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1 Optimized combinations of statins and azoles against *Acanthamoeba*
2 trophozoites and cysts *in vitro*

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22

23 **Abstract**

24 **Subheadings:** The effective treatment of *Acanthamoeba* infections is complicated by the
25 resistant cyst stage in the life cycle of these opportunistic parasitic protozoans. In previous
26 studies, we demonstrated the high activity of statins and voriconazole against clinical
27 strains of *Acanthamoeba*.

28 **Objective:** this study aimed to evaluate the evaluation of the combination of several
29 statins (atorvastatin, fluvastatin and simvastatin) and various azoles (voriconazole,
30 posaconazole and itraconazole) against *Acanthamoeba* spp.

31 **Methods:** The efficiency of the different drugs combinations against the trophozoite
32 stage of different *Acanthamoeba* strains was evaluated by Alamar Blue assay. Nevertheless,
33 the effect on the cyst stage was made by Inverted Microscope. As for the cytotoxicity of
34 these combinations of azoles and statins was evaluated by measuring the release of lactate
35 dehydrogenase from a murine macrophage cell line.

36 **Results:** We found that combinations of any of the tested statins and voriconazole or
37 posaconazole were more efficient at inhibiting *Acanthamoeba* compared to statins or
38 azoles when tested individually. We found lower toxicity of these drug combination at
39 the combined IC₅₀s compared to that of the compounds alone.

40 **Conclusions:** We report that the combination of statins together with voriconazole and
41 posaconazole is more efficient than these drugs by themselves, and these combinations
42 have lower cytotoxicity in mammalian cell lines.

43

44 **Keywords:**

45 Keratitis; voriconazole; posaconazole; atorvastatin; fluvastatin; simvastatin

46

47 **1. Introduction**

48 Among several genera of free-living amoebae, *Acanthamoeba* genus are responsible for
49 different diseases such as, “*Acanthamoeba* Keratitis” (AK), a sight-threatening ulceration
50 of the cornea, Granulomatous Amoebic Encephalitis (GAE) and various disseminated
51 infections (mostly cutaneous). [1, 2] Current therapy against AK is based on topical
52 applications of antimicrobials including various combinations of propamidine isethionate
53 and neomycin or biguanides. [1, 2] However, *Acanthamoeba* can form a double wall cyst
54 stage which is highly resistant to external agents including those mentioned above which
55 significantly complicates therapy. [3] This means that there is an urgent need to discover
56 drugs and drug regimens that are active against both trophozoites and cysts of
57 *Acanthamoeba* [1, 2, 4-6].

58 Statins are hypolipidemic agents widely used to lower the cholesterol levels and to
59 prevent atherosclerotic cardiovascular disease. [7] Our previous studies have also
60 demonstrated the *in vitro* efficacy of statins against *Acanthamoeba* trophozoites and cysts,
61 highlighting them as a novel effective therapeutic approach against these pathogens. [5]
62 Another agent which has shown high activity against both trophozoites and cysts of
63 *Acanthamoeba* is voriconazole. [6, 8] Voriconazole belongs to the triazole family which
64 has been shown to inhibit 14- α -demethylase resulting in the reduced production of
65 ergosterol in *Acanthamoeba*. [9] Ergosterol and 7-dehydrostigmasterol have been reported
66 as the major sterols presented in *Acanthamoeba*. [10, 11] The high efficacy of
67 voriconazole against *Acanthamoeba* strains *in vitro* and in clinical cases has been reported
68 in previous studies. [6, 8, 12] Other members of the family of triazoles that have been
69 used as antifungals are posaconazole and itraconazole. Posaconazole has been previously
70 reported to have a wide microbial activity spectrum and has recently been evaluated
71 against *Acanthamoeba* cysts *in vitro*. [9, 13] Although itraconazole, has been included in

72 successful treatment combinations against skin lesions caused by *Acanthamoeba*, it is
73 reported to be less active compared to other azoles. [3, 14] In many cases combinations
74 of therapeutic drugs are found to be more efficient than the individually applied drugs. In
75 the case of *Acanthamoeba*, this phenomenon has been recently demonstrated recently
76 using combination of biguanides. [4]

77 Although the activity of statins and triazoles has been already described against
78 *Acanthamoeba* strains, they have not been tested in combination. In the present study,
79 combinations of these molecules were tested for their amoebicidal and cysticidal activity
80 as well as for their cytotoxicity in a mammalian cell line. We report that these drugs are
81 more efficient in combination, which suggests a better approach for the treatment of
82 *Acanthamoeba* infections.

