Poor *in vivo* efficacy of caspofungin, micafungin and amphotericin B against wild-type *Candida krusei* clinical isolates does not correlate with *in vitro* susceptibility results

Tamás Kardos[1,2], Renátó Kovács[2,3], Gábor Kardos[2], Istvan Varga[4], Aliz Bozó[2], Zoltán Tóth[2], Fruzsina Nagy[2], László Majoros[2*]

[1] University of Debrecen, Faculty of Medicine, Department of Pulmonology, Hungary

[2]University of Debrecen, Faculty of Medicine, Medical Microbiology, Hungary

[3]University of Debrecen, Faculty of Pharmacy, Hungary

[4]University of Debrecen, Faculty of Dentistry, Hungary

Running title: Echinocandins against *C. krusei*

*corresponding author: László Majoros, University of Debrecen, Faculty of Medicine, Medical Microbiology, 4032 Debrecen, Nagyerdei krt. 98. Hungary

Phone: 00-36-52-255-425, Fax: 00-36-52-255-424;

e-mail: major@med.unideb.hu

Abstract

We determined micafungin, caspofungin and amphotericin B (AMB) MICs and killing rates in RPMI-1640 ~~with ~~and in RPMI-1640 with~~out~~ 50% serum against three *C. krusei* bloodstream isolates. MIC ranges in RPMI-1640 were 0.125-0.25, 0.25 and 0.125-0.5

mg/L, ~~respectively;~~ in RPMI-1640 with 50% serum~~with serum~~, MICs were 64-128-, 8- and 4-16-fold higher, respectively. In RPMI-1640 micafungin and caspofungin at 1, 4, 16 and 32 mg/L as well as AMB at 2 mg/L were fungicidal against all isolates in ≤3.96, ≤4.42 and 14.96 hours, respectively. In RPMI-1640 with 50% serum, caspofungin was fungicidal for all ~~three~~ isolates only at 32 mg/L, micafungin and AMB were fungistatic. In ~~a~~ neutropenic ~~murine model~~mice 5 mg/kg caspofungin and 1 mg/kg AMB were ineffective against two of the three isolates. Thus, *in vivo* efficacy of echinocandins and AMB is weak or absent against *C. krusei*. Prescribers treating *C. krusei* infections with echinocandins should ~~be on the~~ watch out for clinical resistance and therapeutic failure.

1. Introduction

The primarily fluconazole resistant *Candida krusei* is one of the most important non-*albicans Candida* species causing life-threatening infections among severely ill patients. Haematological malignancies, neutropenia, solid tumors and recent gastrointestinal surgery are well-known risk factors for invasive infections caused by *C. krusei*, it is a major pathogen in breakthrough fungemia in patients with fluconazole chemoprophylaxis.[1,2] For many decades, amphotericin B was the only systematically used antifungal agent for the treatment of invasive *C. krusei* infections; however, *in vitro* and *in vivo* data suggest that efficacy of amphotericin B is strongly questionable against *C. krusei*.[3-6] As echinocandins (anidulafungin, caspofungin and micafungin) show relatively low MIC values and concentration-dependent fungicidal activity against *C. krusei in vitro*, currently echinocandins are among the preferred antifungals against *C. krusei*, besides amphotericin B and voriconazole.[7,8] However, mortality rate due to invasive infections by *C. krusei* among intensive care unit patients is still unacceptably high (50-70%) even with the widely used echinocandin therapy.[9-11]

formázott: Betűtípus: Dőlt, Betűszín: Automatikus

We cultured 43 *C. krusei* bloodstream isolates from thirteen patients between 2011 and 2016 in our laboratory serving an 1665-bed tertiary care center. After initiation of micafungin or caspofungin therapy, *C. krusei* repeatedly cultured from the bloodstream of four echinocandin-treated patients for up to 18 days (persistent candidemia) suggesting insufficient eradication of this fungus by echinocandins. Notably, all isolates were fully susceptible to echinocandins and amphotericin B by the routinely used Etest interpreted using the CLSI breakpoints.[12,13]

As echinocandins are highly protein-bound antifungal drugs, the free (thus active) drug concentration may be low, which may, at least partly, be responsible for the poor sterilizing ability of echinocandins.[7,13,14] This inspired this study in which we determined the *in vivo* efficacy of micafungin, caspofungin and amphotericin B in a neutropenic murine model against *C. krusei* clinical isolates and compared it to *in vitro* activity using killing rates in RPMI-1640 and RPMI-1640 plus 50% serum to model protein binding.

