Single dose pharmacodynamics of amphotericin B against *Aspergillus* species in an in vitro pharmacokinetic/pharmacodynamic model

Running title: Single-dose AMB pharmacodynamics for *Aspergillus* spp

Rafal Al-Saigh[1], Maria Siopi[1], Nikolaos Siafakas[1], Aristea Velegraki[2],

Loukia Zerva[1], Joseph Meletiadis[1]

Clinical Microbiology Laboratory, Attikon Hospital[1], Mycology Research Lab, Department of Microbiology[2], Medical School, National and Kapodistrian University of Athens, Athens, Geece

Correspondence: Joseph Meletiadis, Ph. D.

Lecturer in Mycology,

Clinical Microbiology Laboratory,

Attikon University Hospital

Rimini 1, Haidari, 124 62 Athens

Tel: 210-583-1909

Fax: 210-532-6421

Email: jmeletiadis@med.uoa.gr

1   **ABSTRACT**

2   Conventional MIC testing of amphotericin B results in narrow MIC ranges challenging the

3   detection of resistant strains. In order to discern amphotericin B pharmacodynamics, the in vitro

4   activity of amphotericin B was studied against *Aspergillus* isolates with the same MIC with a new

5   in vitro pharmacokinetic/pharmacodynamic (PK/PD) model that simulates amphotericin B human

6   plasma levels. Clinical isolates of *A. fumigatus*, *A. terreus* and *A flavus* with the same CLSI modal

7   MICs of 1 mg/l were exposed to amphotericin B concentrations following the plasma

8   concentration-time profile after single bolus administration with C_{max} 0.6, 1.2, 2.4 and 4.8 mg/L.

9   Fungal growth was monitored up to 72h based on galactomannan production. Complete growth

10 inhibition was observed only against *A. fumigatus* with amphotericin B C_{max} ≥2.4 mg/L. At lower

11 C_{max}s 0.6 and 1.2 mg/L, a significant growth delay of 34h and 52h was observed, respectively

12 (p<0.001). For *A. flavus*, there was no complete inhibition but a progressive growth delay of 1h-50h

13 at amphotericin B C_{max} 0.6-4.8 mg/L (p<0.001). For *A. terreus*, the growth delay was modest (up to

14 8h) at all C_{max}s (p<0.05). The C_{max} (95% confidence interval) associated with 50% activity for *A.*

15 *fumigatus* was 0.60 (0.49-0.72) mg/L, significantly lower than for *A. flavus* 3.06 (2.46-3.80) and for

16 *A. terreus* 7.90 (5.20-12.29) (p<0.001). A differential in vitro activity of amphotericin B was found

17 among *Aspergillus* species despite the same MIC in the order of *A. fumigatus*>*A flavus*>*A. terreus*

18 in the in vitro PK/PD model possibly reflecting the different concentration- and time-dependent

19 inhibitory/killing activities amphotericin B exerting against these species.

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22 **Keywords**: amphotericin B, *A. fumigatus, A. flavus, A. terrus,* pharmacodynamics,

23   pharmacokinetics, simulation, in vitro

24

INTRODUCTION

26   Amphotericin B (AMB) is an antifungal drug of major importance in the treatment of

27   invasive aspergillosis (1). It is a highly lipophilic and amphoteric molecule that interacts with

28   fungal cell membrane forming pores and disrupting its integrity (2). Due to its unique mechanism of

29   action, it demonstrates a wide range of pharmacodynamic effects and broad spectrum of antifungal

30   activity. However, conventional MIC testing of amphotericin B resulted in narrow MIC ranges

31   within 1-2 twofold dilutions challenging the detection of resistant strains (3-5). Efforts to develop in

32   vitro assays that separate susceptible and resistant strains using richer media or gradient drug

33   concentrations strips were unsuccessful (3, 5). Species-specific epidemiological cutoff values

34   (ECV) have been proposed for amphotericin B and *Aspergillus* spp. based on CLSI broth

35   microdilution methodology with *A. terreus* ECV being one dilution higher than *A. fumigatus* and *A.*

36   *flavus* ECV (6).

37   In addition to inhibitory activity captured by the MIC, amphotericin B exerts a range of

38   different pharmacodynamic effects such as post-antifungal effect and concentration-dependent

39   killing (7). All these effects are usually determined after fungal exposure to constant drug

40   concentrations (2). However, in vivo, fungus is exposed to non-constant amphotericin B

41   concentrations as the drug undergoes metabolism, distribution and excretion. In particular, its

42   plasma levels follow a triphasic time-concentration profile characterized by the alpha-phase

43   observed within the first 4h after administration with a half-life of <1h, the beta-phase observed

44   within 4-24h after administration with a half life of 6-10h, and the gamma phase observed >24h of

45   administration with a half-life of >120h (8). Simulating this time-concentration profile in vitro is a

46   challenge because amphotericin B binds to plastic surfaces and degrades over time (9).

47   We recently developed an in vitro model that simulated human pharmacokinetics of

48   antifungal drugs and enabled to study the pharmacodynamics of decreasing drug concentrations as

49   in human plasma (10). This pharmacokinetic/pharmacodynamic (PK/PD) model showed

50   considerable differences of voriconazole activity against *Asperillus* species which had the same

51 MICs indicating that studying the in vitro activity of decreasing drug concentrations provides

52 unique information of pharmacodynamic effects of antifungal drugs (11). With this model, the time-

53 and concentration-dependent pharmacodynamic properties of antifungal drugs can be studied and

54 PK/PD analysis simulating human pharmacokinetics can be performed.

55 We therefore studied the activity of amphotericin B against *A. fumigatus*, *A. flavus* and *A.*

56 *terreus* strains with similar MICs with the new in vitro PK/PD model simulating single-dose

57 pharmacokinetics of amphotericin B in human plasma and monitoring *Aspergillus* growth over time

58 with galactomannan production. Despite the same MICs, important pharmacodynamic differences

59 were found among the three species with amphotericin B being less active against *A. flavus* and *A.*

60 *terreus* than against *A. fumigatus* reflecting differences in inhibitory, killing and post-drug exposure

61 effects.

