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Inhibition of 3-Hydroxy-3-Methylglutaryl–Coenzyme A Reductase and Application of Statins as a Novel Effective Therapeutic Approach against *Acanthamoeba* Infections

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***Acanthamoeba* is an opportunistic pathogen in humans, whose infections most commonly manifest as *Acanthamoeba* keratitis or, more rarely, granulomatous amoebic encephalitis. Although there are many therapeutic options for the treatment of *Acanthamoeba*, they are generally lengthy and/or have limited efficacy. Therefore, there is a requirement for the identification, validation, and development of novel therapeutic targets against these pathogens. Recently, RNA interference (RNAi) has been widely used for these validation purposes and has proven to be a powerful tool for *Acanthamoeba* therapeutics. Ergosterol is one of the major sterols in the membrane of *Acanthamoeba*. 3-Hydroxy-3-methylglutaryl–coenzyme A (HMG-CoA) reductase is an enzyme that catalyzes the conversion of HMG-CoA to mevalonate, one of the precursors for the production of cholesterol in humans and ergosterol in plants, fungi, and protozoa. Statins are compounds which inhibit this enzyme and so are promising as chemotherapeutics. In order to validate whether this enzyme could be an interesting therapeutic target in *Acanthamoeba*, small interfering RNAs (siRNAs) against HMG-CoA were developed and used to evaluate the effects induced by the inhibition of *Acanthamoeba* HMG-CoA. It was found that HMG-CoA is a potential drug target in these pathogenic free-living amoebae, and various statins were evaluated *in vitro* against three clinical strains of *Acanthamoeba* by using a colorimetric assay, showing important activities against the tested strains. We conclude that the targeting of HMG-CoA and *Acanthamoeba* treatment using statins is a novel powerful treatment option against *Acanthamoeba* species in human disease.**

Free-living amoebae of the genus *Acanthamoeba* are ubiquitous protozoa that pervade the entire environment and include amphizoic strains that are pathogenic to humans and other animals (1). In humans, these protozoa are opportunistic causal agents of sight-threatening ulcerations of the cornea called *Acanthamoeba* keratitis (AK), disseminated infections (mostly cutaneous and nasopharyngeal), and fatal granulomatous amoebic encephalitis (GAE) (1–4).

Present therapeutic measures for *Acanthamoeba* keratitis rely on topical applications of antimicrobials, including the combination of propamidine isothionate and neomycin or chlorhexidine. Moreover, the length of these treatments makes the process arduous. Furthermore, as current treatments are poorly effective against the cyst form of these amoebae, residual infection often remains even after treatment. No treatment against GAE has been established, although therapeutic measures have been used with apparent satisfactory effects, even saving the patient's life or at least slowing down the progression of the amoebic infection (1–3, 5). Chlorhexidine and polyhexamethylene biguanide (PHMB) as monotherapy agents have been proven not to be sufficient against clinical or environmental strains of acanthamoebae, hence the importance of multiple-strain testing of drugs against *Acanthamoeba*, as their effectiveness might depend on the *Acanthamoeba* isolate (6–9). There is a need to search for and validate new therapeutic targets against *Acanthamoeba*, focusing mostly on key proteins related to cellular viability and the pathogenesis of *Acanthamoeba*.

3-Hydroxy-3-methylglutaryl–coenzyme A (HMG-CoA) reductase is an enzyme involved in the conversion of HMG-CoA to mevalonate, a precursor of cholesterol in humans and ergosterol in plants, fungi, and protozoa (10, 11). In *Acanthamoeba*, ergosterol and 7-dehyrostigmasterol have been reported to be the main sterols of the membrane in both the trophozoite and the cyst forms (12–15). Therefore, if this enzyme is blocked or inhibited, defective membrane architecture as well as an increased permeability and leakage of ions from the cell should be expected. However, the presence of this enzyme in *Acanthamoeba* has not been previously demonstrated until the present study.

