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

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

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Abstract

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

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

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

Results: We found that combinations of any of the tested statins and voriconazole or posaconazole were more efficient at inhibiting *Acanthamoeba* compared to statins or azoles when tested individually. We found lower toxicity of these drug combination at the combined IC_{50}s compared to that of the compounds alone.

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

Keywords:

Keratitis; voriconazole; posaconazole; atorvastatin; fluvastatin; simvastatin

1. Introduction

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

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

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

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

2. Materials and Methods

2.1. Acanthamoeba strains.

We have used three *Acanthamoeba* isolates in this study. One type strain *Acanthamoeba castellanii* Neff (ATCC 30010, genotype T4) and two clinical strains previously isolated by our group CLC-16, (genotype T3) and CLC-51. l (genotype T1). [15] All strains were culture axenically at room temperature in PYG medium supplemented with 40 μg/ml gentamicin (Sigma-Aldrich Chemistry Ltd., Madrid, Spain).

2.2. Chemicals.

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

2.3. Activity assays.

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

2.4. Evaluation of the cysticidal activity of posaconazole.

The effects of posaconazole against cysts were evaluated against the three strains of *Acanthamoeba.* Mature cysts were prepared as previously described. [17] Briefly, trophozoites were transferred from PYG medium to Neff's encystment medium (NEM) where they were cultured for a week under slight agitation to obtain mature cysts. After that, the cells were harvest and washed twice with PYG medium. A concentration of 10^4 cysts/ml was transferred to plates containing fresh PYG medium and incubated with posaconazole at the previously calculated IC_{50}s and IC_{90}s. A Neubauer chamber, was used to count the number of of trophozoites, cysts, and non-viable each 24 hours for a week as previously described by Martín-Navarro et al, 2013a; 2013b. [5, 6] A negative control including only mature cysts in Neff's encystment medium was included to the 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 IC_{50}s compared to the IC_{50} 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 IC_{50} and IC_{90} of

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

We tested the toxicity of the drugs, simvastatin IC_{50} (S50), posaconazole IC_{50} (P50), voriconazole IC_{50} (V50) and the following combinations of molecules: posaconazole and atorvastatin (P + A), posaconazole and fluvastatin (P + F), posaconazole and simvastatin (P + S), and voriconazole with atorvastatin, fluvastatin and simvastatin (V + A, V + F and V + S) did not induce cytotoxicity against macrophages. Whereas in the case of atorvastatin IC_{50} and IC_{90} (A50 and A90), fluvastatin IC_{50} and IC_{90} (F50 and F90), posaconazole IC_{90} (P90) and voriconazole IC_{90} (V90), low cytotoxicity values against macrophages were observed. Furthermore, moderate toxicity was shown in the case of cells incubated with simvastatin IC_{90} (S90) and Amphotericin B IC_{90} (AnfB90) and chlorhexidine IC_{90} (Chx90), induced high levels of toxicity in the tested cell line (Figure 2). The statistical analysis revealed statistical differences ($p < 0.001$) in the cytotoxicity produced by the reference drugs (IC_{90} of Chx and AnfB) and all treatments, excepting in the comparison between Chx90 and S90 (Figure 2).

4. Discussion

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

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

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

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

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

5. Conclusion

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

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Additional Information

The author(s) declare no competing financial interests.

References

1. Lorenzo-Morales J, Martín-Navarro CM, López-Arencibi, A, Arnalich-Montiel F, Piñero JE, Valladares B. Acanthamoeba keratitis: an emerging disease gathering importance worldwide?. Trends Parasitol, 2013 ; **29**(4), 181-187.
2. Lorenzo-Morales J, Martín-Navarro CM, López-Arencibia A, Santana-Morales MA, Afonso-Lehmann RN, Maciver SK, Valladares B, Martínez-Carretero E. Therapeutic potential of a combination of two gene-specific small interfering RNAs against clinical strains of Acanthamoeba. *Antimicrob Agents Chemother*, 2010; **54**(12), 5151-5155.
3. Schuster FL, Visvesvara GS. Free-living amoebae as opportunistic and non-opportunistic pathogens of humans and animals. Int. J. Parasitol 2004; **34**(9), 1001-1027
4. Mafra CSP, Carrijo-Carvalho LC, Chudzinski-Tavassi AM, de Carvalho Taguchi FM, Foronda AS, de Souza Carvalho FR, de Freitas D. 2013. Antimicrobial Action of Biguanides on the Viability of Acanthamoeba Cysts and Assessment of Cell ToxicityBiguanides on Acanthamoeba Cysts and Cytotoxicity. *Invest Ophthalmol Vis Sci* 2013; **54**(9), 6363-6372
5. Martín-Navarro M, Lorenzo-Morales J, Machin RP, López-Arencibia A, García-Castellano JM, de Fuentes I, Loftus B, Maciver SK, Valladares B, Piñero JE. Inhibition of 3-Hydroxy-3-Methylglutaryl–Coenzyme A Reductase and Application of Statins as a Novel Effective Therapeutic Approach against Acanthamoeba Infections. Antimicrob Agents Chemother, 2013ª; **57**(1), 375-381.
6. Martín-Navarro CM, López-Arencibia A, Arnalich-Montiel F, Valladares B, Piñero JE, Lorenzo-Morales J. Evaluation of the in vitro activity of commercially

