

1 **Title:** *In Vitro* Activity of Combinations of Zinc Chelators with  
2 Amphotericin B and Posaconazole Against Six Mucorales Species.

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22 **Running Title:** Zn-chelators and antifungals against Mucorales

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26 **Abstract:** Mucormycosis is an emerging disease with high mortality  
27 rates. Few antifungal drugs are active against Mucorales.  
28 Considering the low efficacy of monotherapy, combination-therapy  
29 strategies have been described. It is known that fungi are  
30 susceptible to zinc deprivation, thus we tested the *in vitro* effect  
31 of the zinc chelators clioquinol, phenanthroline and TPEN combined  
32 with either amphotericin B or posaconazole, against 25 strains of  
33 Mucorales. Clioquinol-posaconazole was the most active combination,  
34 although results were strain dependent.

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37 **Text**

38 Mucormycosis is a rapidly progressing fungal disease associated  
39 with high mortality (1). Few antifungal drugs are active against  
40 Mucorales and fewer are being used as mono-drug therapy to treat  
41 these mycoses. Mucormycosis treatment is currently limited to  
42 amphotericin B (AMB) (specially in its lipid formulations) and  
43 isavuconazole, while posaconazole (POS) is being used as salvage  
44 and de-escalation therapy (1-3). As the efficacy of monotherapy is  
45 suboptimal, combination-therapy strategies have been described, not  
46 only between antifungal drugs but also combining antifungals with  
47 other non-antifungal agents (2, 4, 5).

48 It is long known that zinc (Zn) starvation inhibits microbial  
49 growth in tissues (6). Zn deficiency induces stress in fungal cells  
50 and hampers fungal development by restricting the activity of Zn-  
51 binding proteins, which are mainly transcription factors involved  
52 in many biological processes (6, 7). Published data about Zn  
53 homeostasis in fungi has made infer that compounds that interfere  
54 with these metabolic processes would inhibit fungal growth. Thus,  
55 the antifungal activity of some Zn-chelators has been tested  
56 against *Aspergillus fumigatus* strains to evaluate their clinical  
57 application, with promising results (8, 9).

58 The aim of this study was to evaluate the *in vitro* effect of the  
59 Zn- chelators 5-chloro-7-iodo-quinolin-8-ol (clioquinol, CLIO),  
60 1,10-phenanthroline (PHEN) and N,N,N',N'-tetrakis(2-  
61 pyridylmethyl)ethane-1,2-diamine (TPEN) in combination with AMB and  
62 POS against clinical Mucorales strains.

63 Twenty-five isolates were tested, including: 18 *Rhizopus*  
64 *microsporus*, 2 *Rhizopus oryzae*, 2 *Syncephalastrum racemosum*, 1  
65 *Mucor circinelloides*, 1 *Lichtheimia corymbifera* and 1  
66 *Cunninghamella bertholletiae*. *Candida parapsilosis* ATCC 22019 was  
67 used as antifungal susceptibility testing quality control strain.  
68 Mucorales were identified by ITS sequencing (10, 11). In order to  
69 choose the range of drug concentrations to be tested in the  
70 combination studies, MICs of the individual Zn-chelators were  
71 firstly determined following the CLSI M38 3ed. document (12).  
72 Interactions between antifungals and Zn-chelators were studied by  
73 calculating the fractional inhibitory concentration index (FICI)  
74 (13) using the same CLSI guidelines (12) but modified for a broth  
75 microdilution checkerboard procedure. The FICI data was interpreted  
76 as synergism ( $FICI \leq 0.5$ ), antagonism ( $FICI > 4$ ) or no interaction  
77 ( $FICI > 0.5-4$ ) (13). All drugs were purchased from Sigma as standard  
78 powders, dissolved in dimethylsulphoxide and stored at  $-70^{\circ}\text{C}$ .  
79 Each isolate was tested at least three times on different days.  
80 Sporangiospore suspensions were counted in a hemocytometer chamber  
81 and then diluted into RPMI to reach a concentration of  $2 \times 10^4$   
82 sporangiospores/ml (equivalent to 0.16 OD at 530 nm). The  
83 concentration ranges tested in checkerboard plates were 0.06-4  
84  $\mu\text{g/mL}$  for AMB, 0.12-8  $\mu\text{g/mL}$  for POS and 0.03-16  $\mu\text{g/mL}$  for the Zn-  
85 chelators. Microplates were incubated at  $35^{\circ}\text{C}$ , and MICs were  
86 determined visually as the lowest drug concentration (tested alone  
87 or in combination) which had no visible growth (100% inhibition).  
88 The incubation time was extended to 48 h in the combination

89 experiment in order to confirm the 100% inhibition. MIC and FICI  
90 values are expressed as the geometric mean (GM) and arithmetic mean  
91 of the results obtained for the triplicates, respectively. Off-  
92 scale MIC values were converted to the following concentration  
93 (e.g. 32 for >16 µg/mL) to obtain the GMs.