RNA interference (RNAi) was previously applied to *Acanthamoeba* for the elucidation of key processes in this genus by using chemically synthesized specific small interfering RNAs (siRNAs) and was also recently proposed as a possible therapeutic approach against acanthamoebae, at least *in vitro*. Therefore, the

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Acanthamoeba castellanii (ACA1_280080)	89	FGRCCENVIGY	120	PMATTEGCLVA	214	RSSTGDAMGMN
Arabidopsis thaliana (P14891.1)	239	LGQCCEMPVGY	270	PMATTEGCLVA	364	CCSTGDAMGMN
Dictyostelium discoideum (XP_646489.1)	211	QGQCCENVIGY	242	PMATTEGCLVA	336	KCDTGDAMGMN
Homo sapiens (1DQ8_A)	112	MGACCENVIGY	143	PMATTEGCLVA	237	QSRSGDAMGMN
Mus musculus (AAH59873.1)	142	MGACCENVIGY	173	PMATTEGCLVA	267	QSRTGDAMGMN
Rattus norvegicus (NP_037266.2)	532	MGACCENVIGY	563	PMATTEGCLVA	657	QSKTGDAMGMN
Trypanosoma cruzi (AAB62280.1)	99	VGQNCENIIGY	122	AMATTEGALVA	212	RATTGDAMGMA
Leishmania major (AAD38406.1)	96	VGQSCENILGY	117	PMATTEGALVA	209	SAFTGDAMGMN

FIG 1 Amino acid sequences that surround the conserved acidic residues of the catalytic domain of the available sequences of HMG-CoA reductases in the GenBank database and the *Acanthamoeba* HMG-CoA reductase. The comparative analysis of the catalytic domains of the HMG-CoA reductases revealed 1 conserved aspartate and 2 conserved glutamate residues (asterisks).

use of siRNAs for *Acanthamoeba* currently presents a powerful tool to validate and evaluate suspected drug targets or develop novel therapeutic approaches. In this work, siRNAs against HMG-CoA were developed and tested in order to evaluate the potential use of this enzyme in future therapies. Once validated, the following step was the search for active compounds that can inhibit the target. For this purpose, statins were used in this study.

Statins are a family of lipid-lowering drugs widely used to control cholesterol levels and to prevent stroke and cardiac failure in patients at a high risk of coronary artery disease. The mechanism of action of statins is the inhibition of HMG-CoA reductase by binding to the active site of this enzyme (16). This process is a process of competitive inhibition with respect to the substrate. Moreover, it has been reported that several residues in the catalytic region of HMG-CoA reductase can participate in substrate catalysis. Especially, the active-site glutamate and aspartate are conserved in all known HMG-CoA reductases, and the changes in activity that accompany their mutagenesis support their proposed roles in catalysis (17).

Different statins have been used against some parasites, such as *Schistosoma mansoni* and *S. haematobium* (18, 19), *Leishmania amazonensis* and *L. donovani* (20, 21), *Trypanosoma cruzi* (22, 23), *Plasmodium falciparum* (24–27), and *Toxoplasma gondii* (28). Statins differ in terms of their chemical structures, pharmacokinetic profiles, and lipid-modifying efficacies (29). For this reason, the efficacies of five different statins (simvastatin, pravastatin, lovastatin, atorvastatin, and fluvastatin) were evaluated against *Acanthamoeba castellanii* Neff and three clinical isolates from contact lens cases.

MATERIALS AND METHODS

***Acanthamoeba* strains.** Three clinical isolates (CLC-16, genotype T3; CLC-41.r, genotype T4; and CLC-51, genotype T1) obtained in a previous study in our laboratory (5) and the *Acanthamoeba castellanii* type strain Neff (ATCC 30010, genotype T4) were used in this study.

The four *Acanthamoeba* strains were axenically grown in PYG medium (0.75% [wt/vol] proteose peptone, 0.75% [wt/vol] yeast extract, and 1.5% [wt/vol] glucose) containing 40 mg/liter gentamicin (Biochrom AG; Cultek, Granollers, Barcelona, Spain) at room temperature. However, experiments were carried out at 28°C.

Statins. Five statins were used in this work: simvastatin, which was kindly provided by Merck Chemical Spain Ltd. (Barcelona, Spain); pravastatin and atorvastatin, which were purchased from Sigma-Aldrich Chemistry Ltd. (Madrid, Spain); and lovastatin and fluvastatin, which were purchased from Enzo Life Sciences Inc. (Taper Group, Spain).

Design of HMG-CoA reductase PCR. The predicted coding sequence of *Acanthamoeba* HMG-CoA (Ac-HMG-CoA) was generated as part of the ongoing *Acanthamoeba* Genome Project (accession number AHJI01000000) being carried out in the laboratory of Brendan Loftus (B. Loftus, personal communication).

A comparative analysis of this sequence was carried out by using the available HMG-CoA reductase sequences in the GenBank database using MEGA5.0 software (30).