2. Materials and methods

2.1. Strains

We used three *C. krusei* isolates, all three isolated prior to antifungal administration from three different intensive care unit patients from different units and years. The first patient (isolate 22910) with acute pancreatitis was treated with caspofungin (Cancidas ®) followed by micafungin (Mycamine ®) for 28 days, and died on day 29 from the first isolation of *C. krusei* from his bloodstream. After the initial isolation of *C. krusei*, positive follow-up cultures were found on four different days within the first 15 days. The total number of the positive blood cultures was eight. The second patient (isolate 26513) with ileus and colon resection was treated with caspofungin (Cancidas ®) for 31 days and survived. His follow-

71 up blood cultures in the next 18 days were positive on five different days. The total number
72 of the positive blood cultures was eight. The third patient (isolate 25193) was neutropenic,
73 suffering from acute myelogeneous leukaemia, and she died on the day the blood was
74 collected and received no antifungals. The first and second patients were not neutropenic at
75 the time of blood culture obtained and they were given standard doses of micafungin or
76 caspofungin (100 mg per day and 70 mg on the first day followed by 50 mg daily,
77 respectively). Isolates were identified with conventional methods (Micronaut-Candida and
78 API ID32C) and MALDI Biotyper (Bruker, Bremen, Germany).[15]

79

80 *2.2 Susceptibility testing*

81 Micafungin pure powder was kindly provided by Astellas while caspofungin and
82 amphotericin B pure powders were purchased from Sigma (Budapest, Hungary). MICs in
83 RPMI-1640 and RPMI-1640 plus 50% serum (serum from a human male, type AB, Sigma,
84 Budapest, Hungary) were determined using the standard broth macrodilution method at 35
85 °C.[16-20] For caspofungin and micafungin in RPMI-1640 and in RPMI-1640 plus 50%
86 serum, drug concentrations ranged between 0.015-8 and 0.5-32 mg/L, respectively.
87 Amphotericin B concentrations were 0.125-8 mg/L for both media. MIC values were read
88 visually after 24 h.[12] For micafungin and caspofungin we used the partial inhibition
89 criterion (the lowest concentration that produced a prominent decrease in turbidity
90 compared to the drug-free control). In case of amphotericin B the total inhibition criterion
91 was used. *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 strains were used as
92 quality control strains.[12]

93

94 *2.3. Time-kill studies*

95 Activity of micafungin and caspofungin against *C. krusei* clinical isolates were determined
96 in RPMI-1640 with and without 50% human serum at 1, 4, 16 and 32 mg/L concentrations
97 using a starting inoculum of ~10^5 cells/ml in a final volume of 10 ml.[17-20] Activity of
98 amphotericin B was determined at 0.5, 1 and 2 mg/L in both media as well.[3,21] Aliquots of
99 100 µl were removed after 0, 4, 8, 12, 24 and 48 hours of incubation, tenfold serial
100 dilutions were prepared, and samples of dilutions (4x30 µl) were plated onto a single
101 Sabouraud dextrose agar (SDA) plate and incubated at 35 °C for 48 hours.[16-20] All
102 experiments were performed twice in both media.

103

104 *2.4. Analysis of* in vitro *data*

105 Antifungal activity was defined as fungicidal when at least 99.9% reduction in viable cell
106 count was observed as compared to the starting inoculum.[6]

107 Killing kinetics was analysed in both media (RPMI-1640 and RPMI-1640 plus 50%
108 serum), as described previously.[6,18,19] Briefly, an exponential equation was fitted to the
109 mean data at each time point: $N_t = N_0 \times e^{-kt}$, where N_t is the number of viable yeasts at time
110 t, N_0 is the number of viable yeasts in the initial inoculum, k is the killing rate, and t is the
111 incubation time. Negative and positive k values indicate growth and killing, respectively.
112 The goodness of fit for each isolate was assessed by the r^2 value ($r^2 > \pm 0.8$). The mean times
113 to achieve the fungicidal endpoint (T99.9=3/k) were calculated from the k values for each
114 isolate and concentrations in both media.[6,18,19]

