Statins and Voriconazole Induce Programmed Cell Death in *Acanthamoeba castellanii*

Carmen M. Martín-Navarro,^a Atteneri López-Arencibia,^a Ines Sifaoui,^b María Reyes-Batlle,^a Basilio Valladares,^a Enrique Martínez-Carretero,^a José E. Piñero,^a Sutherland K. Maciver,^c Jacob Lorenzo-Morales^a

University Institute of Tropical Diseases and Public Health of the Canary Islands, University of La Laguna, Canary Islands, Spain^a; Laboratoire Matériaux-Molécules et Applications, IPEST, La Marsa, University of Carthage, Tunis, Tunisia^b; Centre for Integrative Physiology, University of Edinburgh, Edinburgh, Scotland, United Kingdom^c

Members of the genus *Acanthamoeba* are facultative pathogens of humans, causing a sight-threatening keratitis and a life-threatening encephalitis. In order to treat those infections properly, it is necessary to target the treatment not only to the trophozoite but also to the cyst. Furthermore, it may be advantageous to avoid parasite killing by necrosis, which may induce local inflammation. We must also avoid toxicity of host tissue. Many drugs which target eukaryotes are known to induce programmed cell death (PCD), but this process is poorly characterized in *Acanthamoeba*. Here, we study the processes of programmed cell death in *Acanthamoeba*, induced by several drugs, such as statins and voriconazole. We tested atorvastatin, fluvastatin, simvastatin, and voriconazole at the 50% inhibitory concentrations (IC₅₀s) and IC₉₀s that we have previously established. In order to evaluate this phenomenon, we investigated the DNA fragmentation, one of the main characteristics of PCD, with quantitative and qualitative techniques. Also, the changes related to phosphatidylserine exposure on the external cell membrane and cell permeability were studied. Finally, because caspases are key to PCD pathways, caspase activity was evaluated in *Acanthamoeba*. All the drugs assayed in this study induced PCD in *Acanthamoeba*. To the best of our knowledge, this is the first study where PCD induced by drugs is described quantitatively and qualitatively in *Acanthamoeba*.

Free-living amoebae (FLA) of the genus *Acanthamoeba* are the causative agents of several opportunistic infections in humans, such as a sight-threatening ulceration of the cornea known as *Acanthamoeba* keratitis (AK), the usually fatal granulomatous amoebic encephalitis (GAE), and also disseminated infections (mostly cutaneous and nasopharyngeal) (1–4). Currently, the genus *Acanthamoeba* is classified into 19 different genotypes (T1 to T19) based on rRNA gene sequence analysis (5–14). The T4 genotype is the most common genotype related to amoebic infections, and also it is the most commonly isolated genotype from the environment (3, 15). Nevertheless, its association with pathogenicity is not explained by its apparent abundance alone (16).

Acanthamoeba species exist in a trophozoite stage (the metabolically active stage) and a cyst stage that is characterized by the presence of a highly resistant double cyst wall (2). Recurrent amoebic infections are a complication of the presence of *Acanthamoeba* cysts, as they are able to survive the currently available treatments (17, 18). Diamidines (propamidine and hexamidine) and biguanides (chlorhexidine and polyhexamethylene biguanide [PHMB]) have been tested against *Acanthamoeba* trophozoites and cysts (3, 4, 19, 20) with successful outcomes. However, it has been reported that about 5% of patients with AK present persistent inflammation due to viable *Acanthamoeba* in the cornea, even after prolonged treatment with these molecules (21). Recently, new approaches have been tested against *Acanthamoeba*. Statins are a family of lipid-lowering drugs widely used to control cholesterol levels in humans. Their mechanism of action is the inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase by binding to the active site of this enzyme in *Acanthamoeba* (22). The enzyme converts HMG-CoA to mevalonate, a precursor of cholesterol in humans and ergosterol in plants, fungi, and protozoa (23, 24). In *Acanthamoeba*, ergosterol and 7-dehyrostigmasterol have been reported to be the main sterols of the membrane in both the trophozoite and the cyst stages (25–28). The *in vitro*

activities of three statins (atorvastatin, fluvastatin, and simvastatin) against *Acanthamoeba* have been demonstrated, showing a 50% inhibitory concentration (IC₅₀) range between 9 and 58 μ M (22). In addition, antifungal agents, such as voriconazole, have been recently demonstrated to be highly active against clinical strains of *Acanthamoeba* (29–31).

