Mulundocandin, an Echinocandin-like Lipopeptide Antifungal Agent:

Biological Activities In Vitro

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Mulundocandin (MCN) is an antifungal lipopeptide which belongs to the echinocandin class of antifungal agents. MCN exhibited good in vitro activity against Candida albicans and C. glabrata isolates with MIC ranges of 0.5 ~ 4.0 μg/ml and 2.0 ~ 4.0 μg/ml, respectively. MCN also exhibited some activity against C. tropicalis isolates (MIC range 1.0 ~ 8.0 μg/ml). However, MCN was poorly active against other non-albicans isolates and was inactive against Cryptococcus neoformans, Aspergillus species and Trichophyton. MCN appeared to exert its antifungal activity through preferential inhibition of germ tube formation (MIC-HY 0.015 ~ 0.03 μg/ml) and was typically less active on the yeast form (MIC 0.5 ~ 4.0 μg/ml). In kill-curve experiments 99.9 % reductions in cell viability were observed following 8 hours exposure to MCN at 4 × MIC and 8 × MIC and after 5 hours exposure to 16 × MIC.

The emergence of azole-resistant isolates of pathogenic yeasts in the clinic, particularly those resistant to fluconazole, and the failure of treatment of systemic mycoses in HIV-positive and full blown AIDS patients, are growing concerns among infectious disease specialists. There is, therefore, an urgent need for new antifungal agents which demonstrate potent, broad spectrum and efficacious activities in the therapy of serious fungal infections, and the potential to overcome problems associated with drug resistance.

One avenue of research that has lead to the discovery and development of new antifungal agents is that of the macrocyclic lipopeptide antifungal agents. Examples of these include the pneumocandins, echinocandins and the aureobasidins. Mulundocandin (MCN) is an echinocandin-like lipopeptide antifungal agent, isolated from a culture of Aspergillus sydowii and as it is the case for other structurally relates antifungal agents is assumed to exert its antifungal activity through inhibition of β-(1,3)-D-glucan synthesis. The purpose of this study was to characterize the biological properties of MCN in vitro.

Materials and Methods

Antifungal Agents
MCN was prepared at Hoechst AG, Frankfurt. Amphotericin B (AMB) and flucytosine (FC) were purchased from Sigma Chemical Co. (St. Louis, MO) and itraconazole (ITZ) and ketoconazole (KTZ) were from Janssen Pharmaceutica (Beersse, Belgium). Fluconazole was synthesized at Hoechst AG (Frankfurt, Germany).

Fungal Isolates
Clinical isolates of Candida spp., C. neoformans, Aspergillus spp. and Trichophyton spp. were obtained from the Center for Medical Mycology, Cleveland, OH, Mycology Reference Laboratory, Glasgow, United Kingdom and from Hoechst AG, Frankfurt, Germany and included C. albicans (n=18), C. tropicalis (n=5), C. lusitaniae (n=5), C. glabrata (n=12), C. parapsilosis (n=6), C. krusei (n=10), and C. neoformans (n=6), A. niger (n=5), A. flamigatus (n=5), A. flavus (n=4), Trichophyton mentagrophytes (n=3) and T. rubrum
(n = 3). C. parapsilosis ATCC 22019 and C. krusei ATCC 6258 were tested concurrently as quality control (QC) isolates. Yeasts were grown and maintained on Sabouraud dextrose agar (SDA, Difco). Filamentous fungi and dermatophytes were grown and maintained on potato dextrose agar (Difco).

Minimum Inhibitory Concentration (MIC) Determination
The MIC of each isolate to the antifungal agents was assessed as follows. Susceptibility testing was performed in 96-well tissue culture plates as described in the M27-A procedure for the susceptibility testing of yeasts. The MIC for yeasts was read visually as described in the NCCLS M27-A document. For MCN, MICs were read in the same way as described for amphotericin B. MICs of filamentous fungi and dermatophytes were performed as above except that an inoculum of 1 x 10^6 CFU/ml was used and the MICs read visually in the same way as for the yeasts after 72 hours incubation at 35°C.

Minimum Fungicidal Concentration (MFC) Determination
The MFC was determined by transferring 10 µl of solution from each well of the MIC plates, by use of disposable 96 spike plastic transfer plates onto agar in rectangular plastic dishes. For yeasts, the MFC was read as the lowest concentration of antifungal agent that prevented the growth of colonies on agar after 48 hours (Candida spp.) or 72 hours (C. neoformans) incubation at 35°C. For the other fungi, the MFC was read after 72 hours incubation at 35°C. Controls included cells that were incubated in the absence of antifungal agent.

Effect of Inoculum Size on the MIC
The effect of inoculum size on the activities of the antifungal agents against C. albicans was assessed. MICs were performed as described above, using C. albicans ATCC 10231, with the exception that inocula of 10^5 CFU/ml were used.

