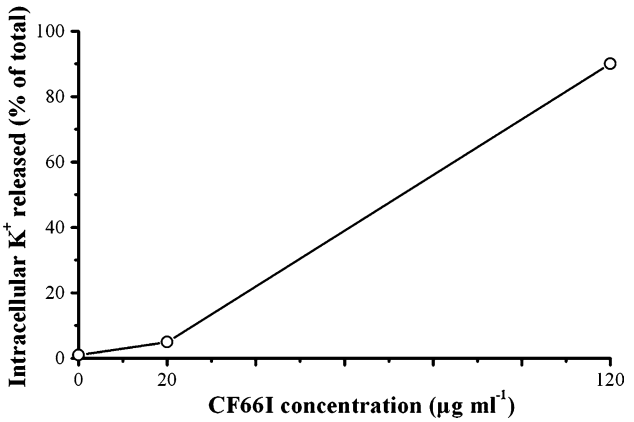

Fig. 3 CF66I-induced K^+ from *F. solani*. Hyphal cell suspensions (10^7 cfu ml^{-1}) were incubated with CF66I for 12 h, and then the intracellular K^+ released from the fungi was determined as described in 'Materials and methods'

deposition of chitin, with prevalent staining of hyphal tips and visualization of septa separating cells at regular distance in the hyphae (Fig. 2b).

Incubation with 120.0 μg ml^{-1} of CF66I revealed collapsed hyphae with visualizations of PI and FDA staining, indicating extensive cell death and membrane permeation (Fig. 1h, i). At this high CF66I concentration, no marked alterations in hyphae morphology and chitin deposition were observed (Figs. 1g and 2e, f). These findings led us to hypothesize that high doses of CF66I might kill *F. solani* by acting primarily on the cell membrane. In accordance with our hypothesis, over 90% of intracellular K^+ from hyphal cells was observed be leaked out after CF66I treatment (Fig. 3).

However, after exposure to 20.0 μg ml^{-1} of CF66I (MIC), fungal growth of *F. solani* was completely inhibited, and profound morphological changes were observed. Hyphae grew abnormally and became swelling, and large amounts of balloon-shaped cells were formed (Figs. 1d, 2c). With this treatment, most cells remained impermeable to PI (Fig. 1f) and the leakage of intracellular K^+ was minimal (Fig. 3), indicating the maintenance of cell viability associated with membrane integrity. But interestingly, our results showed that the compound significantly impaired the intracellular esterases activity of *F. solani*, as the treated hyphae were not stained with FDA (Fig. 1e). In addition, abnormal chitin deposition of the hyphae was observed, as determined by CFW staining. Chitin was not concentrated in regions of high chitin content (as shown in control cells), but instead, occurred preferentially on balloon-shaped cells located in the middle of hyphae (Fig. 2d). Similar findings were described by Muñoz et al. (2006), which demonstrated that this phenomenon was due to the alterations in cell wall structure. These results indicated that CF66I might be translocated across the cell membranes and located inside the cells, wherein they induced a diversity of inhibitory activities that disrupted normal cell functions, which included the alterations of cell wall synthesis and inhibition of intracellular esterases activities. As we all know, intracellular enzyme activity is often used as an indicator of cell metabolic activity. Therefore, these findings led us to conclude that CF66I inhibited the growth of *F. solani* by interfering with cell metabolic pathways, rather than membrane permeation. This action pattern was similar to those of some lipophilic antimicrobial agents, such as butenafine and ibupropen (Iwatani et al. 1993; Pina-Vaz et al. 2000), which were reported to inhibit the fungal growth by interfering with cell metabolic pathways. Likewise, Brogden (2005) demonstrated that several antimicrobial peptides acted in this mode against pathogenic fungi and bacteria.

Results from the present study indicated that high doses of CF66I (120.0 μg ml^{-1}) were fungicidal, whereas low doses (20.0 μg ml^{-1}) were fungistatic. Different morphological effects, as well as modes of action, of CF66I against *F. solani* at different concentrations were revealed, which probably related with its complicated structural characteristics (Quan et al. 2006). Therefore, further studies on this compound might be useful for us to develop a novel class of antifungal agents.