62

63 **MATERIAL AND METHODS**

64 **Strains**. Three clinical strains of *A. fumigatus*, *A. flavus* and *A. terreus* isolated from patients

65 with invasive pulmonary aspergillosis were studied. The minimal inhibitory concentrations (MIC)

66 as determined thrice with the CLSI broth microdilution method were 1-1 mg/L for *A. fumigatus*, 1-1

67 mg/L for *A. flavus* and 1-2 mg/L (mode 1 mg/L) for *A. terreus* (12, 13). The *A. terreus* strain was

68 included because of its known reduced susceptibility to amphotericin B. The strains were

69 maintained at-70°C in 10% glycerol and cultured twice in Sabouraud Dextrose agar at 30°C for 5-7

70 days. A conidial suspension was prepared in normal saline with 1% Tween 20. Conidia were

71 counted with a Newbauer chamber in order to obtain a final suspension 1x10^{5}CFU/ml and their

72 concentration was confirmed by quantitative cultures on Sabouraud Dextrose Agar.

73 **Antifungal susceptibility testing.** In order to explore the in vitro susceptibility of the three

74 isolates with other methodologies, the isolates were also tested with the gradient concentration strip

75 method Liofilchem™ MIC Test Strips (MTS) (Varelas SA, Athens, Greece) according to

76 manufacturer's instructions and the XTT methodology as previously described (14). Briefly, for

MTS method agar plates with RPMI1640+MOPS+2% glucose were inoculated in three directions with a cotton swab dipped into a 0.5MacFarland conidial inoculum and the MTS was applied and incubated at 35°C for 24h and 48h. The MIC was determined as drug concentration at which the border of the elliptical inhibition zone corresponding to 100% inhibition intersected the strip. For XTT methodology, twofold serial dilutions of amphotericin B in RPMI1640+MOPS in 96-flatbottom well microtitration plates were inoculated with $1-5 \times 10^4$CFU/ml, incubated for 48h when 0.1 mg/ml XTT + 25 μM Menadione was added in each well, further incubated for 2h at 35°C when absorbance at 450nm was measured and % growth in each well was calculated in comparison to growth in the drug-free control. The MIC was determined as the lowest drug concentration with <10% growth. Furthermore, the minimal fungicidal concentration (MFC) was determined with an XTT methodology as previously described (13). Briefly, after XTT MIC determination, fresh medium was added to all clear wells after washed with saline and after incubation for 24h at 35°C XTT+MEN was added and % growth was calculated based on absorbance at 450nm. The MFC was determined as the lowest drug concentration showing <10% growth. All tests were performed three times.

Antifungal drug and medium. Amphotericin B (AMB, Fungizone, Bristol-Myers) was reconstituted at 10,000 mg/L according to manufacturer's instructions and stored at-70°C. The medium contained 10.4 g/L RPMI1640 with glutamine without sodium bicarbonate (Sigma-Aldrich, St. Luis, MO) and 0.165M buffer MOPS (Invitrogen, Carlsbad, CA), pH 7.0, with 100 mg/L chloramphenicol (Sigma -Aldrich, St. Luis, MO).

In vitro pharmacokinetic/pharmacodynamic model. The in vitro pharmacokinetic simulation model consists of a) a glass beaker containing 700ml medium (external compartment EC) in which is placed, b) a dialysis tube of 10 ml volume (internal compartment IC) the wall of which consists of cellulose permeable membrane allowing the free diffusion of molecules with a molecular weight <20kD, and c) a peristaltic pump (Minipuls Evolution, Gilson, France), which removes the content of EC and adds medium within it at a rate equivalent to drug removal from

103   human serum (10). The conidial suspension is inoculated in the IC within which the growing fungus

104   and its derivative galactomannan (molecular weight 20-60kD) remain trapped, while nutrients and

105   drug diffuse freely between IC and EC. The concentration of the galactomannan increases with

106   fungal growth. The drug is injected into the EC and its concentration is adjusted by the pump to

107   correspond to the average half-life observed in human plasma after intravenous administration of

108   amphotericin B. The EC was covered with aluminum foil in order to minimize light exposure and

109   placed on a heated magnetic stirrer (37°C). Before starting each experiment, temperature and flow

110   rate were controlled. All experiments were repeated twice.

111   **Determination of amphotericin B concentrations**. The drug levels in the IC were

112   determined by a microbiological method using the strain *Paecilomyces variotii* ATTC 22319,

113   susceptible to AMB (15). Specifically, *P.variotii* conidia at final concentration 5×10^{5}CFU/ml were

114   inoculated into prewarmed at 54°C RPMI1640 medium + MOPS with 15 g/L agar and poured to

115   plastic plates 10x10cm. After solidification of the agar, 1 cm-diameter holes were opened and filled

116   with 100μl of known drug dilutions (range 0.25-16 mg/L), as well as 100μl of IC samples. The

117   plates were incubated at 37°C for 24h when diameters of inhibition zones were measured. Unknown

118   drug concentrations in the IC samples were determined using the standard curve constructed from

119   known drug concentrations and corresponding diameters of inhibition zones.

120   **Pharmacokinetic analysis**. Several clinically relevant AMB doses (0.25, 0.5, 1 and 1.5

121   mg/kg) were simulated in the in vitro model with maximum concentrations in human plasma C_{max}

122   of 0.6, 1.2, 2.4 and 4.8 mg/L and AUC values of 9.4, 21, 46.3 and 57.3 (8, 16, 17). After taking

123   into account any loss of amphotericin B during the experiments due to degradation and surface

124   binding, the flow rate was adjusted in order to approximate the plasma concentration profile of

125   amphotericin B in humans with an alpha phase with a short half life of <1h observed within 4h after

126   drug administration followed by a beta phase with a longer half life of 6-10h observed 4-24h after

127   drug administration and a gamma phase with a half life of 120h observed >24h after drug

128   administration (8). Amphotericin B concentrations were determined at 0h, 4h, 6h, 8h, 20h, 24h,

129   44h, 48h and 72h after the introduction of the drug GI the IC using the bioassay. The data were

130   analyzed by nonlinear regression based on a three-compartment model described by the equation

131    $C= C_\alpha e^{k\alpha t}+C\beta e^{k\beta t}+C_\gamma e^{k\gamma t}$ where k_α, k_β and k_γ are the rate constants, C_α, C_β and C_γ are the Y-

132   intercepts for alpha, beta and gamma phase, respectively, and C is the concentration at a given time

133   t. The half-lives of alpha, beta and gamma phases were calculated for EC and IC separately using

134   the equations $t_{1/2.\alpha}=k_\alpha/\ln(2)$, $t_{1/2.\beta}=k_\beta/\ln(2)$, and $t_{1/2.\gamma}=k_\gamma/\ln(2)$, respectively, and were compared with

135   the corresponding values observed in human plasma.