83 **2. Materials and Methods**

84 *2.1. Acanthamoeba strains.*

85 We have used three *Acanthamoeba* isolates in this study. One type strain *Acanthamoeba*
86 *castellanii* Neff (ATCC 30010, genotype T4) and two clinical strains previously isolated
87 by our group CLC-16, (genotype T3) and CLC-51.1 (genotype T1). [15] All strains were
88 culture axenically at room temperature in PYG medium supplemented with 40 µg/ml
89 gentamicin (Sigma-Aldrich Chemistry Ltd., Madrid, Spain).

90 *2.2. Chemicals.*

91 The azoles posaconazole and voriconazole were purchased from Sigma-Aldrich
92 Chemistry Ltd. (Madrid, Spain) and itraconazole was purchased from the Cayman
93 Chemical Company (Vitro, Madrid, Spain). Simvastatin, atorvastatin, and fluvastatin
94 were from the Cayman Chemical Company.

95 *2.3. Activity assays.*

96 The anti-*Acanthamoeba* activities of the drugs were determined by the AlamarBlue®
97 assay, as previously described. [15, 16] Briefly, 50µl of *Acanthamoeba* trophozoite cells
98 (10^4 cells/ml) were seeded in 96-well microtiter plates. Amoebae were allowed to adhere
99 for 15 min and 50 µl of different dilutions of the drugs in were then added. Finally, 10µl
100 of the Alamar Blue Assay Reagent (Bioresource, Europe, Nivelles, Belgium) to each well.
101 Plates were then incubated up to 120 h at 28°C with slight agitation and measured using
102 an EnSpire® Multimode Plate Reader (Perkin Elmer, Madrid, Spain). We used a test
103 wavelength of 570 nm and a reference wavelength of 630 nm. Inhibitory concentration
104 50% (IC₅₀) and 90% (IC₉₀) were calculated by non-linear- regression analysis with 95%
105 confidence limits. The IC₅₀s of each drug alone was calculated and drug combinations
106 were then tested at various concentration to determine the lowest IC₅₀s of each drug
107 combination.

108 2.4. Evaluation of the cysticidal activity of posaconazole.

109 The effects of posaconazole against cysts were evaluated against the three strains of
110 *Acanthamoeba*. Mature cysts were prepared as previously described. [17] Briefly,
111 trophozoites were transferred from PYG medium to Neff's encystment medium (NEM)
112 where they were cultured for a week under slight agitation to obtain mature cysts. After
113 that, the cells were harvest and washed twice with PYG medium. A concentration of 10^4
114 cysts/ml was transferred to plates containing fresh PYG medium and incubated with
115 posaconazole at the previously calculated IC₅₀s and IC₉₀s. A Neubauer chamber, was used
116 to count the number of of trophozoites, cysts, and non-viable each 24 hours for a week as
117 previously described by Martín-Navarro et al, 2013a; 2013b. [5, 6] A negative control
118 including only mature cysts in Neff's encystment medium was included to the
119 experiments. After 7 days of incubation, the supernatant was replaced with fresh PYG

120 medium and the cultures were observed for a second week to confirm the cysticidal
121 activity.

122 2.5. Cytotoxicity evaluation.

123 The murine macrophages cell line (ATCC TIB-67) was used to measure the cytotoxicity
124 of the drugs individually and in combinations. This assay was evaluated by the measure
125 of the release of lactate dehydrogenase by Cytotoxicity Detection Kit (LDH) (Roche
126 Applied Science, Barcelona, Spain) was used, according to the manufacturer's
127 instructions. Levels of cytotoxicity less than 10 % were considered as being non-
128 cytotoxic, levels between 10–25 %, low toxicity, levels between 25–40 %, medium
129 toxicity and higher than 40 %, highly cytotoxic. were compared by one-way ANOVA
130 using Sigma Plot 12.0 software (Systat Software Inc., London, UK). [2, 18].