Necrosis is characterized by a series of morphological changes, such as increased cellular volume and rupture of the cytoplasmic membrane early in the process, which results in the release of the cellular content and induction of an inflammatory response in the host (32). Therefore, it is important to avoid the use of drugs which could induce a necrotic process in *Acanthamoeba* therapy, since these necrotic cells could be the cause of an inflammation process in the cornea or other infected organs.

Induction of programmed cell death (PCD) in parasites for drug development is a novel possibility that has been explored in great detail in several unicellular parasitic protozoa (33) but has not been evaluated so far against the *Acanthamoeba* genus. PCD is a very complex type of genetically controlled death. Moreover, the morphological features that define this type of death occur in different stages. First, cell dehydration causes changes in cellular

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Address correspondence to Carmen M. Martín-Navarro, cmmartin@ull.es.

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shape and size. Another characteristic event of this process is the condensation of nuclear chromatin. Importantly, the structural integrity and most of the functions of the cell membrane remain intact at least in the initial stages of the process. After this, certain changes start to appear, such as a lack of phospholipid asymmetry and exposure of phosphatidylserine (PS) on the cell exterior, which labels these cells as a target for phagocytic cells (34, 35). Most of these morphological changes are the results of the activity of caspases. These proteins are highly conserved cysteine proteases specific to aspartic acid (36). Other members of this family of proteins with homologous activities, such as paracaspases and metacaspases, have been commonly reported in unicellular organisms (37).

PCD in unicellular organisms has been reported so far in yeast, *Dictyostelium discoideum*, *Peridinium gatuense*, *Euglena gracilis*, *Tetrahymena thermophila*, trypanosomatids like *Trypanosoma* and *Leishmania*, *Plasmodium*, *Blastocystis hominis*, and *Entamoeba histolytica* (33, 38–41). In these organisms, these apoptotic processes occur as a phenomenon that presumably benefits the rest of the population in some way. Kin selection may limit the spread of amoebic parasites through colonies or under conditions where limited nutrients may mean the survival of some cells at the expense of others (33, 40–42) or cell damage, such as that induced by chemotherapy (41). The fact that there is evidence that apoptotic processes exist in protozoan parasites has provided new strategies for the development of tools in the study of these diseases, comparing these processes to the one in humans (42).

At present, the literature on the existence of apoptosis in *Acanthamoeba* is limited. A type 1 metacaspase has been reported in *Acanthamoeba*, although its function seems to be more related to the encystation process and with osmoregulation than to apoptosis (43, 44). However, it is known that not all members of the caspase family are involved in PCD (43–46). Evidence for PCD in *Acanthamoeba* infected with *Salmonella* has been observed (47), although the specific pathways and the molecules involved are still unknown.

MATERIALS AND METHODS

Acanthamoeba strains. The *Acanthamoeba castellanii* Neff (ATCC 30010, genotype T4) type strain was used in this study. This strain was grown axenically in PYG medium (0.75% [wt/vol] proteose peptone, 0.75% [wt/vol] yeast extract, and 1.5% [wt/vol] glucose) containing 40 µg/ml gentamicin (Biochrom AG, Cultek, Granollers, Barcelona, Spain) at room temperature.

Chemicals. Four drugs were selected for the different experiments: atorvastatin (Sigma-Aldrich Chemistry Ltd., Madrid, Spain) and simvastatin (Merck Chemical Spain Ltd., Barcelona, Spain), which are lipophilic statins; fluvastatin (Enzo Life Sciences Inc., Farmingdale, NY, USA), which is a hydrophilic statin; and voriconazole (Fluka; Sigma-Aldrich Chemistry Ltd., Madrid, Spain).

Their activity against *Acanthamoeba* strains has been demonstrated in previous studies from our laboratory (22, 30). Briefly, the IC₅₀s obtained in *Acanthamoeba castellanii* Neff for atorvastatin, fluvastatin, simvastatin, and voriconazole were 15.12 ± 2.19, 9.19 ± 0.98, 10.24 ± 1.09, and 13.14 ± 0.69 µM, respectively. In addition, the IC₉₀s obtained in the same strain for atorvastatin, fluvastatin, simvastatin, and voriconazole were 41.09 ± 0.01, 20.70 ± 2.15, 21.37 ± 1.51, and 30.43 ± 1.32 µM, respectively.

