In Vitro Activity of a New Oral Glucan Synthase Inhibitor (MK-3118) Tested Against *Aspergillus* spp. by CLSI and EUCAST Broth Microdilution Methods

Short running title: Note

Michael A. Pfallera*, Shawn A. Messera, Mary R. Motylb, Ronald N. Jonesa, and Mariana Castanheiraa

aJMI Laboratories, North Liberty, IA 52317, USA; and

bMerck Sharp & Dohme Corp., Kenilworth, NJ

*Corresponding author: Michael A. Pfaller, M.D.
JMI Laboratories
345 Beaver Kreek Centre, Suite A
North Liberty, IA 52317
Phone: 319-665-3370
Fax: 319-665-3371
michael-pfaller@uiowa.edu
MK-3118, a glucan synthase inhibitor derived from enfumafungin, and comparator agents were tested against 71 Aspergillus spp., including itraconazole-resistant strains (MIC, ≥4 µg/ml), using CLSI and EUCAST reference broth microdilution methods. CLSI MEC/MIC90 values (µg/ml) for MK-3118, amphotericin B, and caspofungin, respectively, were: Aspergillus flavus species complex (SC), 0.12, 2, 0.03; A. fumigatus SC, 0.25, 2, 0.06; A. terreus SC, 0.12, 2, 0.06 and A. niger SC, 0.06, 1, 0.03. Essential agreement between CLSI and EUCAST (±2 log2 dilution steps) was 94.3%. MK-3118 was determined to be a potent agent regardless of the in vitro method applied, with excellent activity against contemporary wild type and itraconazole-resistant strains of Aspergillus spp.

Keywords: MK-3118, Aspergillus, resistance, glucan synthase inhibitor
Mould-active azoles (itraconazole, posaconazole, and voriconazole) are the primary class of antifungal agents used for the prevention and treatment of invasive aspergillosis (IA) (1). Echinocandins may play a role as alternatives to the azoles (1, 2); however, the lack of an oral formulation limits the role of these glucan synthase (GS) inhibitors of for the prevention or treatment of IA. Echinocandin resistance among clinical isolates of Aspergillus is considered uncommon, however, both increased resistance and breakthrough infections have been reported and may occur in at-risk patient groups with or without long-term exposure to these agents (3-10). In particular, azole resistance in Aspergillus spp. may be associated with a high probability of treatment failure (11) and a recent report from the Netherlands found that the case-fatality rate of patients with azole-resistant IA was 88% (9). These observations have prompted a call for an expanded search for new antifungal agents with novel mechanisms of action, as well as an expanded role for antifungal susceptibility testing of Aspergillus spp. (5, 9-17).

MK-3118 is an orally active, semi-synthetic derivative of the natural product enfumafungin with in vitro and in vivo activity against Aspergillus spp. (18-22). MK-3118 and other derivatives of enfumafungin are potent inhibitors of fungal GS, yet these compounds are structurally distinct from the echinocandins (20-22). The sites of mutations in fks that are associated with resistance to the echinocandins are distinctly different from those causing decreased susceptibility to the enfumafungin derivatives; likewise, echinocandin-resistant isolates remain susceptible to these agents (21).

In the presented study, we used a collection of Aspergillus spp. isolates selected to contain both wild type (WT) as well as azole-resistant strains to examine the activity of MK-3118 as determined by both CLSI and EUCAST methods (23, 24).

A total of 71 isolates of Aspergillus spp. obtained from centers participating in the 2008-2010 ARTEMIS and SENTRY Antimicrobial Surveillance Programs (25, 26) were evaluated. The collection included 23 isolates of A. flavus species complex (SC), 21 A. fumigatus SC, 18 A. terreus SC and nine A. niger SC. Phenotypically resistant isolates (as determined by CLSI methods) (23) included eight itraconazole-resistant (MIC, $\geq$4 µg/ml) isolates including A. fumigatus SC (6 isolates), A. niger SC (1 isolate) and A. terreus SC (1 isolate). The isolates were obtained from a variety of sources, including sputum, bronchoscopy, and tissue biopsy specimens, and represented individual infection episodes.

Isolates were identified by standard microscopic morphology (27) and DNA sequencing of 28S and β-
tubulin genes, as previously described (28). Before testing, each isolate was subcultured at least twice on potato dextrose agar (Remel, Lenexa, Kansas, USA) to ensure viability and purity.

All isolates were tested against MK-3118, amphotericin B, and caspofungin using both CLSI and EUCAST BMD methods (23, 24). MIC values for amphotericin B were determined visually as the lowest concentration of drug that caused complete inhibition of growth (first clear well) relative to that of the growth control. MEC values for MK-3118 and caspofungin were determined as described previously (19, 26) and by the CLSI (23). Quality control was assured by testing the following strains recommended by CLSI and by EUCAST (29): *Candida parapsilosis* ATCC 22019, *C. krusei* ATCC 6258, and *A. flavus* ATCC 204304 (23, 29).