136   **Determination of fungal growth.** Fungal growth in the IC was assessed in samples of

137   100μl at regular time intervals by determining galactomannan production using an ELISA (Platellia,

138   Biorad, Athens, Greece). Samples were diluted with 200μl saline in order to reach the final volume

139   of 300μl before processing. Results were expressed as a galactomannan index (GI) according to the

140   manufacturer's instructions. Galactomannan levels were also determined in the EC in order to

141   ensure that no galactomannan was escaped from the IC.

142   Real time PCR conidial equivalent was used as an alternative biomarker of fungal growth

143   and killing. *Aspergillus* DNA was extracted from 200μl samples from the IC of the in viro PKPD

144   model after 0h and 72h with the Qiagen DNA Blood Mini kit (Roche Diagnostics, Athens, Greece)

145   after enzymatic (incubation with protenase K at 56°C for 10 min) and mechanical (1 min vortex

146   with glass beads) extraction as previously described (18). Real time PCR was performed with a

147   previously described assay (2Asp assay) using *Aspergillus* specific primers (ASF1 and ADR1) and

148   probe (ASP28P) (19). The threshold cycle (Ct) of each sample, which identifies the cycle number

149   during PCR when fluorescence exceeds a threshold value determined by the software, was

150   converted to conidial equivalent (CE) *A. fumigatus* DNA by interpolation from a 6-point standard

151   curve of Ct values obtained with 10^3-10^8 *Aspergillus* CFU/ml. The reduction of the PCR CE after

152   72h of incubation compared to 0h was calculated for each species and amphotericin B doses.

153   **Pharmacodynamic analysis.** In vitro pharmacodynamics of each amphotericin B dose and

154   *Aspergillus* species were determined based on the GI-time relationship analyzed with the Emax

155 model: $E = E_{min} + E_{max} * T^{\gamma} / (T^{\gamma} + T_{50})$, where E is the GI (dependent variable), E_{max} and E_{min}, the

156 maximum and minimum GI, respectively, T the incubation time (independent variable), T_{50} the

157 time corresponding to 50% of E_{max} and γ the slope of the curve. In addition, the area under the

158 galactomannan index-time curve (AUC_{GI}) was calculated for each amphotericin B dose. As shown

159 previously, the parameters E_{max}, γ and T_{50} describe the extent, rate and time of galactomannan

160 production, respectively, whereas the AUC_{GI} is a surrogate marker of fungal growth. The higher the

161 AUC_{GI}, the greater is the fungal growth. The percentage of fungal growth at each dose was

162 calculated based on the AUC_{GI} of each doses divided by the AUC_{GI} of the growth control. Based on

163 all these parameters, the in vitro activity of amphotericin B dose against each *Aspergillus* species

164 was estimated. Finally, the in vitro PKPD relationship AUC_{GI}-C_{max} was plotted for each species and

165 analyzed with the Emax model.

166 **Statistical analysis.** All analysis was performed with the software Prism 5.01 (GraphPad

167 Inc., La Jolla, CA). All Emax models were globally fitted to the data with E_{max} and E_{min} shared

168 among data sets. Comparisons between Emax model parameters of different amphotericin B doses

169 and *Aspergillus* species were assessed using extra sum-of-squares F test. A p value <0.05 was

170 considered statistically significant.

171

172 **RESULTS**

173 **Antifungal susceptibility testing.** The MTS MICs for *A. fumigatus, A. flavus* and *A. terreus*

174 were 0.75-0.75, 2-3 and 1-1.5 mg/l after 24h and 2-3, >32, and >32 mg/l after 48h, respectively.

175 The XTT MICs and MFCS were 1-2 and 1-2 mg/l for *A. fumigatus*, 2-2 and 2-4 mg/l for *A. flavus*

176 and 1-2 and 8-16 mg/l for *A. terreus*.

177 **Bioassay for amphotericin B.** The amphotericin B concentrations detected with the

178 bioassay ranged from 0.25 mg/L to 16 mg/L and lowest limit of detection was 0.25 mg/L. Across

179 all experiments performed on the same and different days, the diameter of inhibition zone correlated

180   linearly with amphotericin B concentration ($r^2 > 0.77$, global $r^2 = 0.84$). The coefficient of variation

181   ranged from 5% to 25% (average 15%) for all concentrations.

182   **Pharmacokinetic analysis**. The C_{max} in the IC were 0.76-0.78, 1.05-1.10, 2.5-2.7 and 3.9-

183   4.4 mg/L and the AUCs 4.5-5, 8-8.6, 31.9-33.2, 64.8-67.9 mg.h/l, respectively, with $t_{1/2,\alpha}$ 0.2-2h,

184      $t_{1/2,\beta}$ 10-17h and $t_{1/2,\gamma}$ 71h for the simulated amphotericin B doses 0.25, 0.5, 1 and 1.5 mg/kg,

185   respectively. Because of the low detection limit of the bioassay, a gamma phase was observed only

186   for the highest dose of amphotericin B with C_{max} 4.8 mg/L. These values were similar to those

187   observed in human plasma after administration of amphotericin B doses 0.25-1.5 mg/kg with the

188   largest deviations observed at lower doses (Figure 1).