94 A wide range of MIC values were obtained when drugs were tested  
95 alone (AMB: 0.25-4 µg/mL, POS: 0.50-4.00 µg/mL, TPEN: 0.25->16  
96 µg/mL, PHEN: 2-8 µg/mL and CLIO: 0.5->16 µg/mL) (Table 1). For AMB  
97 and POS similar GM were obtained: 1.12 µg/mL and 1.21 µg/mL,  
98 respectively. TPEN was the most active Zn-chelator (MIC GM: 0.47  
99 µg/mL), followed by PHEN (MIC GM: 3.68 µg/mL) and CLIO (MIC GM: 8  
100 µg/mL). The studied polyene showed lower MIC values than POS for  
101 all the species but *R. microsporus* (AMB GM: 1.36 µg/mL vs POS GM:  
102 1.08 µg/mL) and *C. bertholletiae* (4.00 µg/mL for both agents).  
103 Regarding the activity of Zn-chelators, TPEN showed low MIC values  
104 for most of the strains. Oppositely, MIC values for PHEN were  
105 elevated for all tested strains, while CLIO acted in a strain-  
106 dependent manner with low MICs only for *S. racemosum* and *L.*  
107 *corymbifera*. *C. bertholletiae* was the only isolate totally  
108 resistant to all the Zn-chelators.

109 Arithmetic means of FICI results and their interpretation are  
110 described in Table 2. AMB exhibited no interaction with the Zn-  
111 chelators against most of the isolates (22 strains out of 25).  
112 Synergism was only observed when the polyene was combined with CLIO  
113 against *M. circinelloides* and two *R. microsporus* isolates. However,  
114 it was remarkable that borderline FICI values were obtained against

115 the *R. oryzae* strains with AMB+CLIO (FICI 0.51 and 0.56), even when  
116 their AMB MICs were not similar (2 and 0.25 µg/mL) and both strains  
117 had their CLIO MIC values above 16 µg/mL. The same was observed for  
118 two *R. microsporus* strains (LMDM-164 and -1127) with borderline  
119 FICI values and high CLIO MICs.

120 When POS was combined with CLIO, PHEN and TPEN both synergism and  
121 antagonism were seen. FICI values  $\leq 0.5$  were observed for 6 (all *R.*  
122 *microsporus*), 3 (*R. microsporus*) and 2 (1 *R. oryzae* and 1 *C.*  
123 *bertholletiae*) strains, respectively. On the other hand,  
124 antagonism was observed for *R. microsporus* when tested against  
125 POS+CLIO (3 strains) and POS+PHEN (1 strain). Again, borderline  
126 FICI values resulted for POS+Zn-chelators combinations, against 5  
127 *R. microsporus* and 1 *S. racemosum* strains.

128 Overall, the results obtained with AMB and Zn-chelators  
129 combinations were discouraging, as no combination showed a clear  
130 synergistic behavior. On the other hand, POS+CLIO combination  
131 showed promising results especially against *R. microsporus*.  
132 However, these POS+Zn-chelators combinations acted in a strain-  
133 dependent manner, as it was described earlier for AMB and POS (14).  
134 However, a Zn-chelator susceptibility pattern could potentially be  
135 established if a larger collection of strains were studied.

136 It is known that metal-chelating agents are able to inhibit  
137 biological processes that are essential in every cellular system.  
138 Zn-chelators concentration-related toxicity was described and  
139 should be taken into consideration (15). It is then clear that Zn-  
140 depletion-based strategy for Mucormycosis therapy would be

141 plausible only if undesired effects of ion sequestration could be  
142 avoided with the development of fungal-specific ion chelators. This  
143 idea would be an item to be added in the drug development pipeline.

