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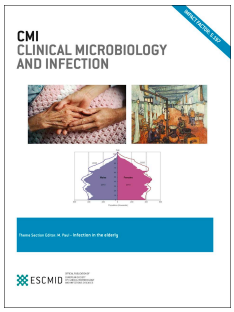

Absent *in vitro* interaction between chloroquine and antifungals against *Aspergillus fumigatus*

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Absent *in vitro* interaction between chloroquine and antifungals against *Aspergillus fumigatus*

Running title: Chloroquine and *Aspergillus fumigatus*

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Potential conflict of interest

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Sir, azole resistance in *Aspergillus fumigatus* is a global and evolving public health threat, which translates into treatment failure in different regions and patient groups worldwide [1] . The increasing trend of azole resistance in *A. fumigatus*, therefore, exemplifies the importance to explore alternative treatment strategies.

The antimalarial drug chloroquine, an acidotropic agent that passively diffuses into acidic organelles, has shown to have a direct antifungal activity against *Histoplasma capsulatum* and *Cryptococcus neoformans* [2]. Moreover, synergistic interaction of chloroquine in combination with various antifungals has been reported on both azole-susceptible and azole-resistant *Candida albicans* isolates [3].

Here, we investigated whether chloroquine potentiates the antifungal activity in combination with polyene and azole antifungals against both azole-susceptible and azole-resistant *A. fumigatus* isolates.

A collection of thirty-one clinical *A. fumigatus* isolates, harboring various substitutions in the cyp-51A gene, was used in this study, including: single point mutations (M220I, M220K, M220I, G138C, G54W, A9T and F219I) and tandem repeat mutations (TR_{34}/L98H and TR_{46}/Y121F/T289A). Voriconazole-susceptible clinical isolates without mutations in the cyp-51A gene were used as wild-type controls (Table 1).

Molecular identification, genotyping, antifungal susceptibility testing and drug interaction studies were performed, as described previously [4]. The final concentrations of the antifungal agents ranged from 0.016 to 16 mg/L for voriconazole and amphotericin B, from 0.06 to 64 mg/L for caspofungin and the chloroquine diphosphate salt (Sigma-Aldrich, St. Louis, MO, USA; molecular weight, 515.9) concentrations ranged from 16 to 1024 mg/L.

The *in vitro* interaction between chloroquine and voriconazole or amphotericin B or caspofungin was determined using a checkerboard microdilution method with spectrophotometric analysis and a viability-based XTT assay. In order to assess the nature of *in vitro* interactions between two drugs, the data obtained as described above were analyzed using fractional inhibitory concentration (FIC) index based on non-parametric Loewe additivity model, as described previously [4].

The MIC and MEC characteristics of the 31 clinical *A. fumigatus* isolates are shown in Table 1.

The mean MICs of voriconazole were 0.45 (0.25 to 0.5) mg/L for the voriconazole-susceptible isolates, whereas higher MICs were observed for isolates harboring mutations in the cyp-51A gene (mean MICs 6.17 mg/L, ranging 0.25 to >16 mg/L).

Amphotericin B and caspofungin showed similar MIC and MEC values for the voriconazole-susceptible and -resistant isolates with the MICs ranging from 0.25 to 1 mg/L and MECs from 0.062 to 0.5 mg/L, respectively. The MICs of chloroquine against all isolates were > 1024 mg/L.

No obvious interactions were found *in vitro* for all the combinations of chloroquine and voriconazole, and amphotericin B or caspofungin tested (Table 1).

Our study showed that chloroquine had no growth-inhibitory antifungal activities against clinical *A. fumigatus* isolates, either voriconazole-susceptible or -resistant isolates. In addition, chloroquine in combination with different classes of antifungal agents, did not show any *in vitro* synergistic antifungal activity against *A. fumigatus*.

The therapeutic actions of chloroquine and its interaction with antifungals have been investigated in a *Saccharomyces cerevisiae* yeast model and several pathogenic fungi, such as; *H. capsulatum, C. neoformans, Candida* spp. and *A. fumigatus* [3] . The mode of action against

Histoplasma and *Candida* spp. is thought to involve alkalinization of the host environment of the fungi through iron deprivation, increasing pH and inhibiting the thiamine transport The iron deprivation enhances the passive diffusion of drugs and causes down regulation of *ERG11*, which results in potentiated drug susceptibility. We previously showed that chloroquine exerts a direct pH-dependent antifungal effect on *A. fumigatus* and significantly increases the antifungal activity of polymorphonuclear cells [5].

Islahudin et al. reported that combination of chloroquine with caspofungin exhibited synergistic activity against *C. albicans*, *C. glabrata*, and *S. cerevisiae in vitro* [2]. By using a genome-wide yeast deletion strain collection of *S. cerevisiae,* it was shown that chloroquine hypersensitivity was associated with the presence of deletion mutants in the cell wall integrity pathway. The MIC of caspofungin against *C. albicans* was decreased 2-fold by 250 µM chloroquine and up to 8-fold at higher chloroquine concentrations. Similar effects were seen in *C. glabrata* and *A. fumigatus*. Of note, only one strain, the AF 293 was used, which is less virulent than all of the clinical isolates tested by us. More recently, Li et al. showed that chloroquine had poor antifungal activity against *Candida* spp., however synergistic interaction between chloroquine and fluconazole against fluconazole-resistant *C. albicans, C. krusei*, and *C. tropicalis* isolates was reported [3].