131 2.6 statistical analysis

132 All experiments were performed three times each in duplicate. Data were analysed using
133 ANOVA, multiple post-hoc analysis, Tukey's test and a paired two-tailed t-test and *p*
134 values of <0.05 were considered significant. Statistical analysis was done with the Sigma
135 Plot 12.0 software program (Systat Software Inc., London, UK).

136 3. Results

137 All three of the *Acanthamoeba* strains tested were found to be sensitive to the statins,
138 posaconazole and voriconazole (Table 1), but the activity of itraconazole was low and
139 this drug was not used in further experiments. All combinations of statins and
140 voriconazole and posaconazole were active against the strains of *Acanthamoeba*.
141 However, the different drug combined, produce lower IC₅₀s compared to the IC₅₀ of the
142 molecules alone (Table 2 and 3). The cysticidal activity of posaconazole was also
143 evaluated and found to reduce the viability of all three strains. The IC₅₀ and IC₉₀ of

144 posaconazole for cysts was calculated (Figure 1). After 168 h no cysts were able to revert
145 into trophozoites.

146 We tested the toxicity of the drugs, simvastatin IC₅₀ (S50), posaconazole IC₅₀ (P50),
147 voriconazole IC₅₀ (V50) and the following combinations of molecules: posaconazole and
148 atorvastatin (P + A), posaconazole and fluvastatin (P + F), posaconazole and simvastatin
149 (P + S), and voriconazole with atorvastatin, fluvastatin and simvastatin (V + A, V + F and
150 V + S) did not induce cytotoxicity against macrophages. Whereas in the case of
151 atorvastatin IC₅₀ and IC₉₀ (A50 and A90), fluvastatin IC₅₀ and IC₉₀ (F50 and F90),
152 posaconazole IC₉₀ (P90) and voriconazole IC₉₀ (V90), low cytotoxicity values against
153 macrophages were observed. Furthermore, moderate toxicity was shown in the case of
154 cells incubated with simvastatin IC₉₀ (S90) and Amphotericin B IC₉₀ (AnfB90) and
155 chlorhexidine IC₉₀ (Chx90), induced high levels of toxicity in the tested cell line (Figure
156 2). The statistical analysis revealed statistical differences ($p < 0.001$) in the cytotoxicity
157 produced by the reference drugs (IC₉₀ of Chx and AnfB) and all treatments, excepting in
158 the comparison between Chx90 and S90 (Figure 2).

159 **4. Discussion**

160 Drugs are often combined in the hope that their activities synergize to increase their
161 therapeutic potency. There are many instances where this applies (*e.g.* HIV therapy) and
162 this has also been found with *Acanthamoeba*, where polyhexamethylene biguanide
163 combined effectively with chlorhexidine. [4] This has also been found with various other
164 drug combinations. [19] It is also an advantage if the cytotoxicity produced by this
165 combination will be the same or even lower than that produced by the individual drugs
166 themselves. Such a decrease in the toxicity has been reported by the combination of
167 PHMB and chlorhexidine on epithelial cells. [4]

168 Statins and voriconazole were used in this study because of their proven amoebicidal and
169 cysticidal activity and their relatively low cytotoxicity. The activity of atorvastatin,
170 fluvastatin, simvastatin and voriconazole against *Acanthamoeba* was previously
171 evaluated by our group. [5, 6, 8, 16] These *in vitro* results can be compared with *in vivo*
172 experiment where voriconazole has been successfully used to treat amoebic infections,
173 even against resistant *Acanthamoeba* strains. [12] Initially, those drugs were chosen
174 because their molecular target, ergosterol was known to be a valid one. Statins inhibit 3-
175 hydroxy-3-methylglutaryl-coenzyme A (HMGCoA) reductase, an enzyme that catalyse
176 the conversion of HMG-CoA to mevalonate, which is a precursor of cholesterol in
177 vertebrates and ergosterol in fungi and some protozoa such as *Acanthamoeba*. [5, 20]
178 Voriconazole, it is a triazole antifungal agent that causes demethylation of ergosterol. As
179 both drugs act to inhibit different parts of the same ergosterol pathway, we suspected that
180 these two drugs may be combined successfully.