189   **Pharmacodynamic analysis (PD)**. The GI–time curves were described very well with the

190   Emax model ($R^2 > 0.86$) and they were characterized by the same E_{max} but different slopes and T_{50}s

191   for the different amphotericin B doses and *Aspergillus* species. Among all species and doses tested,

192   complete inhibition of galactomannan production was observed only against *A. fumigatus* with

193   amphotericin B doses corresponding to $C_{max} \geq 2.4$ mg/L (Figure 2). At lower doses, a significant

194   delay in galactomannan production was observed with a mean±SEM T_{50} of 38.1±2.3h for C_{max} 0.6

195   mg/L and 57.9±2.7h for C_{max} 1.2 mg/L compared to 4.2±0.4h for the drug free control ($p < 0.001$).

196   For *A. flavus*, there was no complete inhibition but a progressive delay of galactomannan

197   production with increasing amphotericin B doses since the T_{50} increased from 8.2±0.6h for the

198   growth control to 9.3±0.6h at amphotericin B dose with C_{max} 0.6 mg/L, 24.3±3.2h at C_{max} 1.2 mg/L,

199   36.7±3.1h at C_{max} 2.4 mg/L and 57.8±2.7h at C_{max} 4.8 mg/L ($p < 0.001$). For *A. terreus*, the delay in

200   galactomannan production was modest since the T_{50} of 4±0.4h for the growth control increased to

201   12.6±3.3h at the highest dose of amphotericin B with C_{max} 4.8 mg/L ($p = 0.013$). The differences

202   among the tree species in galactomannan production with the two high doses of amphotericin B

203   with C_{max} 2.4 and 4.8 mg/l were confirmed with real time PCR results with PCR CE at 72h being

204   reduced by 0.7 and 0.8 $log_{10}CE$ of *A. fumigatus*, 0.1 and 0.4 $log_{10}CE$ of *A. flavus* and increased by

205   1.5 and 0.1 $log_{10}CE$ of *A. terreus*, respectively (data not shown).

206   Finally, the in vitro activity of amphotericin B against the three *Aspergillus* species was

207   compared by constructing PK/PD curves. In order to quantify the effect of each amphotericin B

208   dose at the entire 72h period of incubation, the AUC_{GI} was calculated for each dose and species as a

209   surrogate marker of fungal growth and plotted against the corresponding C_{max}s (Figure 3). The in

210   vitro PK/PD relationship followed a sigmoid pattern (global $R^2=0.99$). The C_{max} (95% confidence

211   interval) associated with 50% activity for *A. fumigatus* was 0.60 (0.49-0.72), which was statistically

212   significant lower than the corresponding C_{max} against *A. flavus* (3.06, 2.46-3.80) and *A. terreus*

213   (7.90, 5.20-12.29) ($p<0.001$).

214

215   **DISCUSSION**

216   Important pharmacodynamic differences of amphotericin B against the three *Aspergillus*

217   species were found in the recently developed in vitro PK/PD model where conidia were exposed to

218   decreasing drug concentrations simulating the plasma concentration-time profile of amphotericin B.

219   Despite the same MICs, the strongest in vitro activity of amphotericin B was found against *A.*

220   *fumigatus* followed by *A. flavus* and *A. terreus*. The XTT and gradient concentration strips methods

221   showed minor differences in the MIC of the three isolates which clustered within 1-2 twofold

222   dilution, emphasizing the problem of narrow amphotericin B MIC ranges with these techniques.

223   However, 48h MTS MICs were similar for *A. flavus* and *A. terreus* and higher for *A. fumigatus*

224   whereas XTT MFCs were similar for *A. fumigatus* and *A. flavus* and higher for *A. terreus*

225   advocating for the different pharmacodynamic effects amphotericin B possessed against different

226   *Aspergillus* species.

227   Studying the effect of decreasing concentrations of antifungal drugs provides information

228   about pharmacodynamic properties related with sub-MIC effect, post-antifungal effect, time- and

229   concentration-dependent activities. These effects can be quantified by a surrogate marker of fungal

230   growth based on galactomannan production kinetics which captures any difference regarding the

231   above antifungal effects. Differential antifungal activity was also previously found with the present

232   in vitro PK/PD model for voriconazole against the three *Apergillus* species with identical MICs

233   emphasizing the importance of studying non-constant drug concentrations (10, 11).

234   The findings of the present study are in agreement with previous time-kill assays where

235   supra-MIC (4x and 20xMIC) concentrations of amphotericin B killed *A. fumigatus* but not *A.*

236   *terreus* (20). Minimal fungicidal concentrations of amphotericin B were similar against *A.*

237   *fumigatus* and *A. flavus* and much higher against *A. terreus*. However, MFC/MIC ratios for *A.*

238   *fumigatus* were lower than those for *A. flavus* differentiating the in vitro activity of amphotericin B

239   against these two species (13). In addition, the three species were previously found to differ also in

240   the post-drug exposure effects since 4x and 1xMIC of amphotericin B demonstrated >4h post

241   antifungal effect against *A. fumigatus* and <4h against *A. flavus* and *A. terreus* (21). Time-

242   dependent activity of amphotericin B inhibition also differed among the three *Aspergillus* species

243   (22). Exposure of *Aspergillus* conidia to supra-MIC concentrations for 8h resulted in significant

244   amount of metabolic activity for *A. terreus* (16%), less for *A. flavus* (8%) and even lesser for *A.*

245   *fumigatus* (5%) isolates. Furthermore, despite the same concentration-effect curves of amphotericin

246   B for *A. fumigatus* and *A. flavus* at 48h, the inhibitory concentration-effect curve after 8h of

247   exposure to amphotericin B were shifted to the left for *A. fumigatus* but not for *A. flavus* indicating

248   that amphotericin B activity is faster against *A. fumigatus* than *A. flavus* species (22).

249   Taking into account all these different effects exerted by amphotericin B, the order of

250   amphotericin B in vitro activity demonstrated by the present model (*A. fumigatus*>*A. flavus*>*A.*

251   *terreus*) could be explained by a fast inhibitory action and increased killing rate against *A.*

252   *fumigatus*, a slower inhibitory action and reduced killing efficiency against *A. flavus* and the

253   slowest inhibitory action and no killing against *A. terreus* as also found with real time PCR results.

