Gentian Violet Exhibits Activity against Biofilms formed by Oral Candida isolates Obtained from HIV-infected Patients

Running title: Anti-biofilm activity of gentian violet

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Abstract
The effect of gentian violet against *albicans* and non-*albicans* *Candida* 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 reduction in dry weight, disruption of biofilm architecture, and a reduced biofilms 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 antibiotic 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%-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 C. albicans to germinate.

Isolates obtained from oral cavities of HIV-infected patients were tested in this study. Susceptibility of planktonic Candida cells to GV (Sigma-Aldrich Co., St. Louis, MO) and fluconazole (Pfizer Pharmaceuticals, New York, N.Y.) was determined using the Clinical and Laboratory Standards Institute M27-A3 method (3). The minimum inhibitory concentrations (MICs) of fluconazole (FLC) and GV, respectively, against each planktonic strain were as follows: C. albicans strain 9105 - 0.25 µg/ml (FLC-susceptible) and 0.06 µg/ml; C. albicans strain 9920 - 16 µg/ml (FLC-resistant) and 0.06 µg/ml; C. glabrata strain 9848 - 16 µg/ml (FLC-resistant) and 0.06 µg/ml; C. glabrata strain 9040 - 2 µg/ml (FLC-susceptible) and 0.25 µg/ml; C. parapsilosis strain 9283 - 0.5 µg/ml and 0.06 µg/ml; and C. parapsilosis strain 8966 - 0.25 µg/ml and 0.06 µg/ml.
Biofilms were formed on polymethylmethacrylate substrate, exposed to GV for 48 hours, 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 (0.5x to 5x 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 software (version 9.2; SAS Institute, Cary, NC). A P value of < 0.05 was considered significant.

Our data showed that all tested Candida isolates formed robust biofilms, with C. albicans forming significantly more biofilms than C. parapsilosis (Mean mass 2.31±0.65 mg and 1.54±0.32 mg, respectively, P=0.0005). C. glabrata trended to form higher biofilms compared to C. parapsilosis (Mean mass, 1.97±0.66 mg, 1.54±0.32 mg, respectively, P=0.05). No significant difference between biofilms formed by C. albicans compared to C. glabrata (P=0.2).

GV at 4 µg/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 \textit{C. albicans} compared to \textit{C. glabrata} ($P=0.31$) and \textit{C. parapsilosis} ($P=0.43$). However, there was a significant difference in the activity GV for \textit{C. glabrata} when compared to \textit{C. parapsilosis} ($P=0.01$). GV significantly reduced the biomass of biofilms formed by both FLC-susceptible and -resistant \textit{C. albicans} strains. Furthermore, GV significantly reduced biofilms formation by \textit{C. parapsilosis} 9283 ($P < 0.0001$) (Table 1). Although exposure of \textit{C. parapsilosis} 8966 to GV resulted in a trend of decreased biofilm biomass, this reduction was not statistically significant ($P=0.06$). Exposure of \textit{C. glabrata} strains to GV significantly reduced the biomass of biofilms formed by strain 9848, $P < .0001$), but had no effect on biofilms formed by the second strain (9040, $P = 1$).

Confocal analyses showed GV treatment significantly reduced biofilm thickness of FLC-susceptible and -resistant \textit{C. albicans} strains ($22 \pm 3.60$ and $46.66 \pm 7.57$ µm, respectively) compared to untreated controls (thickness $= 98\pm14.73$ µm, ($P =0.008$), and $62.33\pm1.52$ µm ($P=0.07$), respectively). GV treatment also led to a decrease in the thickness of biofilms formed by \textit{C. glabrata} 9848 and \textit{C. parapsilosis} 9283 ($27.33 \pm 7.09$ µm and $41 \pm 3.60$ µm, respectively), compared to untreated controls ($64 \pm 10.44$ µm ($P=0.06$) and $62.66 \pm 1.15$ µm ($P=0.004$), respectively).

Our data showed that GV treatment inhibited the ability of \textit{C. albicans} FLC-susceptible and -resistant strains to germinate in a dose-dependant manner (Fig.1, A 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: (a) production of hydroxyl/perhydroxy radicals, which induce cell penetration and DNA binding of positively charged GV, (b) induction of permeability leading to dissipation of mitochondrial membrane potential and cell lysis, (c) alteration of redox potential, (d) inhibition of microbial cell wall formation, and (e) 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 1- the effect of GV on biofilms formed by C. tropicalis, C. dubliniensis and C. krusei even though they were reported to be present in HIV patients, was not investigated (8), 2- the effect of GV on biofilm formation was only assessed by dry weight analysis and not XTT or viability assays, 3- detailed investigations regarding the mechanisms by which GV inhibits biofilm was not undertaken.
Gentian violet may play a potential role in the treatment of oral candidiasis due to its antibiofilm and anti-germination activity. Clinical studies to determine the efficacy of GV in the treatment of this disease are warranted.
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REFERENCES


Table 1: The effect of gentian violet (4 µg/ml) on biomass of *Candida* biofilms

<table>
<thead>
<tr>
<th><em>Candida</em> species</th>
<th>Strain number</th>
<th>Biofilm mass (mg)</th>
<th>P value*</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>GV</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>9105§</td>
<td>2.39±0.69</td>
<td>1.06±0.48</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>9920†</td>
<td>2.17±0.61</td>
<td>0.79±0.27</td>
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<tr>
<td><em>C. parapsilosis</em></td>
<td>9283</td>
<td>1.53±0.33</td>
<td>0.69±0.19</td>
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<tr>
<td><em>C. parapsilosis</em></td>
<td>8966</td>
<td>1.58±0.33</td>
<td>0.76±0.11</td>
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<tr>
<td><em>C. glabrata</em></td>
<td>9848</td>
<td>1.91±0.68</td>
<td>1.04±0.41</td>
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<tr>
<td><em>C. glabrata</em></td>
<td>9040</td>
<td>2.43±0.24</td>
<td>1.92±0.49</td>
</tr>
</tbody>
</table>

§fluconazole-susceptible, †fluconazole-resistant, *P-value for each strain was determined by comparing biofilm mass of cells grown in the absence (control) or presence of GV.
Figure 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 ± SD.