VOL. 52, NO. 3, MAR. 1999 THE JOURNAL OF ANTIBIOTICS pp. 305 — 310

Mulundocandin, an Echinocandin-like Lipopeptide Antifungal Agent: Biological Activities *In Vitro*

STEPHEN HAWSER[†,*], MONICA BORGONOVI[†], ASTRID MARKUS[††] and DIETER ISERT[††]

[†] Hoechst Marion Roussel,
102 Route de Noisy, F-93235 Romainville, France
[††] Hoechst Marion Roussel, Core Research Functions,
H811, D-65926 Frankfurt/Main, Germany

(Received for publication November 5, 1998)

Mulundocandin (MCN) is an antifungal lipopeptide which belongs to the echinocandin class of antimycotic 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[1,2] 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[3~5]. Mulundocandin (MCN) is an echinocandin-like lipopeptide antifungal agent, isolated from a culture of *Aspergillus sydowii*[6] 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[5]. 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 (Beerse, Belguim). 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. fumigatus* (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[7]. For MCN, MICs were read in the same way as described for amphotericin B[7]. MICs of filamentous fungi and dermatophytes were performed as above except that an inoculum of 1×10^5 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 $10^{2\sim6}$ cfu/ml were used.

Effect of Serum on the MIC

MICs 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).

Morphogenetic Transformation Experiments

The effects of different antifungal agents on the morphogenetic transformation by *C. albicans* were assessed by phase-contrast microscopy (Olympus CK microscope & Zeiss phase-contrast microscope) as described previously[8]. Briefly, 96 well microtiter plates were prepared in the same manner as for MIC experiments (see above) and the effects of antifungal agents were viewed by microscopy after 3 hours incubation at 35°C and morphology of cells scored as described previously[8]. By microscopy the entire population in each well was examined and the lowest concentration of antifungal agent used at which the morphogenetic transformation was inhibited was recorded (MIC-HY). All MIC-HY experiments were performed at least three times.

Time Kill Curves

The killing activities of MCN and AMB against actively metabolizing yeast cells were determined in RPMI-1640 medium in shaking flasks at 35°C. In brief, an overnight culture of *C. albicans* ATCC 10231 was prepared as previously described[8] and inoculated at 1×10^6 cfu/ml into 100 ml of RPMI-1640 medium. MCN or AMB were added to the log-phase cultures at 0.5 × MIC, 1 × MIC, 2 × MIC, 4 × MIC, 8 × MIC and 16 × MIC or 0.5 × MIC, 1 × MIC, 2 × MIC, 4 × MIC and 8 × MIC, respectively. A separate group of cells were allowed to grow in the absence of antifungal agent (drug free control). Samples were taken for up to 24 hours and growth curves were established following plating of the samples on drug free SDA plates and enumeration of colony forming units.

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.

Isolate (number)	Minimum inhibitory concentration (μg/ml)					
	MCN	AMB	FLZ	ITZ	KTZ	FC
C. albicans (18)	0.5～4.0	0.25～1.0	0.5～>128	0.125～32	0.03～16	0.125～128
C. glabrata (12)	2.0～4.0	0.5～2.0	4.0～>128	0.5～32	0.5～4.0	0.03～0.5
C. krusei (10)	16～64	1.0～2.0	32～128	0.125～16	0.25～8.0	2.0～4.0
C. parapsilosis (6)	16～128	0.125～1.0	1.0～16	0.125～1.0	0.125～0.5	0.125～0.5
C. tropicalis (5)	1.0～8.0	0.25～2.0	1.0～4.0	0.06～16	0.015～8.0	0.03～64
C. lusitaniae (5)	8.0～32	0.125～0.5	2.0～8.0	0.06～1.0	0.03～1.0	0.03～8.0
C. neoformans (6)	32～>128	0.125～2.0	2.0～>128	0.125～16	0.5～16	0.25～0.5
NCCLS QC strains						
C. parapsilosis ATCC 20019	16	0.5	1	0.25	0.125	0.5
C. krusei ATCC 6258	32	1	32	0.25	0.5	4

Table 2. Susceptibility of filamentous fungi and dermatophytes to mulundocandin (MCN) and comparison with other antifungal agents.