115 Killing kinetics for different isolates was compared using one-way ANOVA with Tukey's
116 post-testing in RPMI-1640 or RPMI-1640 plus 50% serum. The effect of the same drug
117 concentration in RPMI-1640 and RPMI-1640 plus 50% serum was analyzed using paired *T*
118 tests (with Welch's correction, where appropriate).[18,19]

119 *2.5. In vivo studies*

120 Groups of seven to nine male BALB/c mice (23-25 g) were immunosuppressed with four
121 doses of cyclophosphamide, i.e. 4 days before infection (150 mg/kg), 1 day before
122 infection (100 mg/kg), 2 days postinfection (100 mg/kg) and 5 days postinfection (100
123 mg/kg).[17-19] The Guidelines for the Care and Use of Laboratory Animals was strictly
124 followed during maintenance of the animals; experiments were approved by the Animal
125 Care Committee of the University of Debrecen (permission no. 12/2014). Mice were
126 inoculated intravenously through the lateral tail vein with an infectious dose of 4-4.5x10^6
127 CFU/mouse. Inoculum density was confirmed by plating serial dilutions onto SDA plates.
128 At the beginning of the therapy (day 1) fungal kidney burden was determined after
129 dissection of 4-5 untreated mice in case of each isolate (day 1 control burden).[19]

130 Five-day intraperitoneal treatment with daily 5, 10 and 1 mg/kg caspofungin (Cancidas®),
131 micafungin (Mycamine®) and amphotericin B (Fungizone), respectively, against wild-type
132 clinical isolates were started after 24 hours postinfection. Five and 10 mg/kg caspofungin
133 and micafungin correspond to 70 mg and 200-300 mg daily doses in humans, respectively,
134 which are higher than the currently recommended daily doses.[22-29]

135 On day six after infection, all mice were sacrificed; both kidneys were removed, weighed
136 and homogenized aseptically. Homogenates were diluted tenfold; aliquots of 0.1 ml of the
137 undiluted and diluted (1:10) homogenates were plated onto SDA plates and incubated at 35
138 °C for 48 h. The lower limit of detection was 50 CFU/g of tissue. Statistical analysis of the
139 kidneys tissue burden was performed using the Kruskal-Wallis test with Dunn's post-test
140 for multiple comparisons.[17-19]

141 **3. Results**

142 ***3.1. In vitro* studies**

143 *3.1.1. MIC results in RPMI-1640 and RPMI-1640+50% serum*

144 In RPMI-1640 MIC values for the quality control strains were within the published
145 acceptable ranges.[12] MICs for clinical isolates are presented in Table 1. In RPMI-1640 all
146 three isolates were susceptible to micafungin and caspofungin (susceptibility breakpoints
147 for *C. krusei* are ≤0.25 mg/L for both drugs).[13] In RPMI-1640 plus 50% serum, micafungin
148 and caspofungin MICs were 64-128 and 8 times higher than in RPMI-1640, respectively.

149 In RPMI-1640, MIC ranges for amphotericin B were 0.12-0.5 mg/L which is lower than
150 the suggested susceptibility break-point (1 mg/L).[8,12] In RPMI-1640 plus 50% serum,
151 amphotericin B MIC values were increased 4-16-fold (Table 1).

152

153 *3.1.2. Killing activity of micafungin in RPMI-1640 and RPMI-1640+50% serum*

154 Growth rate (i.e. the times to achieve 1 log increase in CFU compared to the starting
155 inoculum) for the control isolates in RPMI-1640 and RPMI-1640 plus 50% serum was
156 similar ($P>0.05$). The mean times to achieve 99.9% (T99.9) growth reduction from the
157 starting inocula at different antifungal concentrations are shown in Table 2.

158 In RPMI-1640, at 1, 4, 16 and 32 mg/L micafungin was rapidly fungicidal against clinical
159 isolates (all three were killed ≤3.96 hours) (Table 2). Killing activity of micafungin was
160 concentration independent in cases of isolates 26513 and 25193 ($p>0.05$). Numerically, the
161 highest k value (1.536 1/h) was found in case of isolate 22910 at 16 mg/L (Fig. 1).