Cellular DNA fragmentation. A cellular DNA fragmentation enzyme-linked immunosorbent assay (ELISA) kit (Roche) was used. This kit is an ELISA for the detection of BrdU-labeled DNA fragments in culture supernatants and cellular lysates.

The procedure for characterization of cell death consists of two parts:

(i) analysis of the supernatant, which will contain DNA fragments at early stages of necrosis and late stages of apoptosis, and (ii) lysing of the remaining cells in order to release apoptotic DNA fragments located in the cytoplasm. The experiment was carried out by following the manufacturer's recommendations. Briefly, 10⁵ cells/ml were incubated with the BrdU labeling solution overnight at 37°C. After that, the culture was washed and cells were resuspended in BrdU-free culture medium. First, 10⁵ BrdU-labeled amoebae/well were seeded in a 96-well microtiter plate, and they were incubated with each of the drugs mentioned above at the previously calculated IC₅₀ and IC₉₀ values. After the incubation time, the plate was centrifuged, and 100 µl of supernatant was removed in order to be analyzed by ELISA.

Second, the remaining supernatant was discarded and an incubation solution was added in order to lyse the cells for 30 min. After the incubation time, the plate was centrifuged, and 100 µl of supernatant was removed in order to be analyzed by ELISA.

Results were statistically analyzed and compared with a control using the Sigma Plot 12.0 software program (Systat Software Inc., London, United Kingdom) with a one-way analysis of variance (ANOVA) test and a multiple *post hoc* Tukey tests. These experiments were carried out in triplicate and at different time points (8, 24, and 48 h).

TUNEL assay. The terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) apoptotic detection kit (GenScript, Piscataway, NJ, USA) was used by following the manufacturer's recommendations. Briefly, the experiment was carried out in a 6-well plate in which 10⁵ amoebae/well were incubated with the IC₅₀ and IC₉₀ values previously calculated for each drug. After 24 h, cells were collected and fixed on a slide using a paraformaldehyde (4%) fixative solution prior to TUNEL assay. This system relies on the binding of terminal deoxynucleotidyl transferase (TdT) to the end of DNA fragments, which are formed after apoptosis fragmentation. To this enzyme, a conjugate of streptavidin and peroxidase (streptavidin-horseradish peroxidase [HRP]) is bound, converting the substrate, a mixture of hydrogen peroxide and 3,3'-diaminobenzidine (DAB), in a brown precipitate. This precipitate can be detected by light microscopy, identifying apoptotic nuclei with a dark-brown color.

Double-stain assay for apoptosis determination. A double-stain apoptosis detection kit (Hoechst 33342/PI) (GenScript, Piscataway, NJ, USA) and an inverted confocal microscope (Leica DMI 4000B) were used. The experiment was carried out by following the manufacturer's recommendations, and thus 10⁵ cells/well were incubated in a 24-well plate for 24 h with the previously calculated IC₅₀. The double-staining pattern allows the identification of three groups in a cellular population: live cells will show only a low level of fluorescence, apoptotic cells will show a higher level of blue fluorescence, and dead cells will show low-blue and high-red fluorescence.

Plasma membrane permeability. Sytox green nucleic acid stain (Invitrogen, Life Technologies SA, Madrid, Spain) is a high-affinity nucleic acid stain (absorption and emission maxima at 504 and 523 nm, respectively) that renders cells with compromised plasma membranes bright-green fluorescent.

The experiment was carried out by following the manufacturer's recommendations. Briefly, 10⁵ cells/ml were seeded in a black-wall 96-well microtiter plate (Nunc; Thermo Fisher Scientific Inc., Massachusetts, USA) with the previously calculated IC₉₀s of each active principle (a negative control and a positive control with 2.5% of Triton X-100 [Sigma] were added in order to obtain fully permeabilized cells). Measurement was performed by using an EnSpire microplate reader (PerkinElmer) every 10 min for 3 h, after which samples of amoebae were removed for fluorescence microscopy analysis. A Student *t* test analysis was done in order to compare each point with the negative control.