Isolate (number)	Minimum inhibitory concentration (μg/ml)				
	MCN	AMB	FLZ	ITZ	FC
A. niger (5)	64～128	0.5～4.0	64～>128	0.125～2.0	0.5～4.0
A. fumigatus (5)	32～>128	0.5～4.0	128～>128	0.5～2.0	0.5～4.0
A. flavus (4)	64～>128	1.0～4.0	>128	0.125～2.0	1.0～4.0
T. mentagrophytes (3)	64～>128	0.5～4.0	32～>128	0.5～4.0	2.0～8.0
T. rubrum (3)	64～>128	0.5～4.0	32～>128	0.5～4.0	2.0～8.0

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×MIC and exerted fungicidal activity against *C. tropicalis* at 8～16×MIC. By contrast, AMB was fungicidal against the different *Candida* species at 2～4×MIC (data not shown).

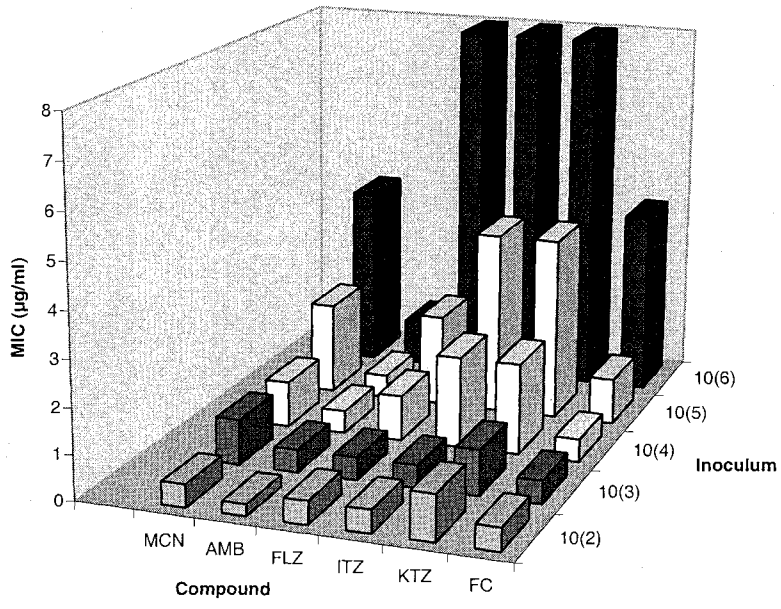
Activity Against the Morphogenetic Transformation

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*.

	MIC-HY					
	MCN	AMB	FLZ	ITZ	KTZ	FC
Fluconazole-susceptible (8)	0.015～0.03	0.06～0.125	32～64	16～64	32～64	32～64
Fluconazole-resistant (8)	0.015～0.03	0.06～0.125	>128	>128	>128	>128
	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

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 $10^2 \sim 10^6$ 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

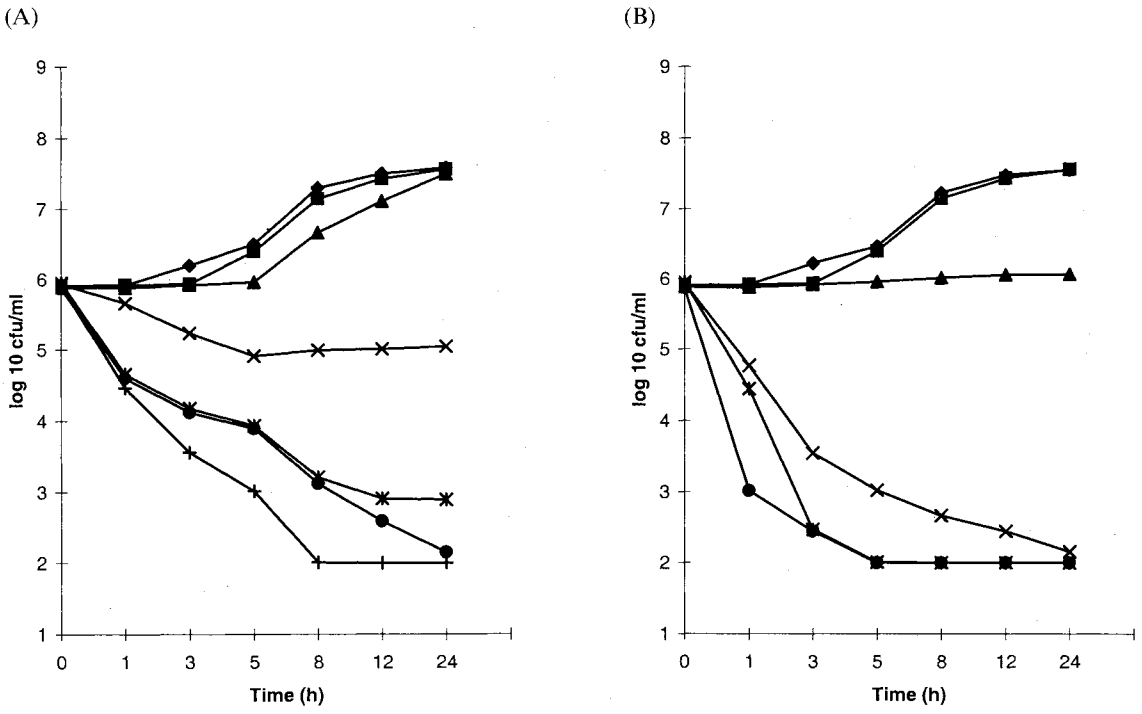
Table 4. Effects of serum on the activity of MCN and AMB against *C. albicans*.