162 In RPMI-1640 plus 50% serum, growth curves were similar to controls at 1 mg/L. At 4 and
163 16 mg/L micafungin produced only transient CFU decreases, but the mean k values were
164 always negative. Positive k values were noticed only at 32 mg/L (CFU decreases were -
165 1.48-2.78 CFU/mL) (Fig. 1).

166

167 *3.1.3 Killing activity of caspofungin in RPMI-1640 and RPMI-1640+50% serum*

168 Caspofungin in RPMI-1640 was fungicidal within 4.42 hours against clinical isolates
169 (Table 2). Killing activity of caspofungin against isolate 22910 was concentration
170 independent. For isolates 25193 and 26513 the highest *k* values were measured at 1 and 4
171 (1.23 1/h for both concentrations) and 4 mg/L (1.30 1/h), respectively. In RPMI-1640 plus
172 50% serum, with the exception of 1 mg/L, killing rates were positive and increased with
173 concentrations (concentration-dependent killing). Killing activity of caspofungin in RPMI-
174 1640 plus 50% serum decreased at 4, but increased at 32 mg/l compared to RPMI-1640 for
175 all clinical isolates.

176

177 *3.1.4. Killing activity of amphotericin B in RPMI-1640 and RPMI-1640+50% serum*

178 In RPMI-1640, 0.5 mg/L amphotericin B produced positive *k* values for isolates 22910 and
179 25193. Though *k* values at 1 mg/L were positive (0.07-0.14 1/h) the CFU decreases were
180 weak (1.06-1.48 CFU/mL). At 2 mg/L, amphotericin B was fungicidal within 14.69 hours
181 against all three isolates (Table 2, Fig. 1).

182 In RPMI-1640 plus 50% serum, at 1 mg/L positive *k* value was observed only in case of
183 isolate 25193 (0.014 1/h), while at 2 mg/L *k* values for all three isolates were positive
184 (0.109-0.168 1/h). However, the *k* values are much lower than in case of echinocandins. In
185 RPMI-1640, amphotericin B killing activity at 2 mg/L was significantly higher than in
186 RPMI-1640 plus 50% serum (Table 2, Fig. 1).

187

188 **3.2. *In vivo* experiments**

189 All three isolates showed weak replication ability in the untreated neutropenic mice. In
190 cases of isolates 22910 and 26513, the mean fungal tissue burdens decreased on day 6 as

191 compared to day 1; the decreases were higher than one log in case of isolate 26513. In case
192 of isolate 25193, the mean fungal tissue burdens on day 6 increased only slightly (less than
193 1 log) comparing to day 1 (Fig 2).

194 Caspofungin and amphotericin B were effective against isolate 25193 ($p<0.05$); the CFU
195 decreases were lower than 1 log compared to untreated controls (on day 6) and were
196 similar to day 1 control burden. Micafungin did not show any activity in this model
197 system. Against isolates 22910 and 26513 none of the tested drugs showed activity.

198

199 **4. Discussion**

200 Theoretically, the *in vitro* fungicidal activity of echinocandins and amphotericin B against
201 *Candida* species is considered advantageous as they expected to eradicate fungal cells
202 rapidly from infected tissues and the bloodstream.[3,6-8,13,14,30] This drives their increasing
203 preference for treating severe fungal infections. However, the high protein binding
204 decreases the unbound, thus active drug concentration in the blood and tissues, leading to
205 lower drug exposure and potentially to therapeutic failure.[7,13,14,21,30,31] Previous *in vitro* data
206 support this hypothesis, because in the protein-free RPMI-1640 medium used in standard
207 susceptibility testing, echinocandins as well as amphotericin B are more frequently
208 fungicidal at low drug concentrations than in RPMI-1640 plus 50% serum, indicating the
209 clinical relevance of the protein binding.[5,16-20,30,31] Moreover, in a preclinical study with
210 micafungin, *in vivo* efficacy showed poorer correlation with MICs by standard RPMI-1640
211 than by serum-based susceptibility tests.[32]

212 It was previously suggested that determination of MIC alone in case of caspofungin is
213 misleading and may misclassify wild-type *C. krusei* or *C. glabrata* clinical isolates as non-
214 wild-type or resistant.[33] Therefore, currently micafungin or anidulafungin MICs in RPMI-
215 1640 are recommended as best predictors for the clinical efficacy of all three
216 echinocandins against *Candida* species, not excepting *C. krusei*. However, in our
217 experiments standard MICs and killing studies with micafungin in RPMI-1640 yielded
218 misleading results, falsely suggesting efficacy against *C. krusei in vivo*.