254   In particular, the delayed galactomannan production of *A. fumigatus* but not *A. flavus* at C_{max}=0.6

255   mg/L indicates a strong sub-MIC effect of amphotericin B against the former species. Although

256   there are no data on sub-MIC effects of amphotericin B against *Aspergillus* spp. such effects were

257   described against *Candida* spp.(23). At C_{max}=1.2 mg/L, galactomannan was detected after 48h

258   incubation for *A. fumigatus* reflecting the minimal fungicidal action at this concentration (usually

259   observed at 2xMIC) (13) and the long post-antifungal effect observed at 1xMC (21) together with a

260   sub-MIC effect possibly occurred after amphotericin B concentrations fell below the MIC. The

261   absence of galactomannan production at concentrations >2xMIC reflects the fungicidal activity

262   amphotericin B demonstrated at time-kill assays (20). For *A. flavus*, galactomannan was detected at

263   C_{max}=4.8, 2.4 and 1.2 mg/L after 48h, 24h and 6h as soon as the concentration fell below MIC

264   reflecting the absence of killing and post-antifungal effects as previously described (21). Of note, at

265   all three doses galactomannan production was detected after 4h despite amphotericin B

266   concentrations being higher than the MIC reflecting the slow inhibitory action of amphotericin B

267   against this species, as previously found (22). Finally, the modest delay in galactomannan

268   production of *A. terreus* at all doses reflects the lack of killing, post-antifungal and possibly sub-

269   MIC effect and the slow inhibitory action against this species. Thus, single-dose pharmacodynamics

270   in the present in vitro PK/PD model where amphotericin B concentrations decrease over time may

271   reflect concentration- and time-dependent inhibitory and killing activities described by MFC, time-

272   kill and post-antifungal effect assays.

273   Amphotericin B was for decades the treatment of choice for aspergillosis. Clinical and

274   animal data indicated different drug efficacy against infections caused by various *Aspergillus*

275   species (24). Lack of in vivo efficacy, however, was not associated with significantly increased

276   MIC values (3, 25, 26), which remained similar for all three species examined in the present study

277   (13, 27). Results obtained by the new in vitro model revealed striking differences in efficacy of

278   amphotericin B against the three *Aspergillus* species despite their similar MICs with the following

279   order: *A. fumigatus*>*A. flavus*>*A. terreus*. These findings are in agreement with previous

280   comparative animal studies where treatment with amphotericin B was more effective against

281   experimental infection caused by *A. fumigatus* than infection with *A. flavus* and less effective

282   against infection with *A. terreus* (4, 20). In particular, amphotericin B treatment of guinea pigs

283   infected with an *A. flavus* or an *A. fumigatus* strain (each with MIC of 1 mg/L), resulted in 0% and

284   80% survival, respectively at the highest dosage of 2.5 mg/kg (4, 20). Furthermore, the in vivo

285   PKPD parameter C_{max}/MIC associated with near maximum survival in an animal model of

286   experimental aspergillosis by *A. fumigatus* was previously found to be 2.4 similar to the C_{max}/MIC

287   ratio found in the present study to be associated with the maximum suppressive effect of

288   amphotericin B against *A. fumigatus* (28). However, differences in pathogenesis and virulence

289   among these species may confound in vitro-in vivo correlation (29, 30). Clinical studies also

290   demonstrated a higher mortality rate of infections by *A. terreus* compared to those by *A. fumigatus*

291   despite amphotericin B therapy (31, 32). It seems that the new in vitro model, described here, may

292   better characterize the pharmacodynamic characteristics of amphotericin B against the most

293   clinically significant *Aspergillus* species than conventional in vitro susceptibility systems.

294   In summary, the in vitro model simulated well amphotericin B human pharmacokinetics

295   and demonstrated a differential in vitro activity against the three *Aspergillus* species that was not

296   reflected by their respective MICs. The effects observed in the in vitro PK/PD model may be the

297   sum of concentration- and time-dependent inhibitory/killing activities exerted by amphotericin B

298   with the greatest activity found against *A. fumigatus* and the lowest against *A. terreus*. Future

299   studies should focus on testing larger collections of isolates in order to describe the distribution of

300   this new pharmacodynamic effect and taking into account protein binding and amphotericin B

301   disposition in human body in order to obtain clinically relevant drug exposures that was not

302   obtained with the current model. A composite pharmacodynamic effect that describes the different

303   in vitro activities of amphotericin B may overcome the MIC clustering, assess better antifungal

304   activity and help distinguish susceptible and from resistant strains.

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REFERENCES

1. **Walsh TJ, Anaissie EJ, Denning DW, Herbrecht R, Kontoyiannis DP, Marr KA, Morrison VA, Segal BH, Steinbach WJ, Stevens DA, van Burik JA, Wingard JR, Patterson TF.** 2008. Treatment of aspergillosis: clinical practice guidelines of the Infectious Diseases Society of America. Clin Infect Dis **46:**327-360.

2. **Moore CB, Sayers N, Mosquera J, Slaven J, Denning DW.** 2000. Antifungal drug resistance in Aspergillus. J Infect **41:**203-220.

3. **Johnson EM, Oakley KL, Radford SA, Moore CB, Warn P, Warnock DW, Denning DW.** 2000. Lack of correlation of in vitro amphotericin B susceptibility testing with outcome in a murine model of Aspergillus infection. J Antimicrob Chemother **45:**85-93.

4. **Odds FC, Van Gerven F, Espinel-Ingroff A, Bartlett MS, Ghannoum MA, Lancaster MV, Pfaller MA, Rex JH, Rinaldi MG, Walsh TJ.** 1998. Evaluation of possible correlations between antifungal susceptibilities of filamentous fungi in vitro and antifungal treatment outcomes in animal infection models. Antimicrob Agents Chemother **42:**282-288.

5. **Verweij PE, Oakley KL, Morrissey J, Morrissey G, Denning DW.** 1998. Efficacy of LY303366 against amphotericin B-susceptible and -resistant Aspergillus fumigatus in a murine model of invasive aspergillosis. Antimicrob Agents Chemother **42:**873-878.