Primers were designed by using primer 3 software in order to verify the validity of these sequences for all the strains to be used in this study (31). The designed primer pair was Ac-HMG-CoA-F (5′-TGACTCGTGGTCC TTGTGTTCGT-3′) and Ac-HMG-CoA-R (5′-TGACCAGCAGCAAGA

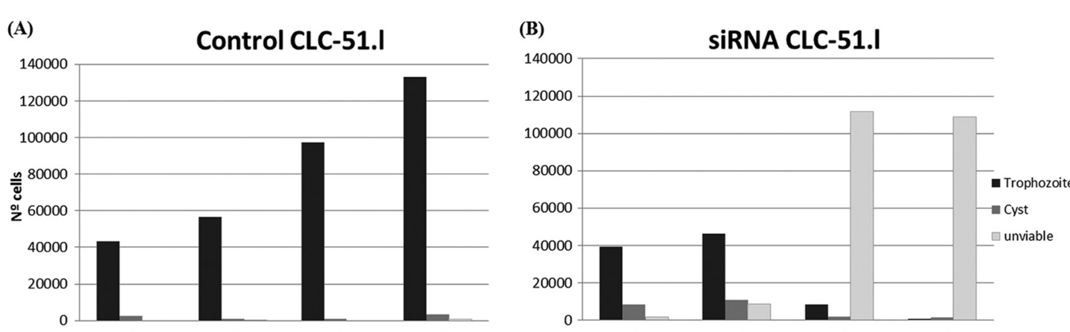

FIG 2 Distribution of *Acanthamoeba* CLC-51.l cell line trophozoites, cysts, and nonviable cells at 6, 12, 24, and 48 h in the absence (A) and in the presence (B) of HMG-CoA reductase siRNA. The cell distribution (number of different cell types) at different time points was determined by using microscopy and cell counting. All experiments were repeated three times. Similar results were obtained with the other strains used in this study.