219 In our study adding 50% serum to RPMI-1640 did not influence the growth rate of the
220 three *C. krusei* isolates, indicating that 50% serum did not inhibit *C. krusei*. In contrast,
221 other authors noticed very poor growth of three *C. krusei* isolates (as well as of *C. glabrata*
222 and *C. lusitaniae*) in RPMI-1640 plus 50% serum even after seven days.[34] The explanation
223 for this difference is unknown. However, the cited study used the CLSI broth microdilution

formázott: Betűtípus: Dőlt, Betűszín: Automatikus

formázott: Betűtípus: Dőlt, Betűszín: Automatikus

224 method for MIC determination, and the smaller final volume (0.2 mL) in their experiment
225 may have influenced the growth of fungi.

226 In this study we have found significant differences in the killing rates of echinocandins in
227 RPMI-1640 and RPMI-1640 plus 50% serum against *C. krusei*. RPMI-1640 plus 50%
228 serum more profoundly decreased killing activity of micafungin than that of caspofungin
229 as compared to RPMI-1640 (Fig. 1., Table 2). The difference is probably due to the
230 difference between the protein binding for micafungin (99.9%) and caspofungin
231 (97.5%).[8,13,14] Our *in vitro* results in RPMI-1640 plus 50% serum (negative *k* values at ≤16
232 mg/L) proved to be a good predictor for the lack of *in vivo* efficacy even of elevated daily
233 micafungin dose, as micafungin never decreased the fungal tissue burden against any of the
234 tested *C. krusei* isolates. This result is concordant to our previous *in vitro* findings with
235 different *C. krusei* bloodstream isolates.[16]

236 In RPMI-1640 plus 50% serum, *in vitro* activity of caspofungin decreased less markedly as
237 positive *k* values were noticed even at 4 mg/L which is the trough concentration using the
238 standard or elevated daily doses.[22-24] However, the efficacy of caspofungin was found to be
239 unreliable against *C. krusei in vivo* in the neutropenic murine model (i.e. only one of three
240 isolates responded to the 5 mg/kg daily dose). A previous study of our group with two
241 different isolates showed a statistically demonstrable efficacy of caspofungin 5 mg/kg
242 daily in a similar model system.[18] However, in the present study the two isolates derived
243 from persistent candidemia showed weak *in vivo* replication ability (as indicated by
244 comparison of fungal tissue burdens on day 1 and 6), and weak efficacy of echinocandins
245 against slowly replicating cells is a well known phenomenon.[7,13,14] Moreover, other factors
246 (i.e. neutropenia, slow drug penetration into inflamed tissues, etc.) may strongly influence
247 therapeutic outcome.[8,35]

248 Amphotericin B used at clinically attainable concentration showed weak *in vitro* killing
249 activity against *C. krusei* regardless of test media, which was confirmed *in vivo* as well.[3-6]
250 Our results correlate with previous *in vitro* and *in vivo* findings that amphotericin B is not
251 superior to echinocandins for the treatment of invasive infections caused by *C. krusei*.[8]
252 However, currently there is no alternative in echinocandin resistant cases.[3,10,13]

253 In conclusion, standard RPMI-1640-based susceptibility tests did not provide reliable
254 information on the *in vivo* efficacy of micafungin or caspofungin against wild-type *C.*
255 *krusei* clinical isolates. Serum based susceptibility testing methods were good predictors of
256 the *in vivo* efficacy of micafungin, while neither the standard not the serum based method
257 were good predictors in case of caspofungin. Our *in vivo* results strongly correlate with the
258 currently reported experience that efficacy of echinocandins may be poor against *C. krusei*
259 in some clinical situations. This does not mean echinocandin resistance, but reflects the
260 lower activity of the cell-wall active echinocandins on a fungal species slowly replicating
261 *in vivo*. Our results suggest that prescribers treating *C. krusei* infections with echinocandins
262 should be on the watchout for clinical resistance and therapeutic failure.