6. **Espinel-Ingroff A, Cuenca-Estrella M, Fothergill A, Fuller J, Ghannoum M, Johnson E, Pelaez T, Pfaller MA, Turnidge J.** 2011. Wild-type MIC distributions and epidemiological cutoff values for amphotericin B and Aspergillus spp. for the CLSI broth microdilution method (M38-A2 document). Antimicrob Agents Chemother **55:**5150-5154.

7. **Dodds ES, Drew RH, Perfect JR.** 2000. Antifungal pharmacodynamics: review of the literature and clinical applications. Pharmacotherapy **20:**1335-1355.

8. **Bekersky I, Fielding RM, Dressler DE, Lee JW, Buell DN, Walsh TJ.** 2002. Pharmacokinetics, excretion, and mass balance of liposomal amphotericin B (AmBisome) and amphotericin B deoxycholate in humans. Antimicrob Agents Chemother **46:**828-833.

336   9.   **Lewis RE, Wiederhold NP, Prince RA, Kontoyiannis DP.** 2006. In vitro

337   pharmacodynamics of rapid versus continuous infusion of amphotericin B deoxycholate

338   against Candida species in the presence of human serum albumin. J Antimicrob Chemother

339   **57:**288-293.

340   10.   **Meletiadis J, Al-Saigh R, Velegraki A, Walsh TJ, Roilides E, Zerva L.** 2012.

341   Pharmacodynamic effects of simulated standard doses of antifungal drugs against

342   Aspergillus species in a new in vitro pharmacokinetic/pharmacodynamic model. Antimicrob

343   Agents Chemother **56:**403-410.

344   11.   **Al-Saigh R, Elefanti A, Velegraki A, Zerva L, Meletiadis J.** 2012. In vitro

345   pharmacokinetic/pharmacodynamic modeling of voriconazole activity against Aspergillus

346   species in a new in vitro dynamic model. Antimicrob Agents Chemother **56:**5321-5327.

347   12.   **CLSI.** 2008. Reference method for broth dilution antifungal susceptibility testing of

348   filamentous fungi; Approved standard. Second edition M38-A2. **28**.

349   13.   **Meletiadis J, Antachopoulos C, Stergiopoulou T, Pournaras S, Roilides E, Walsh TJ.**

350   2007. Differential fungicidal activities of amphotericin B and voriconazole against

351   Aspergillus species determined by microbroth methodology. Antimicrob Agents Chemother

352   **51:**3329-3337.

353   14.   **Meletiadis J, Mouton JW, Meis JF, Bouman BA, Donnelly JP, Verweij PE.** 2001.

354   Colorimetric assay for antifungal susceptibility testing of Aspergillus species. J Clin

355   Microbiol **39:**3402-3408.

356   15.   **Shadomy S, McCay JA, Schwartz SI.** 1969. Bioassay for hamycin and amphotericin B in

357   serum and other biological fluids. Appl Microbiol **17:**497-503.

358   16.   **Ayestaran A, Lopez RM, Montoro JB, Estibalez A, Pou L, Julia A, Lopez A, Pascual B.**

359   1996. Pharmacokinetics of conventional formulation versus fat emulsion formulation of

360   amphotericin B in a group of patients with neutropenia. Antimicrob Agents Chemother

361   **40:**609-612.

362   17.   **Sanders SW, Buchi KN, Goddard MS, Lang JK, Tolman KG.** 1991. Single-dose

363   pharmacokinetics and tolerance of a cholesteryl sulfate complex of amphotericin B

364   administered to healthy volunteers. Antimicrob Agents Chemother **35:**1029-1034.

365   18.   **Griffiths LJ, Anyim M, Doffman SR, Wilks M, Millar MR, Agrawal SG.** 2006.

366   Comparison of DNA extraction methods for Aspergillus fumigatus using real-time PCR. J

367   Med Microbiol **55:**1187-1191.

368   19.   **White PL, Barton R, Guiver M, Linton CJ, Wilson S, Smith M, Gomez BL, Carr MJ,**

369   **Kimmitt PT, Seaton S, Rajakumar K, Holyoake T, Kibbler CC, Johnson E, Hobson**

370   **RP, Jones B, Barnes RA.** 2006. A consensus on fungal polymerase chain reaction

371   diagnosis?: a United Kingdom-Ireland evaluation of polymerase chain reaction methods for

372   detection of systemic fungal infections. J Mol Diagn **8:**376-384.

373   20.   **Walsh TJ, Petraitis V, Petraitiene R, Field-Ridley A, Sutton D, Ghannoum M, Sein T,**

374   **Schaufele R, Peter J, Bacher J, Casler H, Armstrong D, Espinel-Ingroff A, Rinaldi**

375   **MG, Lyman CA.** 2003. Experimental pulmonary aspergillosis due to Aspergillus terreus:

376   pathogenesis and treatment of an emerging fungal pathogen resistant to amphotericin B. J

377   Infect Dis **188:**305-319.

378   21.   **Vitale RG, Mouton JW, Afeltra J, Meis JF, Verweij PE.** 2002. Method for measuring

379   postantifungal effect in Aspergillus species. Antimicrob Agents Chemother **46:**1960-1965.

380   22.   **Antachopoulos C, Meletiadis J, Sein T, Roilides E, Walsh TJ.** 2007. Use of high

381   inoculum for early metabolic signalling and rapid susceptibility testing of Aspergillus

382   species. J Antimicrob Chemother **59:**230-237.

383   23.   **Garcia MT, Llorente MT, Minguez F, Prieto J.** 2002. Postantifungal effect and effects of

384   sub-MIC concentrations on previously treated Candida sp. influence of growth phase. J

385   Infect **45:**263-267.

386   24.   **Denning DW, Marinus A, Cohen J, Spence D, Herbrecht R, Pagano L, Kibbler C,**

387   **Kcrmery V, Offner F, Cordonnier C, Jehn U, Ellis M, Collette L, Sylvester R.** 1998. An

388   EORTC multicentre prospective survey of invasive aspergillosis in haematological patients:

389   diagnosis and therapeutic outcome. EORTC Invasive Fungal Infections Cooperative Group.

390   J Infect **37:**173-180.

391 25. **Lionakis MS, Lewis RE, Chamilos G, Kontoyiannis DP.** 2005. Aspergillus susceptibility

392   testing in patients with cancer and invasive aspergillosis: difficulties in establishing

393   correlation between in vitro susceptibility data and the outcome of initial amphotericin B

394   therapy. Pharmacotherapy **25:**1174-1180.