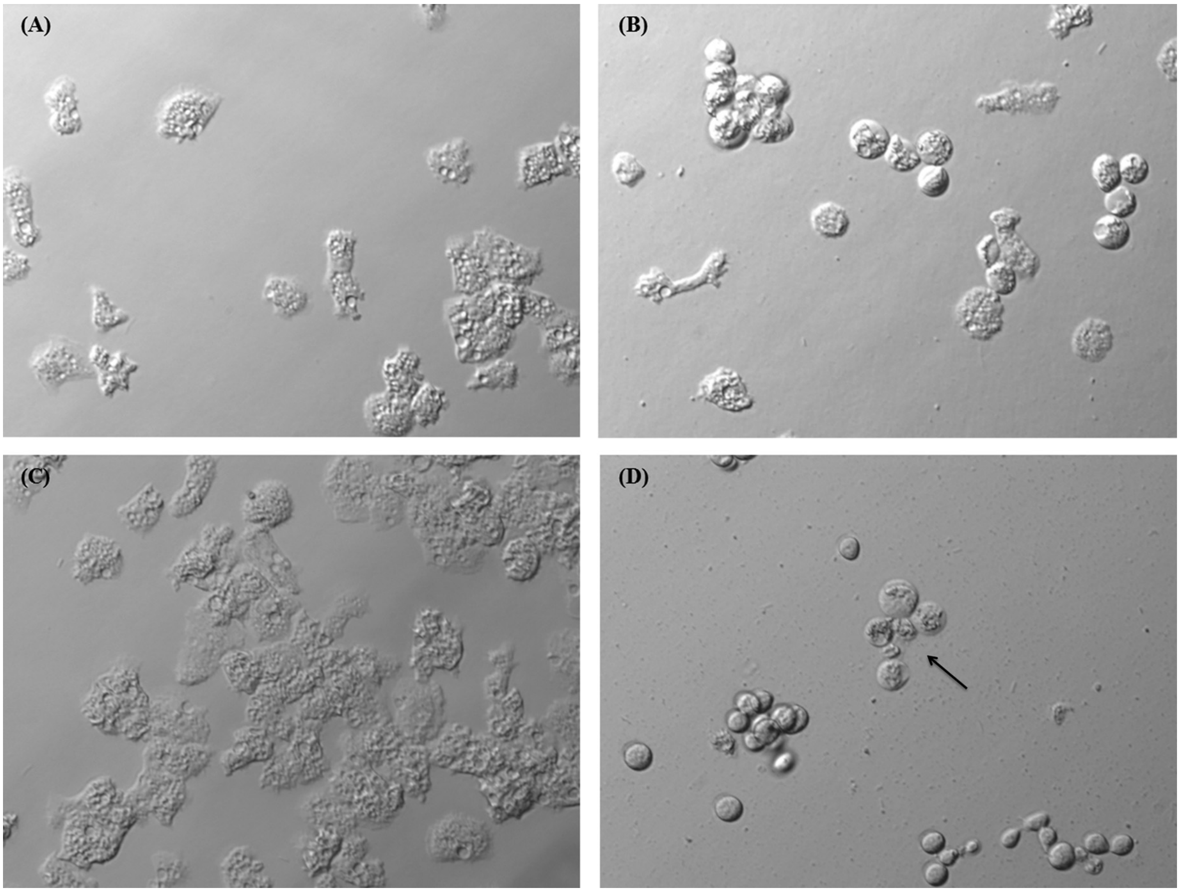

FIG 3 Light microscopy (magnification, ×20) images corresponding to the assay that was carried out with *Acanthamoeba castellanii* Neff. Similar results were obtained with the other clinical strains included in this study. (A and C) Controls at 24 h (A) and 48 h (C) are shown as trophozoites. (B and D) Treated cells at 24 h (B) and 48 h (D) are shown as rounded and lysed cells (arrow).

GCAGCC-3′). The PCR mixture included 5 pmol each primer, 40 ng of DNA (from clinical isolates and the type strain), and 0.25 U of *Taq* polymerase (Bioline; Ecogen Biologia Molecular, Spain). The amplification cycles used were 94°C for 5 min; 94°C for 30 s, 50°C for 15 s, and 72°C for 15 s (for 35 cycles); and 72°C for 7 min.

HMG-CoA reductase silencing. Gene silencing was performed with the following Stealth RNAi siRNAs specifically designed against HMG-CoA reductase by using BLOCK-it RNAi designer software (Invitrogen): ST-siRNA-1 (UGCUUCUACUCAUCGUGGUUGUAAA) and ST-siRNA-2 (UCUUCAUGUUAAAGGUGCUUCUGAA). X-treme Gene siRNA transfection reagent (Roche) was used in order to improve the silencing efficacy without induced cytotoxicity problems (32). The experiment was performed with the four strains mentioned above and was carried out in triplicate starting with 10^4 cells/ml and adding 15 μg/ml of the ST-siRNAs to the medium, as previously described (33). The effect induced by the siRNA treatment was evaluated by using microscopy and cell counting. After 96 h, the cells were incubated in fresh PYG medium in order to check cell viability (capacity of amoebae to excyst).

Effects of the transfection reagent and siRNA were checked by carrying out control experiments with siRNAs which encode green fluorescence protein (scrambled siRNA), GFP-siRNA-1 (UUUACAACCACGAUGAG UAGAAGCA) and GFP-siRNA-2 (UUCAGAAGCACCUUUAACAUGA AGA), as previously described (33).

Activity assays. The anti-*Acanthamoeba* activities of the assayed drugs were determined by the alamarBlue assay, as previously described (5, 34, 35). Briefly, *Acanthamoeba* trophozoites were seeded into a 96-well microtiter plate with 50 μl from a stock solution of 8×10^4 cells/ml. After that, 50 μl of serial dilutions of statins in PYG medium were added to each well, and finally, alamarBlue assay reagent (Biosource Europe, Nivelles, Belgium) was placed into each well at an amount equal to 10% of the medium volume. Test plates containing alamarBlue were then incubated for 120 h at 28°C with slight agitation.

Subsequently, the plates were analyzed during an interval of time between 72 and 120 h on a model 680 microplate reader (Bio-Rad, Hercules, CA), using a test wavelength of 570 nm and a reference wavelength of 630 nm. Percentages of growth inhibition, 50% inhibitory concentrations (IC_{50}) and 90% inhibitory concentrations (IC_{90}), for each molecule were calculated by linear regression analysis with 95% confidence limits. All experiments were performed three times each in duplicate, and the mean values were also calculated. A paired two-tailed *t* test was used for analyses of the data. P values of <0.05 were considered significant. The statistical analysis of the inhibition curves was undertaken by using the Sigma Plot 12.0 software program (Systat Software Inc.).

Cysticidal activity. The effects of statins against cysts were evaluated by incubating 10^4 cysts of *A. castellanii* Neff with the previously calculated IC_{50}s and IC_{90}s of the statins in PYG medium. The numbers of trophozoites, cysts, and nonviable cysts were counted with a Neubauer chamber at 96, 120, 144, and 168 h.