Image-based cytometry analysis for apoptosis determination. The Tali apoptosis kit (Invitrogen, Life Technologies SA, Madrid, Spain) enables identification of apoptotic cells and discrimination of apoptotic from necrotic and live cells in a population. The kit stains apoptotic cells

FIG 1 Amount of DNA detected over time in the culture supernatant and cell lysate (absorbance versus time). (A) Atorvastatin; (B) fluvastatin; (C) simvastatin; (D) voriconazole. Statistical differences (*, $P < 0.05$; ***, $P < 0.001$) are shown, comparing results obtained for supernatants (filled symbols) and cell lysates (empty symbols). IC_{50} s (circles) for atorvastatin, fluvastatin, simvastatin, and voriconazole: 15.12 ± 2.19 , 9.19 ± 0.98 , 10.24 ± 1.09 , and 13.14 ± 0.69 μ M, respectively; IC_{90} s (triangles) for atorvastatin, fluvastatin, simvastatin, and voriconazole: 41.09 ± 0.01 , 20.70 ± 2.15 , 21.37 ± 1.51 , and 30.43 ± 1.32 μ M, respectively.

with green annexin V-Alexa Fluor 488, stains necrotic cells with both red propidium iodide and green annexin V-Alexa Fluor 488, and does not stain live cells. Annexin V showed high affinity with phosphatidylserine (PS). In normal viable cells, PS is located on the cytoplasmic surface of the cell membrane. However, in the intermediate stages of apoptosis, PS is translocated from the inner to the outer side of the membrane so it can be detected using annexin V.

The experiment was carried out by following the manufacturer's recommendations: 10^5 cells/well were incubated on a 24-well plate for 24 h with the drug concentrations previously calculated. After this time, cells were recovered and incubated with the dyes. Finally, 25 μ l of stained cells was loaded into a Tali cellular analysis slide and analyzed using the Tali image-based cytometer (Invitrogen). In order to differentiate dead cells from apoptotic cells, statistical analyses (t test) comparing both cell population types were carried out.

Caspase-like activity detection. A caspase-3 colorimetric assay kit (GenScript, Piscataway, NJ, USA) was used by following the manufacturer's recommendations. The experiment was carried out in a 24-well plate with 10^5 cells/ml incubated with the previously calculated IC_{50} and IC_{90} values. Results were statistically analyzed and compared with a control using the Sigma Plot 12.0 software program (Systat Software Inc., London, United Kingdom) with a one-way ANOVA test and multiple *post hoc* Tukey tests. The experiment was done in triplicate and at different time points (2, 8, 12, 24, 48, and 72 h).

RESULTS

Atorvastatin, fluvastatin, simvastatin, and voriconazole induce cellular DNA fragmentation in *Acanthamoeba castellanii* Neff. When amoebae were treated with the IC_{50} and IC_{90} values of atorvastatin, fluvastatin, simvastatin, and voriconazole, a larger amount of DNA was observed in the cell lysate than in the supernatant (Fig. 1). Therefore, a larger amount of intracellular DNA was detected in all cases, with significant differences between the detected DNA in the lysate and the supernatant, regardless of the concentration tested. Moreover, it is interesting to mention that in the case of atorvastatin (Fig. 1A) and voriconazole (Fig. 1D), significant differences between the used concentrations were also observed.

The TUNEL assay confirmed the observed results using the ELISA kit, since all tested compounds caused the appearance of brown precipitate in the nucleus (Fig. 2).

Atorvastatin-, fluvastatin-, simvastatin-, and voriconazole-treated cells stained positive in the double-stain assay. When double staining was performed, all statins investigated caused nuclei staining with Hoechst, demonstrating the presence of condensed chromatin (Fig. 3). Moreover, the differences between the three cell populations were clear, and thus live cells were detected under fluorescence microscopy, as they showed faint-blue nuclei, whereas cells displaying PCD presented bright-blue nuclei due to karyopyknosis and chromatin condensation. The nuclei of cells that were dead did not stain.

Atorvastatin, fluvastatin, and simvastatin caused plasma membrane permeability in treated cells. Amoebae treated with atorvastatin, fluvastatin, and simvastatin induced cellular membrane damage after 1 h of treatment. In the case of voriconazole-treated cells, no statistical differences compared to the negative control were observed. None of the tested products induced the same level of fluorescence observed in the positive control (Fig. 4F). Nevertheless, cellular membrane disruption was checked and confirmed using fluorescence microscopy in statin-treated cells (Fig. 4A to E).

