Involvement of calcium inhibitable binding to the cell wall in the fungicidal activity of CAN-296

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CAN-296 is a heat stable, complex carbohydrate (molecular mass 4300 Da) isolated from the cell wall of the filamentous fungus \textit{Mucor rouxii}. It possesses potent in-vitro fungicidal activity against a wide spectrum of pathogenic yeasts, including azole-resistant isolates of \textit{Candida albicans} and \textit{Candida glabrata}. As a preliminary step in the study of the mode of action of this novel antifungal agent, we investigated the effect of various cations on the antifungal activity as well as the binding of CAN-296 to intact cells and cell-wall fractions of \textit{C. albicans}. The antifungal activity of CAN-296 was inhibited by low concentrations of calcium, magnesium and lithium and by high concentrations of barium, cobalt and manganese, but not by potassium and copper. The calcium-mediated inhibition of the antifungal activity of CAN-296 was readily reversible by the removal of calcium by dialysis, and the fungicidal activity of the inhibited compound was fully restored. The uptake/binding of CAN-296 to intact cells and to the cell-wall fraction of \textit{C. albicans} was time and concentration dependent. Maximum uptake/binding was obtained at 5 mg/L within 60 min and was associated with the aggregation of intact cells. Washing intact cells and the cell-wall fraction preincubated with radiolabelled CAN-296 with 150-fold excess of unlabelled compound failed to remove CAN-296 associated with the intact cells and the cell-wall fraction, suggesting that the binding of CAN-296 to \textit{C. albicans} is tight. The uptake/binding of CAN-296 and the drug-mediated aggregation of intact cells were inhibited by calcium in a concentration-dependent manner. The fact that CAN-296 is a fungicidal agent that binds to intact cells and the cell-wall fraction of \textit{C. albicans} very tightly, together with the observation that calcium was able to inhibit the fungicidal activity as well as the uptake/binding of CAN-296, suggests that the mode of action of this novel antifungal agent may involve interaction with the cell wall of \textit{C. albicans}.

\textbf{Introduction}

CAN-296 is a naturally occurring heat stable complex carbohydrate isolated from the cell wall of the fungus \textit{Mucor rouxii}. It consists mostly of 1,4-, 3,4- and 4,6-linked, and terminal \textit{N}-acetylglucosamine residues. We have previously shown that CAN-296 possesses excellent in-vitro fungicidal activity against a wide spectrum of pathogenic yeasts including azole-resistant isolates of \textit{Candida albicans} and \textit{Candida glabrata}.\textsuperscript{1} Compared with the azoles and polyene compounds, it has a narrow MIC range and a rapid onset of fungicidal action (99.9% killing within 60 min). CAN-296 appears to have no cross-resistance with any currently used antifungal agents.\textsuperscript{1}

The mode of action of this novel compound is unknown. In recent years, the role of carbohydrate in biological interactions has increasingly been recognized.\textsuperscript{2} Carbohydrate-binding receptors, initiating diverse signal transduction pathways, have been described in hepatocytes,\textsuperscript{3} alveolar macrophages\textsuperscript{4} and other systems.\textsuperscript{5} As the yeast cell wall is a dynamic system composed mostly of complex carbohydrates and proteins, we hypothesized that the mechanism of action of CAN-296 may involve interaction of this compound with the fungal cell wall. In this paper, we present data to suggest that CAN-296 binds to fungal cell wall and mediates aggregation of \textit{C. albicans}. The CAN-296-elicited aggregation of the fungal cells may be the initial step that leads to the potent fungicidal action of this compound.

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Materials and methods

Organism and culture condition

*C. albicans* 90028 obtained from the American Type Culture Collection (ATCC) was used throughout. To prepare a working culture, approximately 0.01 mL of the stock culture (stored at −70°C in litmus milk; Becton Dickinson Microbiology Systems, Cockeysville, MD, USA), was plated on to Sabouraud Dextrose Agar (SDA) and incubated for 48 h. From this primary culture a single colony was subcultured (24 h) on to SDA, and used as the source of inoculum for all experiments.