263

264 **7. References**

265 1 Pfaller MA, Diekema DJ. Epidemiology of invasive candidiasis: a persistent public
266 health problem. Clin Microbiol Rev. 2007;20(1):133-163.
267 2 Pfaller MA, Neofytos D, Diekema D, Azie N, Meier-Kriesche HU, Quan SP, et al.
268 Epidemiology and outcomes of candidemia in 3648 patients: data from the Prospective
269 Antifungal Therapy (PATH Alliance®) registry, 2004-2008. Diagn Microbiol Infect
270 Dis. 2012;74(4):323-331.

271 3 Aversa F, Busca A, Candoni A, Cesaro S, Girmenia C, Luppi M, et al. Liposomal
272 amphotericin B (AmBisome®) at beginning of its third decade of clinical use. J
273 Chemother. 2017;29(3):131-143.
274 4 Baptista MI, Nona J, Ferreira M, Sampaio I, Abrantes M, Tomé MT et al. Invasive
275 fungal infection in neonatal intensive care units: a multicenter survey. J Chemother.
276 2016;28(1):37-43.
277 5 Majoros L, Szegedi I, Kardos G, Erdesz C, Konya J, Kiss C. Slow response of
278 invasive *Candida krusei* infection to amphotericin B in a clinical time-kill study. Eur J
279 Clin Microbiol Infect Dis. 2006;25(12):803-806.
280 6 Cantón E, Pemán J, Gobernado M, Viudes A, Espinel-Ingroff A. Patterns of
281 amphotericin B killing kinetics against seven *Candida* species. Antimicrob Agents
282 Chemother. 2004;48(7):2477–2482.
283 7 Perlin DS. Echinocandin resistance, susceptibility testing and prophylaxis:
284 implications for patient management. Drugs. 2014;74(14):1573-1585.
285 8 Pappas PG, Kauffman CA, Andes DR, Clancy CJ, Marr KA, Ostrosky-Zeichner L et
286 al: Executive summary: Clinical Practice Guideline for the management of candidiasis:
287 2016 Update by the Infectious Diseases Society of America. Clin Infect Dis. 2016,
288 62(4):e1-50.
289 9 Lortholary O, Renaudat C, Sitbon K, Madec Y, Denoeud-Ndam L, Wolff M et al.
290 Worrisome trends in incidence and mortality of candidemia in intensive care units
291 (Paris area, 2002-2010). Intensive Care Med. 2014;40(9):1303-1312.
292 10 Wang E, Farmakiotis D, Yang D, McCue DA, Kantarjian HM, Kontoyiannis DP et
293 al. The ever-evolving landscape of candidaemia in patients with acute leukaemia: non-
294 susceptibility to caspofungin and multidrug resistance are associated with increased
295 mortality. J Antimicrob Chemother. 2015;70(8):2362-2368.

296 11 Colombo AL, Guimarães T, Sukienik T, Pasqualotto AC, Andreotti R, Queiroz-
297 Telles F et al. Prognostic factors and historical trends in the epidemiology of
298 candidemia in critically ill patients: an analysis of five multicenter studies sequentially
299 conducted over a 9-year period. Intensive Care Med. 2014;40(10):1489-1498.
300 12 Clinical and Laboratory Standards Institute. 2008: Reference method for broth
301 dilution antifungal susceptibility testing of yeasts. Approved standard, 3rd ed. M27-A3.
302 Clinical and Laboratory Standards Institute, Wayne, PA.
303 13 Pfaller MA, Diekema DJ, Andes D, Arendrup MC, Brown SD, Lockhart SR. et al.
304 Clinical breakpoints for the echinocandins and *Candida* revisited: Integration of
305 molecular, clinical, and microbiological data to arrive at species-specific interpretive
306 criteria. Drug Resist Updat. 2011;14(3):164-176.
307 14 Chen SC, Slavin MA, Sorrell TC. Echinocandin antifungal drugs in fungal
308 infections: a comparison. Drugs. 2011;71(1):11-41.
309 15 Szabó Zs, Tóth B, Kovács M, Kardos G, Maráz A, Rozgonyi F. et al. Evaluation of
310 the new Micronaut-Candida system compared to the API ID32C method for yeast
311 identification. J Clin Microbiol. 2008;46(5):1824-1825.
312 16 Földi R, Szilágyi J, Kardos G, Berényi R, Kovács R, Majoros L. Effect of 50%
313 human serum on the killing activity of micafungin against eight *Candida* species using
314 time-kill methodology. Diagn Microbiol Infect Dis. 2012;73(4):338-342
315 17 Földi R, Kovács R, Gesztelyi R, Kardos G, Berényi R, Juhász B. et al. Comparison
316 of *in vitro* and *in vivo* efficacy of caspofungin against *Candida parapsilosis*, *C.*
317 *orthopsilosis*, *C. metapsilosis* and *C. albicans*. Mycopathol. 2012;174(4):311-318.
318 18 Kovács R, Gesztelyi R, Berényi R, Domán M, Kardos G, Juhász B. et al. Killing
319 rates exerted by caspofungin in 50% serum and its correlation with *in vivo* efficacy in a