Discrepancies of more than ±two log₂ dilution steps among MEC results were used to calculate the essential agreement (EA) between the two methods. High off-scale MEC results were converted to the next highest concentration and low off-scale results were left unchanged.

Although clinical breakpoints for antifungal agents and *Aspergillus* spp. have not been officially designated by either CLSI or EUCAST, we have used published criteria to classify the strains used in this study as either resistant to itraconazole (MIC, ≥4 µg/ml) (10, 17) or as WT versus non-WT to amphotericin B (MIC, ≤2/≥2 µg/ml for *A. fumigatus*, *A. flavus*, and *A. niger*; MIC, ≤4/≥4 µg/ml for *A. terreus*) (30) and caspofungin (MEC, ≤0.5/≥0.5 for *A. fumigatus*, and ≤0.25/≥0.25 for *A. flavus*, *A. niger*, and *A. terreus*) (31).

Table 1 summarizes the in vitro susceptibilities of tested *Aspergillus* spp. to MK-3118 as determined by the CLSI and EUCAST BMD methods. The MEC endpoint was chosen for MK-3118 due to the fact that, similar to the echinocandins, MK-3118 inhibits GS and hyphal extension of *Aspergillus* spp., resulting in aberrant hyphal growth (short, stubby, highly branched hyphae) but not complete growth inhibition. Preliminary data (19) found that as with the echinocandins, when a MIC for MK-3118 is determined (complete growth inhibition), an endpoint was not achieved for *Aspergillus* spp.

All *Aspergillus* spp. were inhibited by ≤0.25 µg/ml as determined by EUCAST and 69/71 (97.2%) were inhibited at this MEC value as determined by CLSI BMD methods (23, 24). The EA between the two reference methods was 94.3% for MK-3118 across all four *Aspergillus* spp. (range, 85.7 to 100.0%; Table 1). The MEC values generated by the EUCAST method tended to be slightly lower than those obtained
by the CLSI method for most species. These results demonstrate a high level of concordance (same
modal MIC, 0.06 µg/ml) between the MEC results produced by both methods when testing *Aspergillus*
spp.

The activity of MK-3118 against this collection of *Aspergillus* spp. was less than that of
caspofungin (MEC90, 0.12-0.25 µg/ml versus 0.03-0.06 µg/ml, respectively; Table 2). Whereas, all of these
strains exhibited WT MIC/MEC results for amphotericin B (30) and caspofungin (31).

MK-3118 and caspofungin were both quite active against the itraconazole-resistant (MIC, ≥4
µg/ml) isolates in the collection (Table 3). The MEC results for caspofungin ranged from 0.015 to 0.06
µg/ml and those for MK-3118 ranged from 0.03 to 0.5 µg/ml. Whereas cross-resistance may be observed
among the mold-active azoles (5, 9, 14, 17), the distinctively different mechanisms of action represented
by MK-3118, amphotericin B, and caspofungin result in activity of these antifungal agents against this
subset of *Aspergillus* species isolates that are of great concern worldwide (5, 9, 14, 15).

The notable observations to be taken from this study include the excellent in vitro potency of MK-3118
tested against contemporary isolates of *Aspergillus* spp., including triazole-resistant strains, and the
high level of agreement between the CLSI and EUCAST methods for testing MK-3118 against *Aspergillus*
spp. Preliminary data obtained using the CLSI BMD method (23) have demonstrated excellent MK-3118
spectrum and potency against both yeasts and molds, including *Aspergillus* spp. (19). With regard to
*Aspergillus* spp., those studies are limited by the small numbers of isolates tested, the lack of antifungal
comparators, and the lack of a comparison of the MK-3118 potency as determined by the reference BMD
methods of the CLSI and the EUCAST (23, 24). Although we have shown previously that both methods
provide concordant results when testing *Aspergillus* spp. against itraconazole, posaconazole,
voriconazole, anidulafungin and caspofungin (32, 33), similar data has not been available for MK-3118.

Given the important role that both methods currently play in antifungal resistance surveillance and
regulatory evaluations of new agents, it is important to demonstrate the comparability of their results in
the preclinical development of this new antifungal agent.

In conclusion, MK-3118 is a potent, novel antifungal agent with impressive activity against both
WT and antifungal-resistant strains of *Aspergillus* species. Echinocandin-resistant *Aspergillus* spp. strains
have been rarely observed in the clinical setting and/or reported in the literature and were not tested
during this study (34-36). The oral bioavailability of this compound coupled with mechanistic studies that suggest a lack of cross-resistance with the echinocandins suggest that it may provide a valuable benefit for the treatment and prophylaxis of invasive fungal infections (21) and can be tested with confidence by the two most used reference BMD methods.
ACKNOWLEDGMENTS

This work was supported by an educational/research grant from Merck.

The authors would like to thank S. Benning and P. Clark for excellent support in the preparation of the manuscript and Dr. D.J. Diekema (University of Iowa) for kindly supplying some Aspergillus spp. with resistance phenotypes.

