Gentian Violet Exhibits Activity against Biofilms Formed by Oral *Candida* Isolates Obtained from HIV-Infected Patients[▽]

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Received 18 November 2010/Returned for modification 2 January 2011/Accepted 15 March 2011

The effect of gentian violet against *Candida albicans* and non-*Candida albicans* biofilms formed on polymethylmethacrylate strips was evaluated using a dry weight assay and confocal laser scanning microscopy. The ability of gentian violet to inhibit *Candida albicans* germination was also assessed. Gentian violet activity against *Candida* biofilms was demonstrated by a reduction in dry weight, disruption of biofilm architecture, and reduced biofilm thickness. Additionally, gentian violet inhibited *Candida* germination in a concentration-dependent manner.

Oral candidiasis is a common infection in HIV-infected patients (1, 9). In addition to host immune status, the ability of *Candida* to form biofilms is believed to be a key determinant of this disease (12). *Candida* biofilms are resistant to commonly used antifungals (5, 6). Development of an antibiofilm agent to treat oral candidiasis is, therefore, crucial. Previously, we showed that gentian violet (GV), a triphenylmethane dye used clinically at concentrations between 0.5% and 1% to treat oral candidiasis in HIV-infected patients, was fungicidal against planktonic *Candida* cells (10). In this study, we investigated the effect of GV on *Candida* biofilm formation. Since germination is a key component of *Candida* pathogenicity and biofilms, we also investigated its effect on the ability of *Candida albicans* to germinate.

(This work was presented in part at the 48th Annual Inter-science Conference on Antimicrobial Agents and Chemotherapy/46th Annual Infectious Diseases Society of America Meeting, Washington, DC, 25 to 28 October 2008 [10a].)

Isolates obtained from the oral cavities of HIV-infected patients were tested in this study. The susceptibility of planktonic *Candida* cells to GV (Sigma-Aldrich Co., St. Louis, MO) and fluconazole (FLC) (Pfizer Pharmaceuticals, New York, NY) was determined using the Clinical and Laboratory Standards Institute M27-A3 method (3). The MICs of FLC and GV, respectively, against each planktonic strain were as follows: 0.25 µg/ml and 0.06 µg/ml for *C. albicans* strain 9105 (FLC susceptible); 16 µg/ml and 0.06 µg/ml for *C. albicans* strain 9920 (FLC resistant); 16 µg/ml and 0.06 µg/ml for *C. glabrata* strain 9848 (FLC resistant); 2 µg/ml and 0.25 µg/ml for *C. glabrata* strain 9040 (FLC susceptible); 0.5 µg/ml and 0.06

µg/ml for *C. parapsilosis* strain 9283; and 0.25 µg/ml and 0.06 µg/ml for *C. parapsilosis* strain 8966.

Biofilms were formed on polymethylmethacrylate substrate, exposed to GV for 48 h, and quantified using a dry weight assay (2). To determine the effect of GV on biofilm architecture, biofilms were exposed to 4 µg/ml of GV (this concentration was selected because it caused significant reduction in biofilm mass [Table 1]). Treated cells were examined using confocal scanning laser microscopy (2). Germination of *C. albicans* strains in the presence of different concentrations of GV (0.5× to 5× the individual MIC of each isolate) was compared to that of cells exposed to fetal bovine serum (FBS) (HyClone, Logan, UT), a known inducer of germination (7).

Each experiment was performed in triplicate on three separate days ($n = 9$ for each isolate). Statistical analysis, including analysis of variance (ANOVA) *post hoc* analysis with the Bonferroni-Dunn calculation, was performed using SAS soft-

TABLE 1. Effect of gentian violet on biomass of *Candida* biofilms^a

<i>Candida</i> species	Strain	Biofilm mass (mg) (mean ± SEM)		<i>P</i> value ^b
		Control	Treated with GV	
<i>C. albicans</i>	9105 ^c	2.39 ± 0.69	1.06 ± 0.48	0.0004
	9920 ^d	2.17 ± 0.61	0.79 ± 0.27	0.002
<i>C. parapsilosis</i>	9283	1.53 ± 0.33	0.69 ± 0.19	<0.0001
	8966	1.58 ± 0.33	0.76 ± 0.11	0.06
<i>C. glabrata</i>	9848	1.91 ± 0.68	1.04 ± 0.41	<0.0001
	9040	2.43 ± 0.24	1.92 ± 0.49	1.00

^a The effect of gentian violet (GV) (4 µg/ml) on biomass of *Candida* biofilms was determined.

^b The *P* value for each strain was determined by comparing biofilm mass of cells grown in the absence (control) or presence of GV.

^c This strain is susceptible to fluconazole.

^d This strain is resistant to fluconazole.

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[▽] Published ahead of print on 28 March 2011.

FIG. 1. Effect of gentian violet on the germination of *Candida* cells. (A) *C. albicans* 9105; (B) *C. albicans* 9920. Data from three separate experiments are shown. All values are means $\pm$ standard deviations (SD) (error bars).

ware (version 9.2; SAS Institute, Cary, NC). A *P* value of <0.05 was considered statistically significant.