181 Itraconazole has been included in some successful treatment regimens used against skin
182 lesion in a lung transplant patient with disseminated acanthamoebiasis and against
183 *Acanthamoeba* keratitis. [14, 21] However, resistance to azoles has been reported.
184 *Acanthamoeba* from skin nodules of a fatal cutaneous infection in an HIV patient, was
185 resistant to a combination of drugs with itraconazole *in vitro*. [21] We confirm that only
186 a weakly amoebicidal effect of itraconazole and, because of this, it was discarded for
187 further experiments. We could find only one study in which posaconazole, has been used
188 against *Acanthamoeba*, showing a cysticidal activity in clinical and culture collection
189 isolates. [9] This previous study reported minimal cysticidal concentrations (43.75 - 52.5
190 μM) that are higher than the IC_{50} found in the present study (2.56 -7.50 μM).

191 We have reported that statins and voriconazole lead to the death of *Acanthamoeba* by the
192 activation of programmed cell death. [8] We have also reported that caffeine and maslinic

193 acid also activate programmed cell death but we do not yet know the molecular targets of
194 either drug. It remains to be seen if either caffeine or maslinic acid treatment will allow
195 even lower concentrations of statins, posaconazole or voriconazole to be effective in
196 human therapy. [18]

197 **5. Conclusion**

198 We report that the combination of statins together with voriconazole and posaconazole is
199 more efficient than these drugs by themselves, and these combinations have lower
200 cytotoxicity in mammalian cell lines. We anticipate that treatments based on
201 combinations of statins and azoles will be more effective and better tolerated than present
202 treatment regimens against *Acanthamoeba* infections of humans.

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212

213 **Additional Information**

214 The author(s) declare no competing financial interests.

215

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290 **Author Contributions statement**

291 JLM, SKM, IS and CMMN designed the study and all authors performed experiments,
292 ALA, MRB, BV, IS analysed data and JLM, SKM, IS and JEPB wrote the paper; All
293 authors discussed the results and implications and commented on the manuscript at all
294 stages.

295 **Competing Financial Interests statement**

296 Nothing to declare

297 **FIGURE LEGENDS:**

298

299 Figure 1. Number of cysts when they were incubated with posaconazole with previously
300 calculated IC₅₀ and IC₉₀ in PYG medium. Cysts were not viable after incubation with this
301 drug, since amoebae were not able to excyst. Moreover, the number of cysts decreased
302 with time and became non-viable cells.

303

304 Figure 2. Cytotoxicity of atorvastatin, fluvastatin, simvastatin, posaconazole,
305 voriconazole, amphotericin B and chlorhexidine at IC₅₀ and IC₉₀ as well the combination
306 between azoles and statins produced in murine macrophages. A50 and A90: atorvastatin
307 IC₅₀ and IC₉₀. F50 and F90: fluvastatin IC₅₀ and IC₉₀. S50 and S90: simvastatin IC₅₀ and
308 IC₉₀. P50 and P90: posaconazole IC₅₀ and IC₉₀. P + A: posaconazole and atorvastatin. P
309 + F: posaconazole and fluvastatin. P + S: posaconazole and simvastatin. V50 and V90:

310 voriconazole IC₅₀ and IC₉₀. V + A: voriconazole and atorvastatin. V + F: voriconazole
311 and fluvastatin. V + S: voriconazole and simvastatin. AnfB50 and AnfB90: amphotericin
312 B IC₅₀ and IC₉₀. Chx50 and Chx90: chlorhexidine IC₅₀ and IC₉₀. The results showed that
313 S50, P50, V50 and the following combinations: P + A, P + F, P + S, and V + A, V + F
314 and V + S were not cytotoxic against macrophages. A50 and A90, F50 and F90, P90 and
315 V90 presented low cytotoxicity against macrophages. The observed cytotoxicity levels
316 were moderate when cells were incubated with S90. AnfB90 and Chx90 showed high
317 levels of cytotoxicity against macrophages. The cytotoxicity values showed significant
318 differences with the cytotoxicity produced by the reference drugs chlorhexidine and
319 amphotericin B (***, p<0.001).