240 available moxifloxacin and voriconazole eye-drops against clinical strains of
241 Acanthamoeba. *Graefes Arch Clin Exp Ophthalmol* 2013b; **251**(9), 2111-2117.
242 7. Collins R, Reith C, Emberson J, Armitage J, Baigent C, Blackwell, L, ... & Evans
243 S. Interpretation of the evidence for the efficacy and safety of statin therapy. The
244 Lancet 2016; **388**(10059), 2532-2561.
245 8. Martín-Navarro CM, López-Arencibia A, Sifaoui I, Reyes-Batlle M, Valladares
246 B, Martínez-Carretero E, Piñero JE, Maciver SK, Lorenzo-Morales J. Statins and
247 voriconazole induce programmed cell death in Acanthamoeba
248 castellanii. *Antimicrobial agents and chemotherapy* 2015; **59**(5), 2817-2824.
249 9. Smith FR, Korn ED. 7-Dehydrostigmasterol and ergosterol: the major sterols of
250 an amoeba. *J. Lipid Res*; 1968; **9**(4), 405-408.
251 10. Iovieno A, Miller D, Ledee DR, Alfonso EC. Cysticidal activity of antifungals
252 against different genotypes of Acanthamoeba. *Antimicrob Agents Chemother*
253 2014; **58**(9), 5626-5628.
254 11. Mehdi H, Garg HS, Garg NK, Bhakuni DS. Sterols of Acanthamoeba culbertsoni
255 strain A-1. *Steroids* 1988; **51**(5), 551-558.
256 12. Arnalich-Montiel F, Martín-Navarro CM, Alió JL, López-Vélez R, Martínez-
257 Carretero E, Valladares B, Piñero JE, Lorenzo-Morales J. Successful monitoring
258 and treatment of intraocular dissemination of Acanthamoeba. *Arch Ophthalmol*
259 2012; **130**(11), 1474-1475.
260 13. Schiller DS, Fung HB. Posaconazole: an extended-spectrum triazole antifungal
261 agent. *Clin. Ther* 2007; **29**(9), 1862-1886.
262 14. Oliva S, Jantz M, Tiernan R, Cook DL, Judson, M. A. Successful treatment of
263 widely disseminated acanthamoebiasis. *South Med J* 1999; **92**, 55-57.

15. McBride J, Ingram PR, Henriquez FL, Roberts, C. W. Development of colorimetric microtiter plate assay for assessment of antimicrobials against Acanthamoeba. *J. Clin. Microbiol* 2005; **43**(2), 629-634.
16. Martín-Navarro CM, Lorenzo-Morales J, Cabrera-Serra MG, Rancel F, Coronado-Alvarez NM, Pinero JE, Valladares B. The potential pathogenicity of chlorhexidine-sensitive Acanthamoeba strains isolated from contact lens cases from asymptomatic individuals in Tenerife, Canary Islands, Spain. *J Med Microbiol* 2008; **57**(11), 1399-1404.
17. Lorenzo-Morales J, Kliescikova J, Martinez-Carretero E, De Pablos LM, Profotova B, Nohynkova E, Osuna A, Valladares, B. Glycogen phosphorylase in Acanthamoeba spp.: determining the role of the enzyme during the encystment process using RNA interference. *Eukaryotic cell* 2008; **7**(3), 509-517.
18. Martín-Navarro CM, López-Arencibia A, Sifaoui I, Reyes-Batlle M, Fouque E, Osuna A, Valladares B, Pinero JE, Héchard Y, Maciver SK, Lorenzo-Morales, J. Amoebicidal Activity of Caffeine and Maslinic Acid by the Induction of Programmed Cell Death in Acanthamoeba. Antimicrob Agents Chemother 2017; **61**(6), e02660-16.
19. Kulsoom H, Baig AM, Siddiqui R, Khan, NA. Combined drug therapy in the management of granulomatous amoebic encephalitis due to Acanthamoeba spp., and Balamuthia mandrillaris. *Exp Parasitol* 2014; **145**, S115-S120.
20. Montalvetti A, Javier PA, Hurtado R, Ruiz-Pérez LM, González-Pacanowska D. Characterization and regulation of Leishmania major 3-hydroxy-3-methylglutaryl-CoA reductase. Biochem J 2000; **349**(1), 27-34.