Compound	Serum concentration			
	0%	20%	40%	60%
MCN	1.0~2.0	4.0~8.0	8.0~>16	>16
AMB	0.125~0.5	0.125~0.5	0.25~0.5	0.5

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 × 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 × MIC, 3 hours exposure to 4 × MIC and 5 hours exposure to 2 × MIC (Fig. 2B).

Discussion

MCN was first isolated in the laboratories of Hoechst India Ltd.[6] from the fermentation broth of *A. sydowii* and was described as an echinocandin-like molecule[5,6]. 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[5]. 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[5].

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[3~5]. 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 4~8 × 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[9] and MK-0991[10] 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

1) Fan Harvard, P.; D. Capana, S. M. Smith, A. Mangia & R. H. K. Eng: Development of resistance in *Candida* isolates from patients receiving prolonged anti-fungal therapy. Antimicrob. Agents Chemother. 35: 2302~2305, 1991
2) Ruhnke, M.; A. Eigler, I. Tennagen, B. Geiseler, E. Engelmann & M. Trautmann: Emergence of fluconazole-resistant strains of *Candida albicans* in patients with recurrent oropharyngeal candidosis and human immunodeficiency virus infection. J. Clin. Microbiol. 32: 2092~2098, 1994
3) Takesako, K.; K. Ikai, F. Haruna, M. Endo, K. Shimanaka, E. Sono, T. Nakamura & H. Yamaguchi: Aureobasidins, new antifungal antibiotics. Taxonomy, fermentation, isolation, and properties. J. Antibiotics 44: 919~924, 1991
4) Schmatz, D. M.; G. Abruzzo, M. A. Powles, D. C. McFadden, J. M. Balkovec, R. M. Black, K. Nollstadt & K. Bartizal: Pneumocandins from *Zalerion arboricola*. IV. Biological evaluation of natural and semisynthetic pneumocandins for activity against *Pneumocystis carinii* and *Candida* species. J. Antibiotics 45: 1886~1891, 1992
5) Balkovec, J. M.: Lipopeptide antifungal agents. Exp. Opin. Invest. Drugs 3: 65~82, 1994
6) Mukhopadhyay, T.; K. Roy, R. G. Bhat, S. N. Sawant, J. Blumbach, B. N. Ganguli & H. W. Fehlhaber: Deoxymulundocandin-A new echinocandin type antifungal antibiotic. J. Antibiotics 45: 618~623, 1992
7) National Committee for Clinical Laboratory Standards. 1997. Reference method for broth dilution antifungal susceptibility testing of yeast; approved standard, M27-A. NCCLS, Wayne, PA
8) Hawser, S. P. & K. Islam: Spectrophotometric determination of the morphogenetic transformation by synchronous *Candida albicans*: effects of antifungal agents. J. Antimicrob. Chemother. 38: 67~73, 1996
9) Klepser, M. E., E. J. Ernst, M. E. Ernst & M. A. Pfaller: Growth medium effect on the antifungal activity of LY303366. Diagn. Microbiol. Infect. Dis. 29: 227~231, 1997
10) Frosco, M.; L. E. Lawrence & J. F. Barrett: Meetings Highlights. 37th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC). Exp. Opin. Invest. Drugs 6: 1951~1968, 1997