320 neutropenic murine model against *Candida krusei* and *C. inconspicua.* J. Med.
321 Microbiol. 2014;63(Pt2):186-194.
322 19 Domán M, Kovács R, Perlin DS, Kardos G, Gesztelyi R, Juhász B. et al. Dose
323 escalation studies with caspofungin against *Candida glabrata*. J Med Microbiol.
324 2015;64(9):998-1007.
325 20 Szilágyi J; Földi R; Bayegan S; Kardos G, Majoros L. Effect of nikkomycin Z and
326 50% human serum on the killing activity of high-concentration caspofungin against
327 *Candida* species using time-kill methodology. J Chemother. 2012;24(1):18-25.
328 21 Lewis RE, Wiederhold NP. The solubility ceiling: a rationale for continuous
329 infusion amphotericin B therapy? Clin Infect Dis. 2003;37(6):871–872.
330 22 Cornely OA, Vehreschild JJ, Vehreschild MJ, Würthwein G, Arenz D, Schwartz S.
331 et al. Phase II dose escalation study of caspofungin for invasive aspergillosis.
332 Antimicrob Agents Chemother. 2011;55(12):5798-5803.
333 23 Flattery AM, Hickey E, Gill CJ, Powles MA, Misura AS, Galgoci AM. et al.
334 Efficacy of caspofungin in a juvenile mouse model of central nervous system
335 candidiasis. Antimicrob Agents Chemother. 2011;55(7):3491-3497.
336 24 Migoya EM, Mistry GC, Stone JA, Comisar W, Sun P, Norcross A. et al. Safety and
337 pharmacokinetics of higher doses of caspofungin in healthy adult participants. J Clin
338 Pharmacol. 2011;51(2):202-211.
339 25 Goto N, Hara T, Tsurumi H, Ogawa K, Kitagawa J, Kanemura N. et al. Efficacy and
340 safety of micafungin for treating febrile neutropenia in hematological malignancies. A
341 J Hematol. 2010;85(11):872-876.
342 26 Undre N, Stevenson P, Baraldi E. Pharmacokinetics of micafungin in HIV positive
343 patients with confirmed esophageal candidiasis. Eur J Drug Metab Pharmacokinet.
344 2012;37(1):31-38.

345 27 Grau S, Luque S, Campillo N, Samsó E, Rodríguez U, García-Bernedo CA. et al. Plasma and peritoneal fluid population pharmacokinetics of micafungin in post-surgical patients with severe peritonitis. J Antimicrob Chemother. 2015;70(10):2854-2861.

349 28 Lempers VJ, Schouten JA, Hunfeld NG, Colbers A, van Leeuwen HJ, Burger DM. et al. Altered micafungin pharmacokinetics in intensive care unit patients. Antimicrob Agents Chemother. 2015;59(8):4403-4409.

352 29 Yamada N, Kumada K, Kishino S, Mochizuki N, Ohno K, Ogura S. Distribution of micafungin in the tissue fluids of patients with invasive fungal infections. J Infect Chemother. 2011;17(5):731-734.

355 30 Tascini C, Sozio E, Di Paolo A, Tintori G, Leonildi A, Bertolino G. et al. Fungicidal activity and PK/PD of caspofungin as tools to guide antifungal therapy in a fluconazole-resistant C. parapsilosis candidemia. J Chemother. 2017;15:1-4. doi: 10.1080/1120009X.2017.1289308.