320

321 Table 1. IC₅₀ and IC₉₀ (μM) values for posaconazole against different strains of *Acanthamoeba* measured by Alamar blue assay after 96h (Mean
 322 concentration ± SD).

<i>IC₅₀ (μM) 96h</i>	<i>AcNeff</i>		<i>CLC-16</i>		<i>CLC-51.1</i>	
	<i>IC₅₀</i>	<i>IC₉₀</i>	<i>IC₅₀</i>	<i>IC₉₀</i>	<i>IC₅₀</i>	<i>IC₉₀</i>
Atorvastatin	15.12 ± 2.19	41.09 ± 0.01	33.34 ± 2.64	78.66 ± 5.85	26.63 ± 1.20	49.76 ± 1.81
Fluvastatin	9.19 ± 0.98	20.70 ± 2.15	54.64 ± 2.69	105.40 ± 5.34	16.50 ± 1.03	32.86 ± 5.18
Simvastatin	10.24 ± 1.09	21.37 ± 1.51	31.44 ± 2.06	63.55 ± 4.15	39.73 ± 4.34	84.16 ± 8.23
Voriconazole	13.14 ± 0.69	30.43 ± 1.32	21.93 ± 5.87	44.18 ± 11.46	13.31 ± 1.69	30.01 ± 3.93
Posaconazole	3.03 ± 0.32	5.78 ± 0.02	2.56 ± 0.28	16.53 ± 1.49	7.50 ± 0.39	23.53 ± 1.35
Itraconazole	59.32 ± 0.78	196.65 ± 13.32	75.45 ± 4.15	151.62 ± 6.11	113.73 ± 10.80	219.09 ± 11.22

323

324

325

326

327 Table 2. IC₅₀ (μM) values for voriconazole and each statin assayed when this compounds were combined and evaluated against different strains of
 328 *Acanthamoeba* measured by Alamar blue assay after 96h (Mean concentration ± SD). (***) p ≤ 0.001 statistical differences between those values
 329 and the IC₅₀ of each product alone.

<i>IC₅₀ (μM) 96h</i>	<i>V + A</i>		<i>V + F</i>		<i>V + S</i>	
	<i>Voriconazole</i>	<i>Atorvastatin</i>	<i>Voriconazole</i>	<i>Fluvastatin</i>	<i>Voriconazole</i>	<i>Simvastatin</i>
AcNeff	1.63 ± 0.11***	1.71 ± 0.13***	1.00 ± 0.09***	0.97 ± 0.09***	1.00 ± 0.03***	0.97 ± 0.07***
CLC-16	8.96 ± 0.89***	12.50 ± 1.24***	4.18 ± 0.34***	3.88 ± 0.32***	7.67 ± 0.20***	7.08 ± 0.15***
CLC-51.1	6.50 ± 0.20***	9.09 ± 0.27***	6.98 ± 0.09***	6.52 ± 0.09***	6.07 ± 0.20***	5.65 ± 0.18***

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332 Table 3. IC₅₀ (μM) values for posaconazole and each statin assayed when this compounds were combined and evaluated against different strains
 333 of *Acanthamoeba* measured by Alamar blue assay after 96h (Mean concentration ± SD). (***) p ≤ 0.001 statistical differences between those values
 334 and the IC₅₀ of each product alone.

<i>IC₅₀ (μM) 96h</i>	<i>P + A</i>		<i>P + F</i>		<i>P + S</i>	
	<i>Posaconazole</i>	<i>Atorvastatin</i>	<i>Posaconazole</i>	<i>Fluvastatin</i>	<i>Posaconazole</i>	<i>Simvastatin</i>
AcNeff	0.65 ± 0.04***	1.79 ± 0.27***	2.27 ± 0.15***	6.05 ± 0.41***	2.17 ± 0.09***	5.79 ± 0.24***
CLC-16	3.75 ± 0.40***	14.09 ± 1.60***	0.62 ± 0.04***	1.66 ± 0.11***	3.00 ± 0.20	8.01 ± 0.54***
CLC-51.1	2.04 ± 0.03***	8.17 ± 0.13***	2.23 ± 0.17***	5.94 ± 0.45***	1.78 ± 0.09***	4.76 ± 0.24***

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