Cell proliferation. In order to study the effects of the tested active compounds on *Acanthamoeba castellanii* Neff cell proliferation, a Cell Proliferation enzyme-linked immunosorbent assay (ELISA) bromodeoxyuridine (BrdU) (colorimetric) kit was used (Roche), according to the manufacturer's recommendations. Briefly, the assay was carried out in 96-well plates with 10^4 cells/ml per well. The concentrations used were the

TABLE 1 IC_{50} and IC_{90} values of statins tested against different strains of *Acanthamoeba* at 96 h

Statin	Mean concn (μM) at 96 h ± SD[a]							
	AcNeff		CLC-16		CLC-41.r		CLC-51.l	
	IC_{50}	IC_{90}	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	13.70 ± 0.81	26.10 ± 1.18	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	24.29 ± 0.97	55.17 ± 2.91	16.50 ± 1.03	32.86 ± 5.18
Lovastatin	17.14 ± 1.85	NA	NA	NA	46.65 ± 4.50	NA	NA	NA
Pravastatin	58.75 ± 11.01	NA	NA	NA	NA	NA	NA	NA
Simvastatin	10.24 ± 1.09	21.37 ± 1.51	31.44 ± 2.06	63.55 ± 4.15	14.60 ± 0.59	29.85 ± 1.16	39.73 ± 4.34	84.16 ± 8.23

[a] NA, no activity.

IC_{50}s and IC_{90}s, and the obtained results were analyzed at 24, 48, and 72 h. The obtained results were compared by one-way analysis of variance (ANOVA) and by multiple *post hoc* analysis and Tukey's test using Sigma Plot 12.0 software (Systat Software).

Cytotoxicity test. The cytotoxicity produced by active compounds was evaluated against the following cell lines from mammals: murine macrophages (ATCC TIB-67) and HeLa cells (ATCC CCL-2). A cytotoxicity detection kit (lactate dehydrogenase; Roche Applied Science) was used according to the manufacturer's recommendations. Results were classified based on previously established parameters: the active principles with percentages of cytotoxicity of between 0 and 10% were not cytotoxic, values between 10 and 25% correspond to low cytotoxicity, values between 25 and 40% are equivalent to moderate cytotoxicity, and values of at least 40% indicate high cytotoxicity (32).

RESULTS

HMG-CoA reductase silencing. The presence of the sequence of HMG-CoA reductase in *Acanthamoeba* was verified in all tested strains. Furthermore, the comparative analysis revealed that the catalytic region of this enzyme is conserved in *Acanthamoeba* (Fig. 1). After that, siRNA-based gene silencing assays were thus carried out by targeting this enzyme.

The results obtained were similar for all the tested strains. Numbers of untreated control trophozoites increased exponentially during the entire experiment (Fig. 2A). Unlike the control, the number of cells treated with siRNA decreased up to 48 h post-treatment. At this time, the cells were no longer viable (Fig. 2B), and cells undergoing lysis were observed (Fig. 3).

In response to treatment with siRNA, some cells began the process of encystation by forming precysts. However, these precysts were not viable, as they were unable to revert to trophozoites when transferred into fresh PYG medium, even after 168 h.

Activity assays. The amoebicidal activities of the tested statins are summarized in Table 1. We observed that the most active statins are simvastatin, fluvastatin, and atorvastatin. With the *A. castellanii* type strain Neff, pravastatin was the least active statin and was no longer tested. However, lovastatin also exhibited a low level of activity against the clinical strains. Effective drug concentrations higher than 100 μM were not considered useful.

In order to check the cysticidal activities of statins against *A. castellanii* Neff, lovastatin, simvastatin, fluvastatin, and atorvastatin (the most active statins) (Table 1) at the previously calculated IC_{90} values were incubated in wells with 10^4 cells/ml.

Excystation did not occur except when the IC_{90} of lovastatin was used (less active molecule from the used ones) (Fig. 4A); however, if we compared it with the control, the amount of cells was small.