359 31 Nasar A, Ryan L, Frei CR, Cota JM, Wiederhold NP. Influence of serum and albumin on echinocandin *in vitro* potency and pharmacodynamics. Curr Fungal Infect Rep. 2013;7(2):89-95.

362 32 Maki K, Matsumoto S, Watabe E, Iguchi Y, Tomishima M, Ohki H. et al. Use of a serum-based antifungal susceptibility assay to predict the *in vivo* efficacy of novel echinocandin compounds. Microbiol Immunol. 2008;52(8):383-391.

365 33 Espinel-Ingroff A, Arendrup MC, Pfaller MA, Bonfietti LX, Bustamante B, Canton E. et al. Interlaboratory variability of caspofungin MICs for Candida spp. using CLSI and EUCAST methods: should the clinical laboratory be testing this agent? Antimicrob Agents Chemother. 2013;57(12):5836-5842.

369 34 Prigitano A, Esposto MC, Tortorano AM. Comparison of effects of human serum
370 and horse serum on in vitro susceptibility testing of echinocandins. J Chemother.
371 2014;26(1):62-63.
372 35 Felton T, Troke PF, Hope WW. Tissue penetration of antifungal agents. Clin
373 Microbiol Rev. 2014;27(1):68-88.
374
375
376

377 **Figure legends**

378

379 **Figure 1**

380 Killing rates of micafungin (MICA), caspofungin (CAS) and amphotericin B (AMB) and the
381 corresponding adjusted regression lines (dashed lines) against three *Candida krusei*
382 bloodstream isolates in RPMI-1640 (RPMI) and RPMI-1640 plus 50% serum (Serum).
383 Positive and negative *k* values indicate the decreases and increases, respectively, in viable cell
384 numbers.

385

386 **Figure 2**

387 Kidney tissue burden of severely neutropenic BALB/c mice infected intravenously with
388 three *C. krusei* isolates. Intraperitoneal caspofungin (CAS), micafungin (MICA) and
389 amphotericin B (AMB) (5, 10 and 1 mg/kg, respectively) daily treatments were started 24
390 hours after the infection. The bars represent the medians. Level of statistical significance
391 compared to the control population (day six) is indicated at $P<0.05$ (*).

392

393

394 **Table 1.** *Candida krusei* isolates and MICs of micafungin (MICA), caspofungin (CAS) and

395 amphotericin B (AMB) in RPMI-1640 and RPMI-1640 plus 50 % serum (50% serum)

396

Isolates	Media	MIC (mg/L)		
		MICA	CAS	AMB
22910	RPMI-1640	0.25	0.25	0.5
	50% serum	16	2	2
26513	RPMI-1640	0.12	0.25	0.5
	50% serum	16	2	2
25193	RPMI-1640	0.25	0.25	0.12
	50% serum	16	2	2

397
398

399

400 **Table 2**. Time (h) to reach 99.9% (T99.9) growth reduction from the starting inocula at
401 different micafungin, caspofungin and amphotericin B (AMB) concentrations (mg/L) in
402 RPMI-1640 (RPMI) and RPMI-1640 plus 50 % serum (serum). NR means that fungicidal
403 effect (99.9% growth reduction) was not reached.

Isolate	Media	Time (hours)										
		Micafungin (mg/L)				Caspofungin (mg/L)				AMB (mg/L)		
		1	4	16	32	1	4	16	32	0.5	1	2
22910	RPMI	3.04	3.03	1.95	3.96	2.94	2.59	2.59	3.04	NR	NR	8.67
	Serum	NR	NR	NR	NR	NR	NR	2.71	2.71	NR	NR	NR
26513	RPMI	2.72	2.76	2.85	2.84	4.25	2.31	2.74	3.81	NR	NR	9.18
	Serum	NR	NR	NR	NR	NR	NR	3.22	3.12	NR	NR	NR
25193	RPMI	3.38	3.91	3.07	2.95	2.43	2.43	4.42	3.04	NR	NR	14.69
	Serum	NR	NR	NR	NR	NR	NR	NR	3.04	NR	NR	NR

404

405

406