21. Ishibashi Y, Matsumoto Y, Kabata T, Watanabe R, Hommura S, Yasuraoka K, Ishii K. Oral itraconazole and topical miconazole with debridement for Acanthamoeba keratitis. Am J Ophthalmol 1990; **109**(2), 121-126.

Author Contributions statement

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

Competing Financial Interests statement

Nothing to declare

FIGURE LEGENDS:

Figure 1. Number of cysts when they were incubated with posaconazole with previously calculated IC_{50} and IC_{90} in PYG medium. Cysts were not viable after incubation with this drug, since amoebae were not able to excyst. Moreover, the number of cysts decreased with time and became non-viable cells.

Figure 2. Cytotoxicity of atorvastatin, fluvastatin, simvastatin, posaconazole, voriconazole, amphotericin B and chlorhexidine at IC_{50} and IC_{90} as well the combination between azoles and statins produced in murine macrophages. A50 and A90: atorvastatin IC_{50} and IC_{90}. F50 and F90: fluvastatin IC_{50} and IC_{90}. S50 and S90: simvastatin IC_{50} and IC_{90}. P50 and P90: posaconazole IC_{50} and IC_{90}. P + A: posaconazole and atorvastatin. P + F: posaconazole and fluvastatin. P + S: posaconazole and simvastatin. V50 and V90:

voriconazole IC_{50} and IC_{90}. V + A: voriconazole and atorvastatin. V + F: voriconazole and fluvastatin. V + S: voriconazole and simvastatin. AnfB50 and AnfB90: amphotericin B IC_{50} and IC_{90}. Chx50 and Chx90: chlorhexidine IC_{50} and IC_{90}. The results showed that S50, P50, V50 and the following combinations: P + A, P + F, P + S, and V + A, V + F and V + S were not cytotoxic against macrophages. A50 and A90, F50 and F90, P90 and V90 presented low cytotoxicity against macrophages. The observed cytotoxicity levels were moderate when cells were incubated with S90. AnfB90 and Chx90 showed high levels of cytotoxicity against macrophages. The cytotoxicity values showed significant differences with the cytotoxicity produced by the reference drugs chlorhexidine and amphotericin B (***, $p<0.001$).

Table 1. IC_{50} and IC_{90} (μM) values for posaconazole against different strains of *Acanthamoeba* measured by Alamar blue assay after 96h (Mean concentration ± SD).

IC_{50} (μM) 96h	***AcNeff***		***CLC-16***		***CLC-51.l***	
	IC_{50}	***IC_{90}***	***IC_{50}***	***IC_{90}***	***IC_{50}***	***IC_{90}***
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

Table 2. IC_{50} (μM) values for voriconazole and each statin assayed when this compounds were combined and evaluated against different strains of *Acanthamoeba* measured by Alamar blue assay after 96h (Mean concentration ± SD). (***) $p \leq 0.001$ statistical differences between those values and the IC_{50} of each product alone.

IC_{50} (μM) 96h	***V + A***		***V + F***		***V + S***	
	Voriconazole	***Atorvastatin***	***Voriconazole***	***Fluvastatin***	***Voriconazole***	***Simvastatin***
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***

Table 3. IC_{50} (μM) values for posaconazole and each statin assayed when this compounds were combined and evaluated against different strains of *Acanthamoeba* measured by Alamar blue assay after 96h (Mean concentration ± SD). (***) $p \leq 0.001$ statistical differences between those values and the IC_{50} of each product alone.

IC_{50} (μM) 96h	***P + A***		***P + F***		***P + S***	
	Posaconazole	***Atorvastatin***	***Posaconazole***	***Fluvastatin***	***Posaconazole***	***Simvastatin***
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***