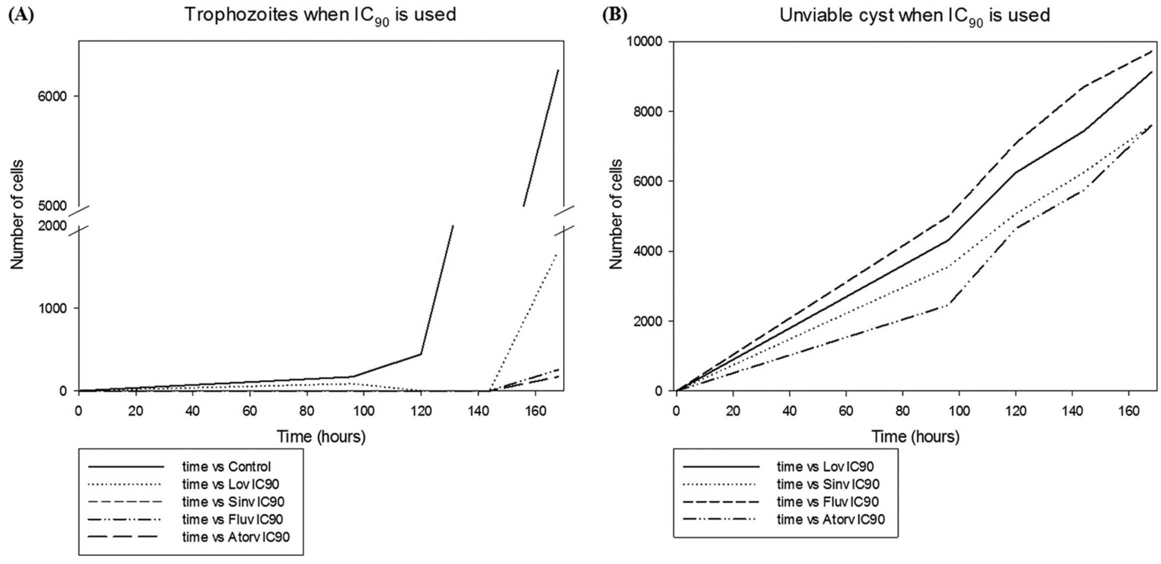

FIG 4 Effects of statins against cysts were evaluated by incubating 10^4 cysts of *A. castellanii* Neff with the previously calculated IC_{50} and IC_{90} values of the selected statins in PYG medium, and cells were counted with a Neubauer chamber at between 96 and 168 h. (A) Number of cysts that reverted to trophozoites in PYG medium after incubation with statins. (B) Number of nonviable cysts when cysts were incubated with the previously calculated IC_{90}s of statins in PYG medium.