Assay of fungicidal activity

A fresh 24 h culture of *C. albicans* 90028 prepared in yeast extract peptone dextrose (YPD) broth (Sigma Chemical Company, St Louis, MO, USA) was diluted approximately 1000-fold to obtain a cell density of $1 \times 10^6$ cfu/mL. One millilitre aliquots of the diluted culture were incubated with an inhibitory concentration (10 mg/L) of CAN-296 for 60 min at 35°C. Aliquots of 0.05 mL of the treated cell suspension were removed, serially diluted ($10^2$- to $10^6$-fold) and triplicate 0.1 mL aliquots were spread on YPD agar (Sigma Chemical Company) plates. The plates were incubated at 35°C for 24 h and the number of cfu/mL of culture was calculated and plotted against different variables. Where applicable, a growth control without CAN-296 was used and compared with the time–kill study as previously described.  

To determine the effect of various cations on the fungicidal activity of CAN-296, approximately $1 \times 10^6$ cells of *C. albicans* 90028 were incubated in 1 mL of YPD broth with the drug (10 mg/L) in the presence of various concentrations (0.312–5 g/L) of CaCl$_2$, BaCl$_2$, CoCl$_2$, CuCl$_2$, KCl, LiCl, MgCl$_2$ and MnCl$_2$ for 60 min, and the fungicidal activity assay was performed as described above.

Dialysis

One millilitre aliquots of CAN-296 solution (250 mg/L) were incubated in duplicate with CaCl$_2$ (20 g/L), at 35°C for 60 min. The calcium-treated CAN-296 was then transferred to sterile dialysis bags (Spectrum, Houston, TX, USA) and dialysed against sterile double-distilled water (1 L) at 4°C overnight. The dialysis solution was changed twice. The contents of the dialysis bags were then collected in microfuge tubes and heated to 65°C for 60 min to kill any contaminant microorganisms. The CAN-296 solution was allowed to cool to room temperature and used in the fungicidal activity assays as described above. Dialysed Ca$^{2+}$-untreated CAN-296 and Ca$^{2+}$-treated but undialysed CAN-296 were used as controls.

Aggregation assay

*C. albicans* 90028 cells (1×10$^7$ cells in 1 mL YPD broth) were incubated with CAN-296 10 mg/L in the presence and absence of calcium chloride (0–2.78 g/L) at 35°C for 60 min. Approximately 2 μL aliquots of the cell suspension were placed on a microscope slide at various time intervals and visually inspected for the formation of aggregates. Intact cells incubated with calcium in the absence of CAN-296 were used as control.

Preparation of cell-wall fragments

*C. albicans* 90028 cells were grown for 18 h in 1 L YPD broth at 35°C, collected by centrifugation (5000 g for 1 min at 4°C) and washed twice with 30 mM Tris buffer (pH 7.0). The resulting pellet was stored at −20°C. The frozen pellet (approximately 2 g wet weight) was transferred to a mortar and ground to a fine powder with a pestle in dry ice with 0.5 g glass beads/g wet weight of the cell pellet. The ground cells were resuspended in 10 mL of 30 mM Tris buffer (pH 7.0) and centrifuged at 5000 g for 10 min at 4°C to remove intact cells, glass beads and other heavy particulate matter. Microscopic examination of the supernatant showed ≤10% intact cells. The clarified supernatant was centrifuged at 17,500 g for 30 min at 4°C to sediment the broken cells and cell-wall fragments. The resulting pellet was resuspended in 30 mM Tris buffer (pH 7.0), washed twice with 30 mM Tris buffer (pH 7.0), resuspended in 6 mL of 30 mM Tris buffer (pH 7.0) and used immediately for $[^3]$H]CAN-296 binding studies.


$[^3]$H]-labelled CAN-296 (specific activity 2 mCi/mL) was obtained from American Radiolabeled Chemicals Inc. (St Louis, MO, USA). *C. albicans* 90028 cells were collected, and washed with and resuspended in YPD broth at a cell density of $2 \times 10^7$ cells/mL. Five mL aliquots of the cell suspension were incubated in duplicate with CAN-296 5 mg/L (specific activity 1.0 μCi/μg CAN-296) at room temperature for 60 min and 0.5 mL aliquots were removed at various intervals. The cells were collected by centrifugation for 1 min in a microcentrifuge, and washed three times rapidly by centrifugation with 30 mM Tris buffer (pH 7.0). The washed cell pellet was resuspended in 0.1 mL of 30 mM Tris buffer (pH 7.0) and the radioactivity associated with the cells was determined by scintillation counting with 5 mL Aquasol (NEN Life Science Products, Boston, MA, USA) as the scintillation fluid.