Effect of Serum on the MIC
MCNs were performed using 4 C. albicans strains as described above. Whole human serum was added to RPMI-1640 medium as final concentrations of 20, 40 or 60% (vol/vol) and MCN and AMB were added to appropriate wells of the tissue culture plates. Controls included cells in medium only (growth control) and cells in the presence of different concentrations of serum (serum effect control).

Results
MIC and MFC Determinations
MCN was tested against a total of 66 yeast isolates. MCN exhibited good activity against 18 C. albicans isolates with an MIC range of 0.5~4.0 µg/ml (Table 1). The compound also showed good activity against 12 C. glabrata isolates with an MIC range of 2.0~4.0 µg/ml, and was also quite active against 5 C. tropicalis isolates (MIC range 1.0~8.0). Furthermore, MCN exhibited a moderate activity against 5 C. lusitaniae isolates (MIC range 8.0~32 µg/ml) (Table 1). However, MCN exhibited variable and poor activities against the remainder of the yeast isolates (Table 1). In terms of its activity against C. albicans, MCN showed...
Table 1. Susceptibility of yeasts to mulundocandin and comparison with different antifungal agents.

<table>
<thead>
<tr>
<th>Isolate (number)</th>
<th>Minimum inhibitory concentration (µg/ml)</th>
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<tr>
<td></td>
<td>MCN</td>
</tr>
<tr>
<td>C. albicans (18)</td>
<td>0.5~&gt;4.0</td>
</tr>
<tr>
<td>C. glabrata (12)</td>
<td>2.0~&gt;4.0</td>
</tr>
<tr>
<td>C. krusei (10)</td>
<td>16~64</td>
</tr>
<tr>
<td>C. parapsilosis (6)</td>
<td>16~128</td>
</tr>
<tr>
<td>C. tropicalis (5)</td>
<td>1.0~8.0</td>
</tr>
<tr>
<td>C. lusitaniae (5)</td>
<td>8.0~32</td>
</tr>
<tr>
<td>C. neoformans (6)</td>
<td>32~&gt;128</td>
</tr>
<tr>
<td>NCCLS QC strains</td>
<td></td>
</tr>
<tr>
<td>C. parapsilosis ATCC 20019</td>
<td>16</td>
</tr>
<tr>
<td>C. krusei ATCC 6258</td>
<td>32</td>
</tr>
</tbody>
</table>

Similar activity to that of AMB and was as active against either azole-susceptible or azole-resistant C. albicans strains (Table 1). Furthermore, its activity against C. glabrata was comparable to that exhibited by AMB (Table 1). MCN was not active against the 6 C. neoformans isolates, though both AMB and FC were typically active and the azole agents showed good to variable activity (Table 1). MCN was typically not active against either the Aspergillus isolates or against Trichophyton isolates (Table 2). The exception to these findings was that several of these isolates were susceptible to MCN at high concentrations (MIC 32 or >32 µg/ml) (Table 2). MCN exhibited a slightly lower fungicidal potential as compared with AMB against C. albicans and C. glabrata at 4~8 x MIC and exerted fungicidal activity against C. tropicalis at 8~16 x MIC. By contrast, AMB was fungicidal against the different Candida species at 2~4 x MIC (data not shown).

Activity Against the Morphogenetic Transformation

MCN was equally active against the morphogenetic transformation in C. albicans in both azole-susceptible and azole-resistant C. albicans isolates (Table 3). Interestingly, MCN inhibited the transformation at sub-MIC values. For example, MCN inhibited the transformation at 0.015~0.03 µg/ml and was more active than AMB (Table 3). By contrast, the azoles and FC were poor inhibitors of the morphogenetic transformation in C. albicans (Table 3). Comparisons of the effects of MCN by MIC and by inhibition of the morphogenetic transformation (MIC-HY) gave MIC/MIC-HY ratios for MCN of 64~128 (Table 3). By contrast, AMB gave ratios of 2.0~4.0 and the other agents gave ratios of <0.02 (Table 3). The data, therefore, suggests that MCN preferentially inhibits growth of C. albicans by blocking germ tube formation and is less able to inhibit growth of the yeast by budding.
Table 3. Effects of the different antifungals on the morphogenetic transformation (MIC-HY) in *Candida albicans*.

<table>
<thead>
<tr>
<th>MIC-HY</th>
<th>MCN</th>
<th>AMB</th>
<th>FLZ</th>
<th>ITZ</th>
<th>KTZ</th>
<th>FC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fluconazole-susceptible (8)</td>
<td>0.015–0.03</td>
<td>0.06–0.125</td>
<td>32–64</td>
<td>16–64</td>
<td>32–64</td>
</tr>
<tr>
<td></td>
<td>Fluconazole-resistant (8)</td>
<td>0.015–0.03</td>
<td>0.06–0.125</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
</tr>
</tbody>
</table>

**Ratio: MIC/MIC-HY**

| Fluconazole-susceptible (8) | 64–128 | 2.0–4.0 | <0.02   | <0.02   | <0.02   | <0.02   |
| Fluconazole-resistant (8)   | 64–128 | 2.0–4.0 | <0.02   | <0.02   | <0.02   | <0.02   |

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Fig. 1. Effect of inoculum size on the activity of MCN and other antifungal agents.