JMI Laboratories, Inc. (MAP, SAM, RNJ and MC) has received research and educational grants in 2009-2011 from American Proficiency Institute (API), Anacor, Astellas, AstraZeneca, Bayer, Cempra, Cerexa, Contrafect, Cubist, Daiichi, Dipexium, Enanta, Furiex, GlaxoSmithKline, Johnson & Johnson (Ortho McNeil), LegoChem Biosciences Inc., Meiji Seika Kaisha, Merck, Nabriva, Novartis, Pfizer (Wyeth), Rempex, Rib-X Pharmaceuticals, Seachaid, Shionogi, The Medicines Co., Theravance, ThermoFisher and some other corporations. Some JMI employees are advisors/consultants for Astellas, Cubist, Pfizer, Cempra, Cerexa-Forest, J&J, and Theravance. MRM is employed by Merck Sharp & Dohme Corp.
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Table 1. In vitro susceptibilities of *Aspergillus* spp. to the oral glucan synthase inhibitor, MK-3118, as determined by CLSI and EUCAST broth microdilution methods.

<table>
<thead>
<tr>
<th>Species (no. tested)</th>
<th>Test Method</th>
<th>No. of isolates at indicated MEC (cumulative %; µg/ml)</th>
<th>% EA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.008</td>
<td>0.015</td>
</tr>
<tr>
<td><em>A. flavus</em> SC (23)</td>
<td>CLSI</td>
<td>14</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>EUCAST</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td><em>A. fumigatus</em> SC (21)</td>
<td>CLSI</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>EUCAST</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td><em>A. terreus</em> SC (18)</td>
<td>CLSI</td>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>EUCAST</td>
<td>7</td>
<td>11</td>
</tr>
<tr>
<td><em>A. niger</em> SC (9)</td>
<td>CLSI</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>EUCAST</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>All <em>Aspergillus</em> spp. (71)</td>
<td>CLSI</td>
<td>7</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>EUCAST</td>
<td>3</td>
<td>23</td>
</tr>
</tbody>
</table>

a. CLSI, Clinical and Laboratory Standards Institute; EUCAST, European Committee for Antimicrobial Susceptibility Testing.
b. MEC, minimum effective concentration
c. EA, essential agreement (MIC ±2 log2 dilutions)
d. SC, species complex
Table 2. In vitro activity of MK-3118 and comparator agents tested against *Aspergillus* spp. as determined by CLSI broth microdilution methods.

<table>
<thead>
<tr>
<th>Species (no. tested)</th>
<th>Antifungal agent</th>
<th>MEC/MIC (µg/ml)$^a$</th>
<th>Range</th>
<th>50%</th>
<th>90%</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. flavus SC (23)</td>
<td>MK-3118</td>
<td>0.06 - 0.12</td>
<td>0.06</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Amphotericin B</td>
<td>1 - 2</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Caspofungin</td>
<td>≤0.008 - 0.03</td>
<td>0.015</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>A. fumigatus SC (21)</td>
<td>MK-3118</td>
<td>0.03 - 0.25</td>
<td>0.12</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Amphotericin B</td>
<td>1 - 2</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Caspofungin</td>
<td>0.015 - 0.25</td>
<td>0.03</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>A. terreus SC (18)</td>
<td>MK-3118</td>
<td>0.03 - 0.25</td>
<td>0.06</td>
<td>0.12</td>
<td></td>
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<tr>
<td></td>
<td>Amphotericin B</td>
<td>1 - 4</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Caspofungin</td>
<td>≤0.008 - 0.06</td>
<td>0.015</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>A. niger SC (9)</td>
<td>MK-3118</td>
<td>0.03 - 0.25</td>
<td>0.06</td>
<td>ND$^c$</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Amphotericin B</td>
<td>1</td>
<td>1</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Caspofungin</td>
<td>≤0.008 - 0.06</td>
<td>0.03</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

*a.* 50% and 90%, MEC/MIC that encompasses 50% and 90% of isolates tested, respectively.

*b.* ND = not determined due to number of isolates <10.
Table 3. In vitro activities of MK-3118 and comparators against itraconazole-resistant (MIC, ≥4 µg/ml) Aspergillus spp. as determined by CLSI broth microdilution methods

<table>
<thead>
<tr>
<th>Species</th>
<th>MIC/MEC(^a) (µg/ml) for:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amphotericin B</td>
<td>Caspofungin</td>
</tr>
<tr>
<td>A. fumigatus SC</td>
<td>1</td>
<td>0.015</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.06</td>
</tr>
<tr>
<td>A. niger SC</td>
<td>1</td>
<td>0.06</td>
</tr>
<tr>
<td>A. terreus SC</td>
<td>1</td>
<td>0.03</td>
</tr>
</tbody>
</table>

\(^a\) MIC, minimum inhibitory concentration; MEC, minimum effective concentration.