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**Table 1.** Antifungal susceptibility testing results for the strains used in this study.

Strains	Species	MICs ( $\mu\text{g/mL}$ ) <sup>a</sup>				
		AMB	POS	CLIO	PHEN	TPEN
LMDM-156	<i>R. microsporus</i>	2.00	1.00	4.00	2.00	0.25
LMDM-157	<i>R. microsporus</i>	1.00	0.50	4.00	4.00	0.50
LMDM-158	<i>R. microsporus</i>	2.00	0.50	8.00	4.00	0.50
LMDM-159	<i>R. microsporus</i>	2.00	1.00	4.00	2.00	0.25
LMDM-164	<i>R. microsporus</i>	2.00	1.00	8.00	4.00	0.25
LMDM-165	<i>R. microsporus</i>	2.00	1.00	8.00	2.00	0.50
LMDM-166	<i>R. microsporus</i>	1.00	2.00	4.00	4.00	0.25
LMDM-167	<i>R. microsporus</i>	2.00	1.00	2.00	4.00	0.25
LMDM-168	<i>R. microsporus</i>	2.00	1.00	8.00	4.00	0.25
LMDM-175	<i>R. microsporus</i>	1.00	2.00	>16	4.00	0.25
LMDM-176	<i>R. microsporus</i>	2.00	1.00	16.00	4.00	0.50
LMDM-184	<i>R. microsporus</i>	2.00	1.00	8.00	4.00	0.25
LMDM-185	<i>R. microsporus</i>	1.00	2.00	>16	4.00	0.25
LMDM-379	<i>R. microsporus</i>	4.00	1.00	>16	4.00	0.50
LMDM-596	<i>R. microsporus</i>	1.00	1.00	8.00	4.00	0.50
LMDM-1073	<i>R. microsporus</i>	0.50	1.00	8.00	4.00	0.50
LMDM-1074	<i>R. microsporus</i>	0.25	1.00	>16	4.00	1.00
LMDM-1127	<i>R. microsporus</i>	1.00	2.00	>16	4.00	0.50
LMDM-597	<i>R. oryzae</i>	2.00	2.00	>16	4.00	0.50
LMDM-1075	<i>R. oryzae</i>	0.25	1.00	>16	4.00	0.25
LMDM-1123	<i>S. racemosum</i>	0.25	1.00	1.00	2.00	0.25
LMDM-1124	<i>S. racemosum</i>	0.50	2.00	1.00	4.00	1.00
LMDM-1019	<i>M. circinelloides</i>	1.00	2.00	4.00	4.00	0.50
LMDM-1121	<i>L. corymbifera</i>	0.25	1.00	0.50	4.00	1.00
LMDM-1291	<i>C. bertholletiae</i>	4.00	4.00	>16.00	8.00	>16.00

AMB: amphotericin B. POS: posaconazole. CLIO: clioquinol (5-chloro-7-iodo-quinolin-8-ol). PHEN: 1,10-phenanthroline. TPEN: N,N,N',N'-tetrakis(2-pyridylmethyl)ethane-1,2-diamine.



159 <sup>a</sup> MIC values were obtained on three different days by using the  
160 protocol published by CLSI (document M38 3<sup>rd</sup> ed.) (12) and are  
161 presented as geometric means.

162 **Table 2.** Fractional Inhibitory Concentration Indexes (FICI) results  
163 for all the isolates and drugs studied.