Our findings stand in contrast to the results as shown for *Candida* spp and suggest that postulated mechanism of fungicidal synergism between cell membrane- and cell wall-active agents and chloroquine, are different between yeasts and molds. Iron deprivation thought to cause a decrease in the level of sterols may lead to less increase in membrane fluidity in molds and therefore less enhancement of passive diffusion of drugs into the fungal cell. A second

mode of action of chloroquine via iron deprivation is thought to be the downregulation of ERG11 in *Candida* spp. leading to increased susceptibility to fluconazole. While *A. fumigatus* is inherently resistant to fluconazole, the regulation of the ERG11 genes involved in the susceptibility to the mold-active azoles clearly differs [5].

In conclusion, although our study showed no *in vitro* activity of chloroquine alone or in combination with voriconazole or amphotericin B or caspofungin against *A. fumigatus*, our previous observation that chloroquine increases the antifungal activity of neutrophils may however suggest that chloroquine exerts different effects *in vivo.* Therefore, investigating the therapeutic efficacy of chloroquine in an experimental murine model of invasive aspergillosis is needed to assess its value in the treatment of infections caused by *A. fumigatus*.

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ID number	Origin	Cyp-51A substitution	MIC/MEC (mg/L)							
			VRC		AmB		CAS		CQ	
			Single	Combined	Single	Combined	Single	Combined	Single	Combined
AZN 8196	Proven IA	None	0.25	0.25	0.25	0.125	0.25	0.25	> 1024	> 1024
V 52-76	Proven IA	None	0.5	0.25	-	-	-	-	> 1024	> 1024
V 28-29	Proven IA	None	0.5	0.25	-	-	-	-	> 1024	> 1024
V 67-38	chronic PA	None	0.5	0.5	-	-	-	-	> 1024	> 1024
V 12-74	Proven IA	None	0.5	0.5	-	-	-	-	> 1024	> 1024
V 54-09	Proven IA	None	0.5	0.5	-	-	-	-	> 1024	> 1024
V 67-37	chronic PA	None	2	2	0.25	0.25	0.25	0.25	> 1024	> 1024
V 67-35	Chronic PA	None	4	4	-	-	-	-	> 1024	> 1024
V 67-36	chronic PA	None	4	2	-	-	-	-	> 1024	> 1024
V 13-09	Probable IA	M220V	2	2	1	0.5	0.25	0.25	> 1024	> 1024
V 59-27	Allergic PA	M220K	2	1	-	-	-	-	> 1024	> 1024
V 28-77	Aspergilloma	M220I	0.5	0.5	-	-	-	-	> 1024	> 1024
V 59-72	Aspergilloma	G138C	2	1	0.25	0.25	0.25	0.25	> 1024	> 1024
V 79-25	Proven IA	G54W	0.25	0.25	-	-	-	-	> 1024	> 1024
V 59-73	Proven IA	G54W	0.25	0.125	-	-	-	-	> 1024	> 1024
V 44-58	Proven IA	TR_{34}/L98H	4	4	-	-	-	-	> 1024	> 1024
V 52-35	Proven IA	TR_{34}/L98H	4	4	0.25	0.25	0.25	0.25	> 1024	> 1024
V 61-76	Proven IA	TR_{34}/L98H	4	4	-	-	-	-	> 1024	> 1024
V 64-51	Proven IA	TR_{34}/L98H	8	8	-	-	-	-	> 1024	> 1024
V 64-72	Proven IA	TR_{34}/L98H	8	8	-	-	-	-	> 1024	> 1024
V 79-79	Proven IA	TR_{34}/L98H	8	8	-	-	-	-	> 1024	> 1024

V 45-07	Proven IA	TR_{34}/L98H	8	8	-	-	-	-	> 1024	> 1024
V 77-40	Proven IA	TR_{34}/L98H	8	8	-	-	-	-	> 1024	> 1024
V 80-01	Proven IA	TR_{34}/L98H	16	16	-	-	-	-	> 1024	> 1024
V 94-10	Proven IA	TR_{46}/Y121F/T289A	>16	> 16	0.25	0.25	0.062	0.062	> 1024	> 1024
V 99-47	Proven IA	TR_{46}/Y121F/T289A	>16	>16	-	-	-	-	> 1024	> 1024
V 107-65	Proven IA	TR_{46}/Y121F/T289A	>16	>16	-	-	-	-	> 1024	> 1024
V 104-35	Proven IA	TR_{46}/Y121F/T289A	>16	>16	-	-	-	-	> 1024	> 1024
V 74-61	Proven IA	A9T	1	1	-	-	-	-	> 1024	> 1024
V 76-03	Proven IA	A9T, F219I	0.25	0.25	0.25	0.25	0.125	0.125	> 1024	> 1024
V 79-063	Proven IA	A9T, F219I	4	4	-	-	-	-	> 1024	> 1024

Table 1: The results of susceptibility tests for voriconazole, amphotericin B and caspofungin alone or in combination with chloroquine against voriconazole-susceptible and voriconazole-resistant- (substitutions in the cyp-51A gene, including single point [M220V, M220K, M220I, G54W, G138C, G54W, A9T, F219I] and tandem repeat [34-bp tandem repeat in the promoter region of the cyp51A gene in combination with substitutions at codon L98 and 46-bp tandem repeat in the promoter region of the cyp-51A gene in combination with mutation at codons Y121 and T289] mutations) clinical *Aspergillus fumigatus* isolates using a checkerboard microdilution method with spectrophotometric analysis.

VRC: Voriconazole, AmB: Amphotericin B, CAS: Caspofungin, CQ: Chloroquine.