395 26. **Mosquera J, Warn PA, Morrissey J, Moore CB, Gil-Lamaignere C, Denning DW.**

396   2001. Susceptibility testing of Aspergillus flavus: inoculum dependence with itraconazole

397   and lack of correlation between susceptibility to amphotericin B in vitro and outcome in

398   vivo. Antimicrob Agents Chemother **45:**1456-1462.

399 27. **Baddley JW, Marr KA, Andes DR, Walsh TJ, Kauffman CA, Kontoyiannis DP, Ito JI,**

400   **Balajee SA, Pappas PG, Moser SA.** 2009. Patterns of susceptibility of Aspergillus isolates

401   recovered from patients enrolled in the Transplant-Associated Infection Surveillance

402   Network. J Clin Microbiol **47:**3271-3275.

403 28. **Wiederhold NP, Tam VH, Chi J, Prince RA, Kontoyiannis DP, Lewis RE.** 2006.

404   Pharmacodynamic activity of amphotericin B deoxycholate is associated with peak plasma

405   concentrations in a neutropenic murine model of invasive pulmonary aspergillosis.

406   Antimicrob Agents Chemother **50:**469-473.

407 29. **Slesiona S, Gressler M, Mihlan M, Zaehle C, Schaller M, Barz D, Hube B, Jacobsen**

408   **ID, Brock M.** 2012. Persistence versus escape: Aspergillus terreus and Aspergillus

409   fumigatus employ different strategies during interactions with macrophages. PLoS One

410   **7:**e31223.

411 30. **Blum G, Perkhofer S, Haas H, Schrettl M, Wurzner R, Dierich MP, Lass-Florl C.**

412   2008. Potential basis for amphotericin B resistance in Aspergillus terreus. Antimicrob

413   Agents Chemother **52:**1553-1555.

414   31.   **Lass-Florl C, Kofler G, Kropshofer G, Hermans J, Kreczy A, Dierich MP,**

415   **Niederwieser D.** 1998. In-vitro testing of susceptibility to amphotericin B is a reliable

416   predictor of clinical outcome in invasive aspergillosis. J Antimicrob Chemother **42:**497-502.

417   32.   **Steinbach WJ, Benjamin DK, Jr., Kontoyiannis DP, Perfect JR, Lutsar I, Marr KA,**

418   **Lionakis MS, Torres HA, Jafri H, Walsh TJ.** 2004. Infections due to Aspergillus terreus:

419   a multicenter retrospective analysis of 83 cases. Clin Infect Dis **39:**192-198.
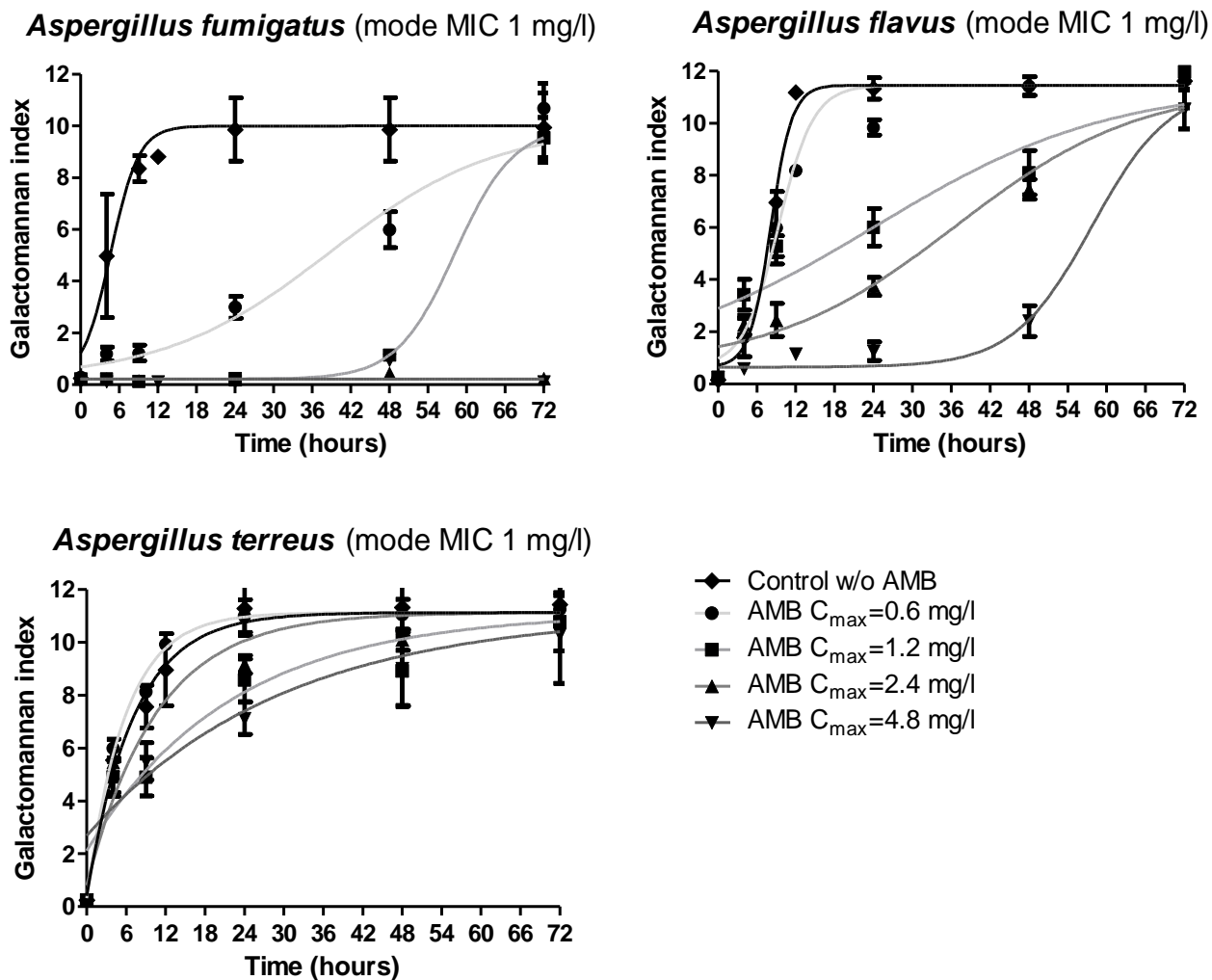
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In vitro pharmacokinetics of Amphotericin B