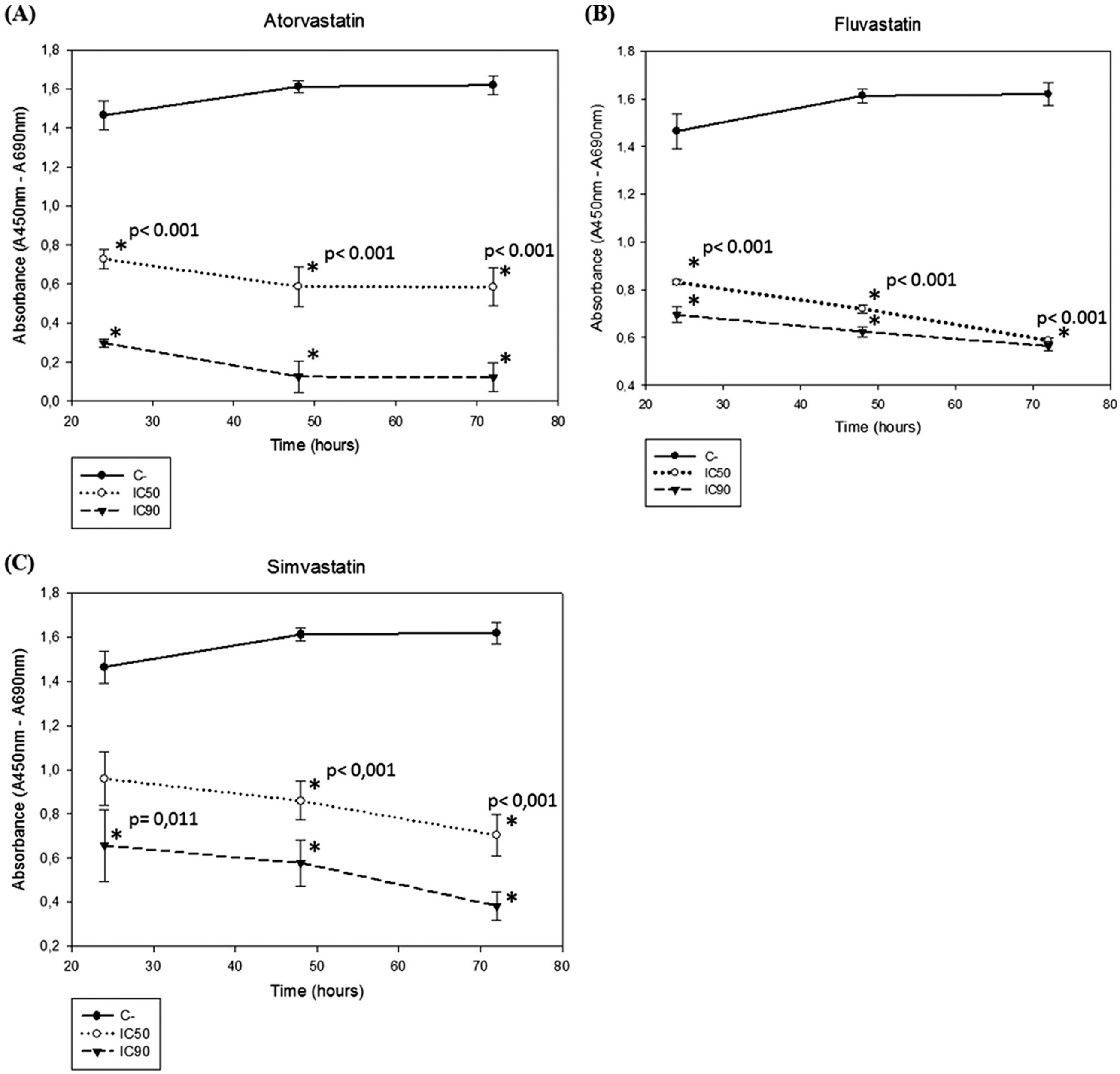

FIG 5 Observed cell proliferation after incubation of *Acanthamoeba castellanii* strain Neff with the selected statins compared to the control using the previously obtained IC_{50} and IC_{90} values. Results were analyzed at 24, 48, and 72 h. Significant differences between the IC_{50}s and IC_{90}s were observed for atorvastatin (A) and simvastatin (C), with the exception of fluvastatin (B).

We observed that the number of cysts decreased during the time period due to reversion to trophozoites (especially when lovastatin was used), and others may have been nonviable. Nonviable cysts were observed with all statins; however, when fluvastatin was used, all initial cysts became nonviable (Fig. 4B).

The effect of each of the statins on *Acanthamoeba castellanii* Neff cell proliferation from 24 to 72 h was checked. It was noted that all active principles decreased the cell proliferation in a dose-dependent manner (Fig. 5). Furthermore, significant differences between the IC_{50}s and IC_{90}s were observed, with the exception of fluvastatin (Fig. 5B), which may serve to establish the IC_{50} as the concentration sufficient to eliminate the cell population.

Cytotoxicity assays. The results showed that the atorvastatin IC_{50} and IC_{90}, the fluvastatin IC_{50} and IC_{90}, and the simvastatin IC_{50} presented low cytotoxicity (the IC_{50} of simvastatin was not cytotoxic to macrophages, but it was cytotoxic to HeLa cells). The simvastatin IC_{90} produced high cytotoxicity. In summary, all IC_{50}s showed null or low cytotoxicity. In the case of the IC_{90}, simvastatin showed only high cytotoxicity (Fig. 6).

DISCUSSION

The enzyme 3-hydroxy-3-methylglutaryl–coenzyme A (HMG-CoA) reductase is widely expressed in vertebrates, and it has been identified in protistan parasites such as *Trypanosoma* and *Leish-*

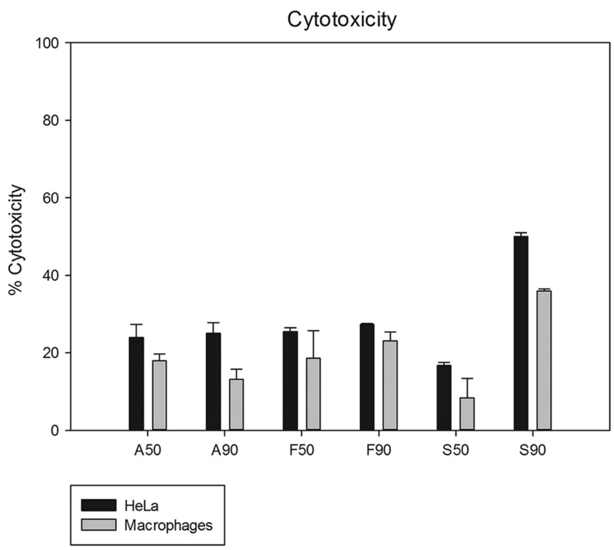

FIG 6 Cytotoxicity levels of the statins tested against *Acanthamoeba* (IC_{50} and IC_{90}) were evaluated against two cell lines, HeLa cells and murine macrophages. Values between 10 and 25% correspond to low cytotoxicity, so the results showed that the atorvastatin IC_{50} (A50) and IC_{90} (A90), the fluvastatin IC_{50} (F50) and IC_{90} (F90), and the simvastatin IC_{50} (S50) presented low cytotoxicity (the IC_{50} of simvastatin was not cytotoxic to macrophages, but it was cytotoxic to HeLa cells). Values of at least 40% correspond to high cytotoxicity, which was the case for the simvastatin IC_{90} (S90). In summary, all IC_{50}s showed null or low cytotoxicity against the tested cell lines. Only in the case of the simvastatin IC_{90} was a high cytotoxicity level observed.