To study the concentration dependence of the uptake/binding of CAN-296 in *C. albicans*, 2×10$^7$ cells were incubated in 1 mL YPD broth in duplicate in the presence of $[^3]$H]CAN-296 0.312–10 mg/L (specific activity 1.0 μCi/μg CAN-296) for 60 min at room temperature. The cells were then collected by centrifugation and washed, and the
The effect of washing with unlabelled CAN-296 on the uptake of \[^{3}H\]CAN-296 in intact cells was examined as follows. Approximately 1 \( \times 10^8 \) cells were incubated in 5 mL YPD broth in duplicate in the presence of \[^{3}H\]CAN-296 5 mg/L (specific activity 1 \( \mu \)Ci/\( \mu \)g CAN-296) at room temperature for 120 min. The washing was initiated by the addition of a 150-fold excess of unlabelled CAN-296 at 60 min of incubation. Aliquots (0.5 mL) of the cell suspension were removed at various time intervals before and after the addition of the unlabelled CAN-296 and the radioactivity associated with the cells before and after the addition of unlabelled CAN-296 was determined as described above.

The effect of washing with unlabelled CAN-296 on the binding of \[^{3}H\]CAN-296 to cell-wall fragments, a 150-fold excess of unlabelled CAN-296 was added at 60 min of incubation. Aliquots of the cell-wall fraction were removed at various time intervals before and after the addition of the unlabelled CAN-296. The cell-wall fragments were collected, washed with 30 mM Tris buffer (pH 7.0) by centrifugation and radioactivity associated with the cell-wall fragments was determined by scintillation counting.

### Results

#### Inhibition of the antifungal activity of CAN-296 by calcium and other divalent cations

The effect of various cations on the antifungal activity of CAN-296 on \( C. \) albicans was examined by the addition of various amounts of the respective metal chlorides to the growth medium along with the addition of an inhibitory concentration of CAN-296 (10 mg/L). As shown in Table I, calcium, magnesium and lithium at concentrations >0.625 g/L inhibited the antifungal activity of CAN-296, and complete inhibition of CAN-296 activity against \( C. \) albicans was obtained at 1.25 g/L. Barium, cobalt and manganese inhibited CAN-296 only at a relatively high concentration (2.5 g/L), whereas copper and potassium had no effect on the activity of CAN-296 at the concentrations used. In these experiments, cations were present simultaneously with the CAN-296 throughout the growth period. Cations alone at the indicated concentrations had no significant effect on the growth of \( C. \) albicans.

Removal of calcium from pretreated CAN-296 solution

<table>
<thead>
<tr>
<th>Metal chloride</th>
<th>0.312</th>
<th>0.625</th>
<th>1.25</th>
<th>2.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{BaCl}_2 )</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>64 ± 1.66</td>
</tr>
<tr>
<td>( \text{CaCl}_2 )</td>
<td>0</td>
<td>17.6 ± 1.06</td>
<td>100 ± 2.51</td>
<td>104 ± 1.52</td>
</tr>
<tr>
<td>( \text{CoCl}_4 )</td>
<td>0</td>
<td>0</td>
<td>55.5 ± 0.44</td>
<td></td>
</tr>
<tr>
<td>( \text{CuCl}_2 )</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>( \text{KCl} )</td>
<td>5.8 ± 0.54</td>
<td>52.9 ± 2.51</td>
<td>118 ± 1.68</td>
<td>127 ± 2.67</td>
</tr>
<tr>
<td>( \text{LiCl} )</td>
<td>0</td>
<td>0</td>
<td>120 ± 1.48</td>
<td></td>
</tr>
<tr>
<td>( \text{MnCl}_3 )</td>
<td>12.6 ± 1.25</td>
<td>100 ± 2.07</td>
<td>114 ± 1.51</td>
<td></td>
</tr>
<tr>
<td>( \text{MgCl}_2 )</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Per cent inhibition = (cfu/cfu) \times 100, where cfu is number of colony forming units obtained in the presence of 10 mg/L CAN-296 with various concentrations of the metal chloride and cfu is number of colony forming units obtained in the absence of the drug and cation (growth control).*

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by dialysis at 4°C overnight completely restored the anti-fungal activity of the compound. Calcium-treated and dialysed as well as calcium-untreated but dialysed CAN-296 killed approximately 99% *C. albicans* cells. In contrast, calcium-treated CAN-296 was inactive (0% kill). The data also suggest that the effect of calcium on CAN-296 is direct and that binding of calcium to CAN-296 is loose enough for calcium to be removed by dialysis.