Our data showed that all *Candida* isolates tested formed robust biofilms, with *C. albicans* forming significantly more biofilm than *C. parapsilosis* (mean mass, 2.31 ± 0.65 mg and 1.54 ± 0.32 mg, respectively; $P = 0.0005$). *C. glabrata* tended to form more biofilms than *C. parapsilosis* (mean mass, 1.97 ± 0.66 mg and 1.54 ± 0.32 mg, respectively; $P = 0.05$). There was no significant difference between biofilms formed by *C. albicans* compared to *C. glabrata* ($P = 0.2$).

GV at 4 μ g/ml reduced the biofilm mass of all *Candida* species (mean reduction was 0.95 ± 0.42 mg, 1.21 ± 0.54 mg, and 0.71 ± 0.17 mg for *C. albicans*, *C. glabrata*, and *C. parapsilosis*, respectively). There was no statistically significant difference in the activity of GV for *C. albicans* compared to *C. glabrata* ($P = 0.31$) and *C. parapsilosis* ($P = 0.43$). However, there was a significant difference in the activity of GV for *C. glabrata* compared to *C. parapsilosis* ($P = 0.01$). GV significantly reduced the biomass of biofilms formed by both FLC-susceptible and -resistant *C. albicans* strains. Furthermore, GV significantly reduced biofilm formation by *C. parapsilosis* 9283 ($P < 0.0001$) (Table 1). Although exposure of *C. parapsilosis* strain 8966 to GV resulted in a trend of decreased biofilm biomass, this reduction was not statistically significant ($P = 0.06$). Exposure of *C. glabrata* strains to GV significantly reduced the biomass of biofilms formed by strain 9848 ($P < 0.0001$) but had no effect on biofilms formed by the second strain (strain 9040) ($P = 1$).

Confocal laser scanning microscopy analyses showed that GV treatment significantly reduced biofilm thickness of FLC-susceptible and -resistant *C. albicans* strains (thickness of 22 ± 3.60 and 46.66 ± 7.57 μ m, respectively) compared to untreated controls (98 ± 14.73 μ m [$P = 0.008$] and 62.33 ± 1.52 μ m [$P = 0.07$], respectively). GV treatment also led to a decrease in the thickness of biofilms formed by *C. glabrata* 9848 and *C. parapsilosis* 9283 (27.33 ± 7.09 μ m and 41 ± 3.60 μ m, respectively) compared to untreated controls (64 ± 10.44 μ m [$P = 0.06$] and 62.66 ± 1.15 μ m [$P = 0.004$], respectively).

Our data showed that GV treatment inhibited the ability of *C. albicans* FLC-susceptible and -resistant strains to germinate in a dose-dependent manner (Fig. 1A and B). As the GV concentration increased, greater inhibition occurred.

The mechanism underlying the inhibition of biofilms by GV

is unknown. Previous studies using planktonic cells suggested multiple mechanisms of action for GV: (i) production of hydroxyl/perhydroxy radicals, which induce cell penetration and DNA binding of positively charged GV; (ii) induction of permeability leading to dissipation of mitochondrial membrane potential and cell lysis; (iii) alteration of redox potential; (iv) inhibition of microbial cell wall formation; and (v) photodynamic reduction to free radicals (4). It is possible that production of the hydroxyl/perhydroxy radicals may facilitate the penetration of GV through the biofilm matrix leading to inhibition of fungal cell wall synthesis. Potential differences in extracellular matrix and cell wall structures of *C. glabrata* and *C. parapsilosis* may be the reason for strain-dependent activity of GV against biofilms formed by these species.

Our data showed that GV inhibited candidal germination in a concentration-dependent manner. Similarly, Ying et al. (11) showed that *Candida* cells exposed to GV reduced germination compared to untreated controls. Given that hyphae are key constituents of *Candida* biofilms, it is possible that the mechanism of action of GV against *Candida* biofilms may also involve inhibition of germination.

This study has a number of limitations, including the following. (i) The effect of GV on biofilms formed by *Candida tropicalis*, *C. dubliniensis*, and *C. krusei* was not investigated, even though they have been reported to be present in HIV patients (8). (ii) The effect of GV on biofilm formation was assessed only by dry weight analysis and not 2,3-bis-(2-methoxy-4-nitro-5-sulphenyl)-(2H)-tetrazolium-5-carboxanilide (XTT) or viability assays. (iii) Detailed investigations regarding the mechanisms by which GV inhibits biofilm were not undertaken.

Gentian violet may play a potential role in the treatment of oral candidiasis due to its antibiofilm and antigermination activity. Clinical studies to determine the efficacy of GV in the treatment of this disease are warranted.

We thank Charles Bark for assistance with statistical analysis.

This work is supported by National Institute of Health (NIH) grants R01-DE017846 and BRS-ACURE Q0600136 (Oral HIV/AIDS Research Alliance [OHARA]) to Mahmoud A. Ghannoum and NIH grant 1R21AI074077-01A2 to Pranab K. Mukherjee.

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