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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.
The 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 AHJI0100000) 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’-TGACTCGTGCTCC TTGTGTTGCT-3’) and Ac-HMG-CoA-R (5’-TGACCACGACGCAAGA...
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 (UGCUUCUACAUCCUGGUUGUAAA) and ST-siRNA-2 (UCUUCAUGUAAAGGUGCUUCUGAA). 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 104 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 (UUUACAACCACGAGAGAGAGAGAGC) and GFP-siRNA-2 (UUCAGAAGCCUUUAAUUGAG), 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 × 10⁴ 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 (IC₅₀) and 90% inhibitory concentrations (IC₉₀), 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 10⁴ cysts of *A. castellanii* Neff with the previously calculated IC₅₀ and IC₉₀ 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 10⁴ cells/ml per well. The concentrations used were the
IC₅₀s and IC₉₀s, 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).

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. 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 IC₉₀ values were incubated in wells with 10⁴ cells/ml. Excystation did not occur except when the IC₉₀ 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 10⁴ cysts of *A. castellanii* Neff with the previously calculated IC₅₀ and IC₉₀ 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 IC₉₀ of statins in PYG medium.

### TABLE 1 IC₅₀ and IC₉₀ values of statins tested against different strains of *Acanthamoeba* at 96 h

<table>
<thead>
<tr>
<th>Statin</th>
<th>AcNeff IC₅₀ (μM) ± SD</th>
<th>AcNeff IC₉₀ (μM) ± SD</th>
<th>CLC-16 IC₅₀ (μM) ± SD</th>
<th>CLC-16 IC₉₀ (μM) ± SD</th>
<th>CLC-41.1 IC₅₀ (μM) ± SD</th>
<th>CLC-41.1 IC₉₀ (μM) ± SD</th>
<th>CLC-51.1 IC₅₀ (μM) ± SD</th>
<th>CLC-51.1 IC₉₀ (μM) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atorvastatin</td>
<td>15.12 ± 2.19</td>
<td>41.09 ± 0.01</td>
<td>33.34 ± 2.64</td>
<td>78.66 ± 5.85</td>
<td>13.70 ± 0.81</td>
<td>26.10 ± 1.18</td>
<td>26.63 ± 1.20</td>
<td>49.76 ± 1.81</td>
</tr>
<tr>
<td>Fluvastatin</td>
<td>9.19 ± 0.98</td>
<td>20.70 ± 2.15</td>
<td>54.64 ± 2.69</td>
<td>105.40 ± 5.34</td>
<td>24.29 ± 0.97</td>
<td>55.17 ± 2.91</td>
<td>16.50 ± 1.03</td>
<td>32.86 ± 5.18</td>
</tr>
<tr>
<td>Lovastatin</td>
<td>17.14 ± 1.85</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>46.65 ± 4.50</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>58.75 ± 11.01</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Simvastatin</td>
<td>10.24 ± 1.09</td>
<td>21.37 ± 1.51</td>
<td>31.44 ± 2.06</td>
<td>63.55 ± 4.15</td>
<td>14.60 ± 0.59</td>
<td>29.85 ± 1.16</td>
<td>39.73 ± 4.34</td>
<td>84.16 ± 8.23</td>
</tr>
</tbody>
</table>

NA, no activity.
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\textsubscript{50} and IC\textsubscript{90} were observed, with the exception of fluvastatin (Fig. 5B), which may serve to establish the IC\textsubscript{50} as the concentration sufficient to eliminate the cell population.

**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*-
was reported previously that statins are also effective in the treat-
ment of certain cancers, but the exact mechanism of this antipro-
liferative 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. falcip-
arium (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 with-
out 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_{50}, the use
of the drug may 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 calcu-
lated concentrations are lower than 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 ex-
periments should be carried out in order to confirm whether st-
atin 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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**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 macro-
phages. 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 cyto-
xicity (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.

**曼** (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 reduc-
tase, 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 re-
ducing its expression through siRNA. However, although siRNA
has been proposed as a therapy (32, 37), it would be a very expen-
sive and controversial treatment. Instead, we have investigated the
effects of inhibiting HMG-CoA reductase activity in Acantham-
oba with a range of statins.

Statins (atorvastatin, fluvastatin, lovastatin, pravastatin, and
simvastatin) have been widely used as a treatment for hypercho-
lesterolemia, as they inhibit HMG-CoA reductase, an enzyme that
converts HMG-CoA to mevalonate, which is a precursor of cho-
lesterol in vertebrates and ergosterol in fungi and some protozoa
(36). In Acanthamoeba, ergosterol and 7-dehydrostigmasterol
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 st-
atins most effective at lowering cholesterol levels in humans are
rosuvastatin, atorvastatin, simvastatin, and pravastatin (29). It
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