**CAN-296-mediated aggregation of *C. albicans***

Intact cells of *C. albicans* were aggregated and formed clumps in the presence of MIC and higher concentrations of CAN-296. Microscopic examination of the intact cells in the presence of CAN-296 at various time intervals revealed that the aggregate formation reached a plateau within 60 min. Addition of calcium (1–5 g/L) completely inhibited the CAN-296-induced aggregation of cells.

**CAN-296 binding studies**

Figure 1a shows the uptake/binding of CAN-296 to intact *C. albicans* cells. The uptake/binding of $[^3]$H]CAN-296 was rapid and reached a plateau within 40 min. Figure 1b shows that the uptake/binding of $[^3]$H]CAN-296 by *C. albicans* cells was concentration dependent in the range of 0.5–3 mg/L, and reached the maximum at 3–5 mg/L. However, at a concentration of 10 mg/L we observed a sharp decline in the radioactivity associated with intact cells.

In addition to intact cells, we also examined the binding of $[^3]$H]CAN-296 to the cell-wall fractions of *C. albicans*, and the results were compared with those obtained for the intact cells. As shown in Figure 2, the uptake/binding of CAN-296 to intact cells and the cell-wall fraction were very similar. Addition of a 150-fold excess of unlabelled CAN-296 at mid-course of incubation failed to decrease significantly the radiolabelled CAN-296 associated with either the intact cells or with the cell-wall fraction. The lack of displacement of bound radiolabelled CAN-296 with an excess of unlabelled CAN-296 suggests that this agent binds to the intact cells and the cell-wall fraction of *C. albicans* very tightly.

The effect of calcium on the uptake/binding of $[^3]$H]CAN-296 to intact *C. albicans* cells is shown in Table II. Calcium inhibited the uptake/binding of radiolabelled CAN-296 to *C. albicans* cells in a concentration-dependent manner with time. At 5.5 g/L, calcium inhibited cell-associated radioactivity by approximately 95% after 60 min of incubation (Table II).

**Discussion**

CAN-296 is a naturally occurring, heat stable complex carbohydrate molecule with a molecular mass of approximately 4300 Da. It has excellent in-vitro activity against a wide spectrum of pathogenic yeast including fluconazole-resistant and amphotericin B-resistant isolates. The effect of CAN-296 on fungal cells is rapid, concentration dependent and lethal. Kill-curve experiments revealed that CAN-296 at 10 mg/L killed $\geq 99.9\%$ of *C. albicans* cells within 60 min and MIC measurements did not differ from minimum lethal concentrations by more than two-fold for all tested *Candida* species.\(^1\)

Because of its relatively large size and the presence of highly charged residues, it is unlikely that this compound would penetrate the cell membrane easily and accumulate inside the cell. Therefore the mode of action of this compound responsible for its antifungal activity may lie in its ability to act on subcellular component(s) or molecules

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Figure 1. Uptake of $[^3]$H]CAN-296 (5 mg/L) in *C. albicans* as a function of time (a), and uptake as a function of concentration of the drug (b).
CAN-296 fungicidal activity

Figure 2. Effect of washing with unlabelled CAN-296 on the uptake/binding of [³H]CAN-296 in intact cells (a) and the cell-wall fraction (b) of C. albicans. The arrow indicates the time of addition of a 150-fold excess of unlabelled CAN-296. Aliquots of the cell suspension and the cell-wall fraction were removed before and after the addition of the unlabelled CAN-296 at the indicated time intervals and the radioactivity associated with intact cells or cell-wall fraction was determined as described in the Materials and methods section. ●, washed with unlabelled CAN-296; ■, unwashed.

Table II. Effect of calcium chloride on the uptake/binding of [³H]CAN-296 to intact C. albicans cells

<table>
<thead>
<tr>
<th>Calcium chloride (g/L)</th>
<th>15</th>
<th>30</th>
<th>45</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>48 ± 0.87</td>
<td>58 ± 1.32</td>
<td>70.5 ± 0.13</td>
<td>80 ± 2.23</td>
</tr>
<tr>
<td>1.1</td>
<td>19.5 ± 0.27</td>
<td>29 ± 1.77</td>
<td>25.9 ± 0.12</td>
<td>26.5 ± 0.64</td>
</tr>
<tr>
<td>2.2</td>
<td>10 ± 0.07</td>
<td>11 ± 0.02</td>
<td>13 ± 0.43</td>
<td>12.75 ± 0.09</td>
</tr>
<tr>
<td>5.5</td>
<td>5.5 ± 0.37</td>
<td>4.5 ± 0.02</td>
<td>4.15 ± 0.02</td>
<td>4.2 ± 0.1</td>
</tr>
</tbody>
</table>

critical for the function of the cell yet accessible from outside the cell. The rapid action of CAN-296 at low concentrations on C. albicans cells also suggests this possibility.