The activity of each antifungal agent was assessed using MIC inocula of $10^2$ to $10^6$ cfu/ml (see Materials and Methods). The bars represent the mean (4 *C. albicans* strains) MIC for each antifungal agent and are expressed as µg/ml.

**Effects of Inoculum and Serum**

When tested against four *C. albicans* isolates, added as inocula of $10^2$, $10^3$, $10^4$, $10^5$ and $10^6$, the activity of MCN was slightly affected by inoculum size (Fig. 1). For example, with inocula of $10^5$ or $10^6$ the activity of MCN was reduced two-fold or four-fold, respectively, in comparison to the activity achieved with an inoculum of $10^3$ (Fig. 1). By contrast, AMB was typically unaffected by inoculum size though the azoles and FC exhibited significantly reduced activities when using inocula of $10^6$ cells/ml (Fig. 1). MCN and AMB were tested against four *C. albicans* isolates in the absence or presence of whole human serum (20–60%). In the presence of 20% serum, the activity of MCN was reduced four-fold and in the presence of 40% serum two isolates gave MICs...
Table 4. Effects of serum on the activity of MCN and AMB against C. albicans.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Serum concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0%</td>
</tr>
<tr>
<td>MCN</td>
<td>1.0~2.0</td>
</tr>
<tr>
<td>AMB</td>
<td>0.125~0.5</td>
</tr>
</tbody>
</table>

The values represent the range of MICs for four C. albicans isolates.

Fig. 2. Killing activity of MCN (A) and AMB (B).

(●) Control, (■) 0.5 × MIC, (▲) 1 × MIC, (×) 2 × MIC, (●) 4 × MIC, (●) 8 × MIC, (+) 16 × MIC.

The killing activities of AMB and MCN were assessed at different concentrations as described in Materials and Methods. The data points represent the log_{10} CFU/ml. The limit of detection is represented by the dotted line.

of 8.0 μg/ml and two gave MICs of >16 μg/ml (Table 4). Furthermore, MCN was inactive when tested in the presence of 60% serum (Table 4). By contrast, the activity of AMB was not significantly affected by the addition of serum (Table 4).

Killing Activity

The killing activity of MCN was compared with the killing activity of AMB (Fig. 2). MCN caused a >99.9% reduction in cell viabilities following exposure of the cells to MCN for 5 hours at 16 × MIC or after 8 hours at 4 × MIC or 8 × MIC (Fig. 2A). Some reductions in cell viability were observed after 5 hours
in the presence of $2 \times MIC$, however, MCN at this concentration was not effective in killing the population (Fig. 2A). By contrast, AMB caused >99.9% reductions in cell viabilities following 1 hour exposure to $8 \times MIC$, 3 hours exposure to $4 \times MIC$ and 5 hours exposure to $2 \times MIC$ (Fig. 2B).

Discussion

MCN was first isolated in the laboratories of Hoechst India Ltd. from the fermentation broth of 

A. sydowii

and was described as an echinocandin-like molecule. Members of the echinocandin class of molecules tend to exhibit good activities against the majority of Candida species, typically lack activity against Cryptococcus and exhibit poor activities against filamentous and dermatophytic fungi. The lack of activity against Cryptococcus shown here for MCN was not surprising given that other members of this class are also poorly active against this yeast.

In terms of its biological properties in vitro, MCN appears to share several characteristics with those exhibited by other members of the lipopeptide class of antifungal agents. For example, MCN exhibited good activities against C. albicans, C. glabrata and C. tropicalis. However, MCN was poorly active against other Candida spp., C. neoformans, filamentous fungi and dermatophytes. The activity of MCN was also attractive in that its activity against both FLZ-susceptible and FLZ-resistant C. albicans strains was good and comparable to those exhibited by AMB. Furthermore, the compound appeared to preferentially exert its action against C. albicans through inhibition of germ tube formation, an attribute which may be important in vivo, rather than by inhibition of growth through budding. Moreover, MCN was fungicidal at $4 \sim 8 \times MIC$ and in kill curve experiments it exhibited a rapid 99.9% kill against C. albicans.

The lipopeptide class of new antifungal agents appears to be a promising class of new antifungal agents. Members of this class including MCN, LY-303366 and MK-0991 exhibit a high fungicidal potential and are clearly differentiated from other antifungal agents in their mechanism of action through inhibition of 1,3-β-D-glucan synthesis. This class of molecules may prove to be useful in treating azole-resistant pathogenic yeasts.

References