mania (36). The amino acids involved in the active site of HMG-CoA reductases have been identified for a number of species, and these have been found to be conserved (17). In this study, we have identified the gene encoding *Acanthamoeba* HMG-CoA reductase, and we have found that these conserved amino acids are also present (Fig. 1), leading us to suspect that statins may also inhibit the amoebal enzyme.

We have demonstrated that the enzyme is a potential target for the development of treatments against *Acanthamoeba* spp. by reducing its expression through siRNA. However, although siRNA has been proposed as a therapy (32, 37), it would be a very expensive and controversial treatment. Instead, we have investigated the effects of inhibiting HMG-CoA reductase activity in *Acanthamoeba* with a range of statins.

Statins (atorvastatin, fluvastatin, lovastatin, pravastatin, and simvastatin) have been widely used as a treatment for hypercholesterolemia, as they inhibit HMG-CoA reductase, an enzyme that converts HMG-CoA to mevalonate, which is a precursor of cholesterol in vertebrates and ergosterol in fungi and some protozoa (36). In *Acanthamoeba*, ergosterol and 7-dehyrostigmasterol are major sterol membrane components of both the nonpathogenic species *A. castellanii* and the pathogenic *A. culbertsoni* strain A-1 (12, 13, 15). We found that statins are amoebicidal and cysticidal, possibly because both stages of the amoeba contain and require ergosterol (15).

Statins are molecules that differ in their chemical structures, pharmacokinetics, and efficacies. From this point of view, the statins most effective at lowering cholesterol levels in humans are rosuvastatin, atorvastatin, simvastatin, and pravastatin (29). It was reported previously that statins are also effective in the treatment of certain cancers, but the exact mechanism of this antiproliferative activity remains unclear (38). The hydrophobicity of the molecules correlates with the anticancer effect of lipophilic statins such as atorvastatin, mevastatin, simvastatin, and rosuvastatin, reducing the risk of progression and prostate cancer mortality (39).

Some statins (atorvastatin, fluvastatin, lovastatin, pravastatin, and simvastatin) were previously tested against various parasitic protozoa, such as *S. haematobium* (19), *S. mansoni* (18), *P. falciparum* (24–27), *T. gondii* (24, 28), *T. cruzi* (23), and *Leishmania amazonensis* and *L. donovani* (20, 21). Although we have shown here that various statins are effective against *Acanthamoeba* in *in vitro* studies, this does not guarantee that it will be effective *in vivo*. For example, although simvastatin is effective against *Plasmodium* (24), the drug by itself showed no inhibition of the growth of the parasite *in vivo* (40).

If the *in vitro* activity of these molecules proves effective without producing cytotoxicity, they can be considered molecules for future treatments. In this sense, atorvastatin and fluvastatin seem suitable for this purpose. However, because of the moderate to high levels of cytotoxicity seen with simvastatin at the IC_{90}, the use of the drug may be not be suitable as a treatment.

The range of concentrations of this class of molecules with activity against trophozoites is between 9 and 58 μM. The calculated concentrations are lower that the dosage of statins (even when bioavailability is taken into account) used for the treatment of hypercholesterolemia (Consejo General de Colegios Oficiales de Farmaceuticos, Spain). It is therefore possible that the present regime for controlling cholesterol levels in patients will be suitable as a treatment for systemic infections by *Acanthamoeba.* This drug regime may also be effective even for the treatment of GAE cases, as statins are able to penetrate the blood-brain barrier (29, 41). However, because of the very serious nature of GAE, higher statin levels may be used, and any side effects must be accepted and lessened to some extent and compensated for by dietary uptake (23). In the case of *Acanthamoeba* keratitis, the application of statins in the form of eye drops may be a better way to deliver statins at high doses when it is required. Additionally, further experiments should be carried out in order to confirm whether statins act differently at different temperatures, since this seems not to have been investigated.

To the best of our knowledge, this is the first time that statins have been tested against *Acanthamoeba*, and our results show the promise of statins as a novel therapy against this facultative pathogen.

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