The uptake/binding of CAN-296 by the C. albicans cells was found to be concentration dependent up to 5 mg/L. At 10 mg/L we observed a decline in the uptake/binding, which was reproduced in three independent determinations. This decline is difficult to explain, but it is possibly related to the rapid cell death known to occur within 30 min at this concentration of the drug. We suspect that at 10 mg/L the cells are killed so rapidly that they do not have sufficient time to accumulate maximal amount of the compound.

Although several likely candidates for the CAN-296 target exist (e.g. cell-wall synthesizing enzymes, proton-pumping ATPase) for the action of this complex carbohydrate, nothing is known about the mode of action at present. Our results demonstrate that CAN-296 binds to the fungal cell wall, which also contains complex carbohydrates, and the action of CAN-296 may involve carbohydrate recognition and interaction. The importance of carbohydrates in the mediation of biological functions is increasingly appreciated. However, most data in glycobiology pertain to the interactions of proteins with oligosaccharides, and relatively little is known with regard to carbohydrate–carbohydrate interactions. On the other hand, many animal lectins contain carbohydrate-recognition domains (CRDs), which recognize a variety of sugars. The mannose-binding protein, for instance, is a serum lectin that binds to the surface of yeasts and bacteria and mediates an innate immune response. It recognizes and binds to mannose, N-acetylgalcosamine and L-fucose with roughly equal affinities. Other lectins, such as the chicken hepatic lectin, exhibit a much higher specificity. This is achieved, in part, by clustering of several CRDs, each with its own sugar binding site. In the case of the macrophage mannose receptor, the
full affinity for yeast mannan can only be attained when five of the eight CRDs are present.\textsuperscript{10}

Calcium was found to inhibit CAN-296 activity on three different levels, with inhibition of the fungicidal activity, the aggregation and the binding of CAN-296 to the cell wall. We have not investigated in detail the relationship between the aggregation induced by CAN-296 and its fungicidal activity. We observed a difference in the kinetics of the cell kill and of aggregation. Cell death was observed within 15–30 min\textsuperscript{1} whereas aggregation reached a plateau only after 60 min, suggesting that CAN-296-induced aggregation of \textit{C. albicans} may be unrelated to its fungicidal activity and the observed reduction in cfu is substantially due to cell death rather than aggregation of cells.

Many lectins require calcium for binding activity (C-type lectins). At the binding site of these C-type lectins, specific oligosaccharide equatorial hydroxyl groups act as coordination ligands and bind to Ca\textsuperscript{2+}. Certain antifungal agents, such as the pradimicins, were found to bind specifically to the yeast wall in the presence of calcium.\textsuperscript{11} This binding, through the \(\text{D-}\text{mannopyranoside-binding site, is similar to the sugar-specific action of C-type lectins.}\textsuperscript{12,13}\n
In contrast to the promotion of pradimicin activity by calcium, the anti-fungal activity of CAN-296 was inhibited by calcium. Nevertheless, the observation that calcium inhibits binding of CAN-296 to the fungal cell wall raises the possibility that, like the pradimicins, CAN-296 may possess a calcium-binding site.

Calcium and magnesium are also known to inhibit a number of aminoglycoside–membrane interactions including those with bacterial, plasma and subcellular membranes.\textsuperscript{14,15} The high-affinity sites for aminoglycoside–membrane interaction were identified as the acidic phospholipids of the membrane,\textsuperscript{16,17} and the binding is due to a charge interaction between these polycationic antibiotics and the anionic head groups of the acidic phospholipids. Not surprisingly, a number of studies have demonstrated that divalent cations can interfere with these reactions and protect against the neurotoxic, nephrotoxic and ototoxic effects of these antibiotics. Similar mechanisms may be operating in the binding of calcium to CAN-296.

In summary, our observations that CAN-296 binds to the cell wall of \textit{C. albicans} and that this binding can be reversibly inhibited by calcium and other divalent cations suggest that CAN-296 may possess lectin- and calcium-binding sites, and that its mode of action may involve interaction with the cell wall or cell membrane component(s).

References