Acknowledgments We are grateful to Dalian Nationalities University for providing supports for this research. This work was also supported by a grant from Scientific Research Foundation for the Returned Overseas Chinese Scholars, State Education Ministry (no. 20052101).

References

Breeuwer P, Dorcourt JL, Bunschoten N, Zwietering MH, Rombouts FM, Abee T (1995) Characterization of uptake and hydrolysis of fluorescein diacetate and carboxyfluorescein diacetate by intracellular esterases in *Saccharomyces cerevisiae*, which result in accumulation of fluorescent product. Appl Environ Microbiol 61:1614–1619

Brogden KA (2005) Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat Rev Microbiol 3:238–250. doi:10.1038/nrmicro1098

Guarro J, Gene J (1995) Opportunistic fusarial infections in humans. Eur J Clin Microbiol Infect Dis 14:741–754. doi:10.1007/BF01690988

Guarro J, Pujol I, Mayayo E (1999) In vitro and in vivo experimental activities of antifungal agents against *Fusarium solani*. Antimicrob Agents Chemother 43:1256–1257

Iwatani W, Arika T, Yamaguchi H (1993) Two mechanisms of butenafine action on *Candida albicans*. Antimicrob Agents Chemother 37:785–788

Li X, Quan CS, Fan SD (2007) Antifungal activity of a novel compound from *Burkholderia cepacia* against plant pathogenic fungi. Lett Appl Microbiol 45:508–514. doi:10.1111/j.1472-765X.2007.02221.x

Muñoz A, López-García B, Marcos JF (2006) Studies on the mode of action of the antifungal hexapeptide PAF26. Antimicrob Agents Chemother 11:3847–3855. doi:10.1128/AAC.00650-06

Nelson PE, Dignami MC, Anaissie EJ (1994) Taxonomy, biology, and clinical aspects of *Fusarium* species. Clin Microbiol Rev 7:479–504

Pina-Vaz C, Sansonetty F, Rodrigues AG, Martinez-De-Oliveira J, Fonseca AF, Mardh PA (2000) Antifungal activity of ibuprofen alone and in combination with fluconazole against *Candida* species. J Med Microbiol 49:831–840

Ponton J, Ruchel R, Clemons KV, Coleman DC, Grillot R, Guarro J et al (2000) Emerging pathogens. Med Mycol 38:225–236. doi:10.1080/714030939

Pore RS (1990) Antibiotic susceptibility testing of *Candida albicans* by flow cytometry. Curr Microbiol 20:323–328. doi:10.1007/BF02091913

Pringle JR (1991) Staining of bud scars and other cell-wall chitin with Calcofluor. Methods Enzymol 194:732–735. doi:10.1016/0076-6879(91)94055-H

Pujol I, Guarro J, Gene J, Sala J (1997) In vitro antifungal susceptibility of clinical and environmental *Fusarium* spp.strains. J Antimicrob Chemother 39:163–167. doi:10.1093/jac/39.2.163

Quan CS, Zheng W, Liu Q, Ohta Y, Fan SD (2006) Isolation and characterization of a novel *Burkholderia cepacia* with strong antifungal activity against *Rhizoctonia solani*. Appl Microbiol Biotechnol 72:1276–1284. doi:10.1007/s00253-006-0425-3

Sud IJ, Feingold DS (1982) Action of antifungal imidazoles on *Staphylococcus aureus*. Antimicrob Agents Chemother 22:470–474

Torres H, Kontoyiannis DP (2003) Hyalohyphomycoses. In: Dismukes WE, Pappas PG, Sobel JD (eds) Oxford textbook of clinical mycology. Oxford University Press, New York, pp 252–270

Watanabe H, Azuma M, Igarashi K, Ooshima H (2005) Analysis of chitin at the hyphal tip of *Candida albicans* using Calcofluor white. Biosci Biotechnol Biochem 69:1798–1801. doi:10.1271/bbb.69.1798