Amoebae treated with atorvastatin, fluvastatin, simvastatin, and voriconazole showed signs of early PCD. *Acanthamoeba castellanii* Neff treated with the four compounds showed externalization of PS (Fig. 5). Therefore, early stages of apoptosis in the treated *Acanthamoeba* cells were demonstrated. In the case of the negative control, only a low degree of fluorescence was observed (Fig. 5A). However, when the statins were added, an increase of the fluorescence caused by annexin V binding to PS was detected (Fig. 5B and C). Moreover, the statistical analyses showed significant differences in the percentage of detected dead and PCD cells after treatment with all of the statins. Furthermore, there were statistically significant differences between the controls and the treated cellular populations (Fig. 5D).

***Acanthamoeba* caspase-3 was activated after treatment with all the tested compounds.** A significant caspase-3-like activity was detected in simvastatin-treated cells after 2 h of treatment (Fig. 6C). Regarding atorvastatin-, fluvastatin-, and voriconazole-treated cells, significant activation of caspase-3 was detected after 24 h (Fig. 6A, B, and D). Furthermore, no significant differences were found between the two tested concentrations in the case of atorvastatin and fluvastatin. In the case of simvastatin and voriconazole, no significant differences were observed between both concentrations at 24 and 48 h. Moreover, the activity of this protein was dose dependent, and thus it showed higher activity when the IC_{90} s of each of the tested compounds were used.

DISCUSSION

It is known that the statins and voriconazole tested in this work induce apoptosis in various mammalian cell lines. Previous investigations have confirmed that in endothelial cells, simvastatin and atorvastatin induced apoptosis through the mitochondrial pathway (48, 49). Assays on endothelial cells from the pulmonary veins of mice showed that lovastatin, simvastatin, atorvastatin, fluvastatin, and cerivastatin induced apoptosis, whereas pravastatin did not (50). In myeloma and leukemia cells, pravastatin did not induce apoptosis, unlike lovastatin, simvastatin, atorvastatin, and cerivastatin, which induce apoptosis via the mitochondrial pathway (51). In prostate cancer cells, the two apoptotic pathways are

FIG 2 TUNEL assay images ($\times 20$). Differences from the negative control can be observed in the rest of the images, showing a brown precipitate in the nuclei. (A) Negative control; (B) positive control; (C) cells treated with atorvastatin (IC_{50}); (D) cells treated with voriconazole (IC_{50}).

activated by the action of lovastatin and simvastatin, although it seems that the main route is the extrinsic one (52). In cholangiocarcinoma cells treated with pitavastatin and atorvastatin, apoptotic processes by the pathway dependent on mitogen-activated

protein kinase (MAPKs) have also been identified (53). In breast cancer cells, some studies have detected the potential antiproliferative effects by statins inducing apoptosis (54). Finally, in lymphoma cells, it has been observed that statins induce apoptosis by promoting reactive oxygen species (ROS) generation and regulating AKT, Erk, and p38 signals (55). Voriconazole has been associated with apoptotic events in corneal endothelial cells, although the activation of this pathway is unknown (56). Regarding protists, it has been demonstrated that atorvastatin and others drugs that interfere with the sterol biosynthetic pathway induce PCD in trypanosomatids (57), and voriconazole induces PCD in *Candida* spp. (58).

Since caspase-3 is a central protein in apoptosis pathways in higher eukaryotes, it can be activated via both the mitochondrial pathway and death receptors. This is an effector caspase, with direct action on DNA fragmentation, chromatin condensation, and membrane disruption. Other members of the family of caspases are the metacaspases and paracaspases; the latter have been found in plants, fungi, and protozoa. The function of metacaspases and paracaspases is related not only to cell death but also to sporulation, embryogenesis, and osmoregulation (43, 44, 59–62). Two types of metacaspases have been identified, 1 and 2. They differ in their protein domains: the type 1 metacaspases have different N-terminal extensions (often rich in proline) and a core proteolytic domain, while the type 2 metacaspases have a proteolytic N-terminal domain and C-terminal regions variable in both

FIG 3 Hoechst staining is different in control cells, where uniformly faint-blue nuclei are observed, and in treated cells, where the nuclei are bright blue. (A to E) Phase contrast: control (24 h) (A), atorvastatin (16 h) (B), fluvastatin (16 h) (C), simvastatin (24 h) (D), voriconazole (24 h) (E). (F to J) Hoechst channel: control (24 h) (F), atorvastatin (16 h) (G), fluvastatin (16 h) (H), simvastatin (24 h) (I), voriconazole (24 h) (J). (K to O) Propidium iodide channel: control (24 h) (K), atorvastatin (16 h) (L), fluvastatin (16 h) (M), simvastatin (24 h) (N), voriconazole (24 h) (O). Scale bars are 25 μm .