Isolates	FICI results for <sup>a</sup> :					
	AMB +			POS +		
	CLIO	PHEN	TPEN	CLIO	PHEN	TPEN
LMDM-156	0.88 (Ni)	0.75 (Ni)	1.06 (Ni)	0.37 (S)	4.01 (A)	1.06 (Ni)
LMDM-157	0.75 (Ni)	0.76 (Ni)	1.06 (Ni)	0.25 (S)	0.32 (S)	0.52 (Ni)
LMDM-158	0.64 (Ni)	0.76 (Ni)	1.02 (Ni)	0.29 (S)	1.38 (Ni)	1.27 (Ni)
LMDM-159	1.00 (Ni)	0.51 (Ni)	1.06 (Ni)	1.00 (Ni)	1.00 (Ni)	1.03 (Ni)
LMDM-164	0.54 (Ni)	0.75 (Ni)	1.09 (Ni)	0.42 (S)	0.26 (S)	1.06 (Ni)
LMDM-165	0.66 (Ni)	0.75 (Ni)	1.00 (Ni)	0.54 (Ni)	2.00 (Ni)	1.28 (Ni)
LMDM-166	1.13 (Ni)	1.00 (Ni)	1.06 (Ni)	1.00 (Ni)	0.50 (S)	1.03 (Ni)
LMDM-167	1.00 (Ni)	1.01 (Ni)	1.06 (Ni)	4.13 (A)	0.56 (Ni)	1.03 (Ni)
LMDM-168	1.28 (Ni)	0.63 (Ni)	0.94 (Ni)	1.01 (Ni)	2.50 (Ni)	1.00 (Ni)
LMDM-175	0.63 (Ni)	1.00 (Ni)	1.06 (Ni)	4.13 (A)	1.00 (Ni)	1.06 (Ni)
LMDM-176	1.38 (Ni)	0.76 (Ni)	0.70 (Ni)	1.36 (Ni)	0.67 (Ni)	1.05 (Ni)
LMDM-184	1.53 (Ni)	0.63 (Ni)	0.84 (Ni)	0.45 (S)	1.00 (Ni)	1.06 (Ni)
LMDM-185	0.25 (S)	2.13 (Ni)	0.59 (Ni)	4.13 (A)	1.00 (Ni)	0.63 (Ni)
LMDM-379	2.05 (Ni)	0.75 (Ni)	1.06 (Ni)	0.39 (S)	2.00 (Ni)	1.06 (Ni)
LMDM-596	0.75 (Ni)	2.06 (Ni)	0.56 (Ni)	0.63 (Ni)	1.50 (Ni)	0.63 (Ni)
LMDM-1073	0.63 (Ni)	1.00 (Ni)	0.63 (Ni)	0.69 (Ni)	2.00 (Ni)	0.53 (Ni)
LMDM-1074	0.34 (S)	0.75 (Ni)	0.83 (Ni)	1.18 (Ni)	1.54 (Ni)	0.52 (Ni)
LMDM-1127	0.55 (Ni)	0.81 (Ni)	0.94 (Ni)	0.75 (Ni)	1.31 (Ni)	0.63 (Ni)
LMDM-597	0.51 (Ni)	0.77 (Ni)	1.03 (Ni)	1.58 (Ni)	2.00 (Ni)	1.38 (Ni)
LMDM-1075	0.56 (Ni)	1.01 (Ni)	1.03 (Ni)	0.69 (Ni)	1.01 (Ni)	0.43 (S)
LMDM-1123	1.03 (Ni)	1.01 (Ni)	0.75 (Ni)	0.77 (Ni)	0.52 (Ni)	1.51 (Ni)
LMDM-1124	1.03 (Ni)	1.00 (Ni)	0.63 (Ni)	2.00 (Ni)	2.00 (Ni)	0.60 (Ni)
LMDM-1019	0.50 (S)	1.01 (Ni)	0.56 (Ni)	0.63 (Ni)	2.00 (Ni)	1.01 (Ni)
LMDM-1121	1.03 (Ni)	1.00 (Ni)	1.03 (Ni)	0.76 (Ni)	1.00 (Ni)	1.57 (Ni)
LMDM-1291	1.00 (Ni)	1.00 (Ni)	0.88 (Ni)	1.38 (Ni)	3.06 (Ni)	0.16 (S)
Average	0.87	0.94	0.90	1.22	1.45	0.92

FICI						
Number (%) synergy <sup>b</sup>	3 (12%)	0 (0%)	0 (0%)	6 (24%)	3 (12%)	2 (8%)

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165 <sup>a</sup> AMB: amphotericin B. POS: posaconazole. CLIO: clioquinol (5-  
166 chloro-7-iodo-quinolin-8-ol). PHEN: 1,10-phenanthroline. TPEN:  
167 N,N,N',N'-tetrakis(2-pyridylmethyl)ethane-1,2-diamine. FICI values  
168 are presented as arithmetic means of at least three repetitions  
169 performed on different days. The interpretation of FICI values are  
170 shown in parenthesis: synergy (S), antagonism (A) and no  
171 interaction (Ni).

172 <sup>b</sup> The numbers of strains for which the combination showed synergism.  
173 Percentage is given in parentheses.

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