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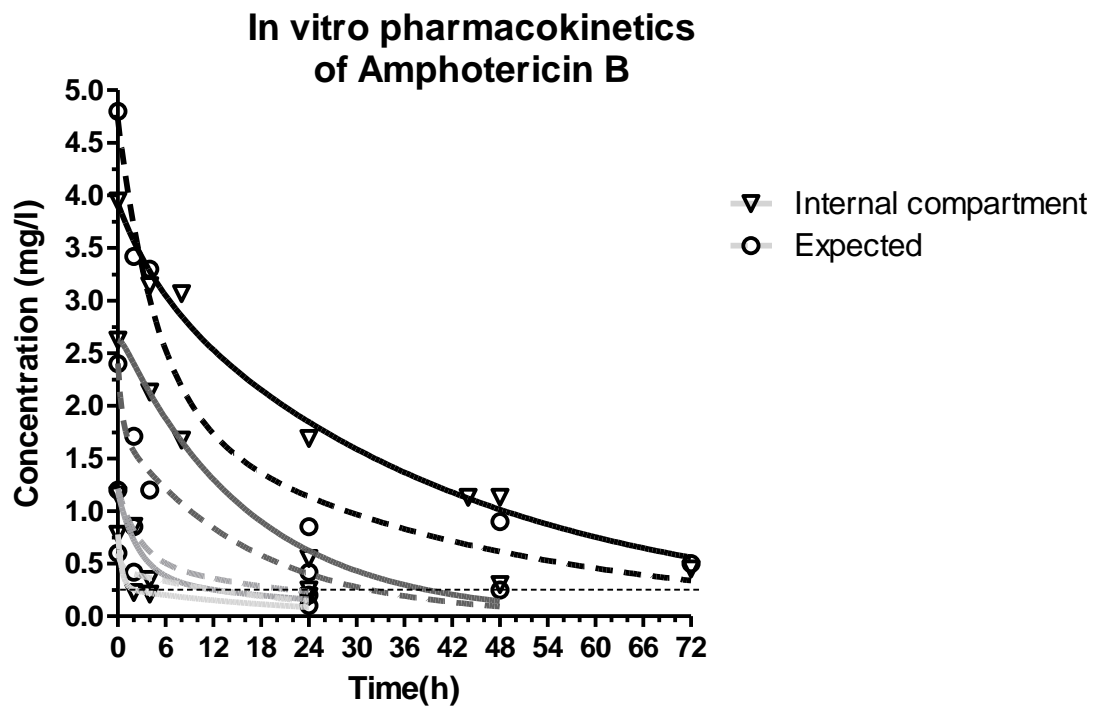
Figure 1. Pharmacokinetic analysis of simulated amphotericin B doses 0.3, 0.5, 1 and 1.5 mg/kg in

humans (dashed lines) and in the in vitro pharmacokinetic/pharmacodynamic model (solid lines)

with C_{max} 0.6 (light gray), 1.2 (medium gray), 2.4 (dark gray) and 4.8 mg/L (black), respectively.

Figure 2. Single-dose pharmacodynamic analysis of simulated amphotericin B doses with C_{max} 0.6, 1.2, 2.4 and 4.8 mg/L against *A. fumigatus*, *A. flavus* and *A. terreus* isolates with mode CLSI MIC of 1 mg/l as determined by galactomannan index in the in vitro PK/PD model.
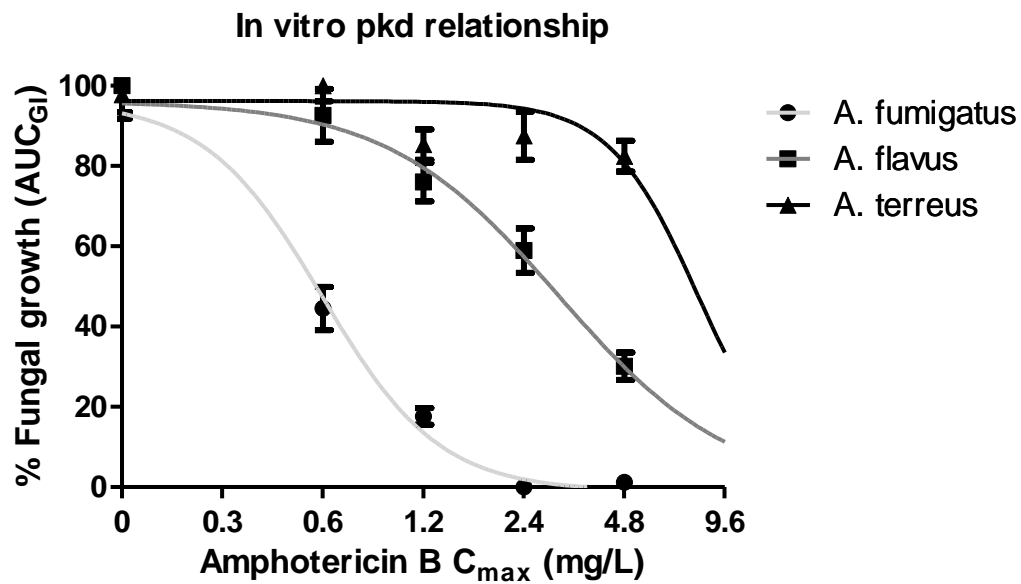
Figure 3. Single-dose exposure-efficacy relationship of amphotericin B against each *Aspergillus* species with modal CLSI MICs 1 mg/L for *A. fumigatus, A. flavus* and *A. terreus* in the in vitro PK/PD system simulating amphotericin B human plasma levels based on the increasing amphotericin B C_{max}s (maximum concentration) and the galactomannan index as a marker of fungal growth.