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

Carmen Maria Martín-Navarro, Jacob Lorenzo-Morales, Rubén P. Machín, Atteneri López-Arencibia, José Manuel García-Castellano, Isabel de Fuentes, Brendan Loftus, Sutherland K. Maciver, Basilio Valladares, José E. Piñero

University Institute of Tropical Diseases and Public Health of the Canary Islands, University of La Laguna, La Laguna, Tenerife, Canary Islands, Spain; Molecular Oncology Group (G-OncMo), Research Unit, University Hospital of Gran Canaria Dr. Negrín, Barranco La Ballena, Las Palmas de Gran Canaria, Canary Islands, Spain; Servicio de Cirugía Ortopédica y Traumatología del Complejo Hospitalario Universitario Insular-Materno Infantil, Laboratorio de Oncología Molecular, Unidad de Investigación C.H.U.I.M.I., Las Palmas de G. C., Spain; Carlos III Health Institute, Microbiology National Center, Majadahonda, Madrid, Spain; School of Medicine and Medical Science, Conway Institute, University College Dublin, Belfield, Dublin, Ireland; Centre for Integrative Physiology, School of Biomedical Sciences, University of Edinburgh, Edinburgh, Scotland, United Kingdom

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 amphiocic 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 isethionate 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-dehydroxymastasterol 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

Received 11 July 2012 Returned for modification 22 September 2012 Accepted 26 October 2012 Published ahead of print 31 October 2012 Address correspondence to Carmen María Martin-Navarro, cmmartin@ull.es. C.M.M.-N. and J.L.-M. contributed equally to this work. Copyright © 2013, American Society for Microbiology. All Rights Reserved. doi:10.1128/AAC.01426-12
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 AHJ101000000) 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).

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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).

FIG 2 Distribution of Acanthamoeba CLC-51.i 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.
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 (UGCUUCUACUUAGUGGUGUAAA) and ST-siRNA-2 (UCUUCAUGUAAAGGUGCUUGAA). 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 (UUUACAACCAGAUGAG UAGAAGCA) and GFP-siRNA-2 (UUUACAACCAGAUGAG UAGAAGCA), as previously described (33).

**Activity assays.** The anti-\textit{Acanthamoeba} activities of the assayed drugs were determined by the alamarBlue assay, as previously described (5, 34, 35). Briefly, \textit{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 \textit{A. castellanii} Neff with the previously calculated IC₅₀s and IC₉₀s 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 \textit{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

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**FIG 3** Light microscopy (magnification, ×20) images corresponding to the assay that was carried out with \textit{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).
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. 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 IC90 values were incubated in wells with 10^4 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](image_url) Effects of statins against cysts were evaluated by incubating 10^4 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 IC90s of statins in PYG medium.

<table>
<thead>
<tr>
<th>Statin</th>
<th>Mean concn (μM) at 96 h ± SD*</th>
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<tr>
<td></td>
<td>AcNeff</td>
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<tr>
<td>IC50</td>
<td>IC90</td>
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<tr>
<td>Atorvastatin</td>
<td>15.12</td>
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<tr>
<td>Fluvastatin</td>
<td>9.19</td>
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<tr>
<td>Lovastatin</td>
<td>17.14</td>
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<tr>
<td>Pravastatin</td>
<td>58.75</td>
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<tr>
<td>Simvastatin</td>
<td>10.24</td>
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*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$_{50}$ and IC$_{90}$ values were observed for atorvastatin (A) and simvastatin (C), with the exception of fluvastatin (B).

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$_{70}$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*-
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-
orum (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
vivo 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 (Consello General de Colegios Oficiales
de Farmaceúticos, 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 activity differs 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.

ACKNOWLEDGMENTS

This work was funded by the RICET project (project no. RD06/0021/0005
of programme Redes Temáticas de Investigación Cooperativa, FIS), Span-
ish Ministry of Health, Madrid, Spain; the Protozoosis Emergentes por
Amebas de Vida Libre: Aislamiento y Caracterización Molecular, Identifi-
cación de Cepas Transportadoras de Otros Agentes Patógenos y
Búsqueda de Quimioterapias project (project no. PI10/01298); and proj-
ect no. PI10/01240 from the Spanish Ministry of Science and Innovation,
Madrid, Spain. C.M.M.-N. was supported by a research technician con-
tract from the University of La Laguna (project no. RYC-2011-08863).
J.L.-M. was supported by the Ramón y Cajal Subprogramme from the
Spanish Ministry of Science and Innovation (grant RYC-2011-08863).
A.L.-A. was funded by the Ayudas del Programa de Formación de Per-
sonal Investigador para la realización de Tesis Doctorales grant from the
Agencia Canaria de Investigación, Innovación y Sociedad de la Infor-

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-
toxicity (the IC$_{90}$ 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$_{90}$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.
mación, from the Canary Islands Government. B.L. was funded by Science Foundation Ireland (grant 05/RPI/B908).

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