FIG 4 Permeabilization of the cellular membrane. Fluorescence from the Sytox green nucleic acid stain can be observed when cells were treated with the different treatments after 2 h. (A) Control; (B) atorvastatin; (C) fluvastatin; (D) simvastatin; (E) voriconazole; (F) differences between control (addition of Triton X-100) and the drug-treated cells were apparent when fluorescence of the cells was measured. Statistical differences (**, $P < 0.01$; ***, $P < 0.001$) are shown, comparing results obtained from the negative control and the different treatments. There is not a statistical difference between the control and the voriconazole treatment. Scale bars are 25 μm.

FIG 5 Image-based cytometer analysis for apoptosis determination (24 h). (A) Control cells; (B) atorvastatin IC₅₀ treatment; (C) voriconazole IC₅₀ treatment; (D) histogram where cells and treatments are compared. Results are represented in percentages, and statistical differences (**, $P < 0.01$; ***, $P < 0.001$) are shown, comparing dead cells and apoptotic cells after treatments with the control.

FIG 6 Caspase-like activity (2 to 72 h) (absorbance versus time). Statistical differences (*, $P < 0.001$; **, $P < 0.01$; ***, $P < 0.05$) are shown, comparing the control with the different concentrations. (A) Atorvastatin; (B) fluvastatin; (C) simvastatin; (D) voriconazole.

length and sequence (37). A type 1 metacaspase has been identified in *Acanthamoeba*. The specificity of this protease has not yet been determined, but its function is related to encystment (43), and activity relating to the osmoregulation processes has been inferred (44).

The experiment carried out in this work in order to study the presence of caspase-like protease detected the activity of cleaving peptides containing the sequence DEVD (Asp-Glu-Val-Asp), which is cleaved by caspase-3 during apoptosis. However, known metacaspases are Arg and Lys specific (63). Moreover, a metacaspase gene thought to be a metacaspase-2 has also been identified in the *Acanthamoeba* genome (64), and its substrate specificity has yet to be established. It is possible that this (or other metacaspases) is responsible for the caspase-3-like activity that we described in this organism for the first time. Future studies will clarify the role of this caspase-3-like activity.

Evidence of apoptosis in *Acanthamoeba* (T4) has been reported 6 h after it has been infected with *Salmonella enterica* serovar Typhimurium. Confirmation of this process was performed by TUNEL assay and flow cytometry (47). Recently, evidence of apoptosis has been described by morphological studies of *Acanthamoeba* species (clinical isolate, T4) when they were treated with methanol extracts of marine sponges (*Aaptos* species). The authors distinguish between early apoptotic cells, late apoptotic cells, and necrotic cells by observing chromatin condensation (65). Furthermore, because this process is produced by methanolic extracts, it is necessary to note that in *Acanthamoeba*, a stress response different from encystment has been described. This phenomenon occurs when amoebae are subjected to low concentrations of methanol, acetone, or dimethyl sulfoxide (DMSO) (organic solvents). This cell stage is called pseudocyst, with a wall that does not protect against other adverse conditions (66). Both phenomena described here could be the same if we consider that the description of apoptosis in the first case was performed only by morphology (65).

In agreement with our findings, others have reported DNA fragmentation detected using the TUNEL assay with *Acanthamoeba* treated with actinomycin K (67). Together with the qualitative and quantitative data presented here, these previous reports

suggest the existence of PCD in *Acanthamoeba*. We aim to further investigate these pathways as targets for therapy against this facultative pathogen.

In order to treat infections caused by *Acanthamoeba*, it is desirable to find agents which induce apoptotic rather than necrotic cell death to minimize the host inflammatory response (68). Our work suggests that statins and voriconazole kill *Acanthamoeba* by nonnecrotic mechanisms, making it a promising drug to combat *Acanthamoeba* infections. We note that statins are a widely prescribed and tolerated group (69), while voriconazole has already been successfully applied as eye drops to treat fungal infections (70).

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