Chloroquine Has a Cytotoxic Effect on *Acanthamoeba* Encystation through Modulation of Autophagy

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Encystation of *Acanthamoeba castellanii* is associated with resistance to chemotherapeutic agents. Blocking the encystation process could potentiate the efficacy of chemotherapeutic agents and biocides. During encystation, autophagy is highly stimulated and required for proper encystation of *Acanthamoeba*. In this study, the cytotoxic effect of chloroquine, a well-known autophagy-inhibitory drug, was tested in *A. castellanii*. Chloroquine was able to selectively reduce cell survival during the encystation of *A. castellanii*. However, *A. castellanii* trophozoites and mature cysts were resistant to chloroquine. Chloroquine treatment led to an increase in the number and size of lysosomes in encysting cells. Moreover, chloroquine inhibited the degradation of long-lived proteins in the encysting cells. Decreased autophagic flux, indicated by an increased number of lysosomes and decreased degradation of long-lived proteins, may be the mechanism by which cell death is induced by chloroquine in encysting *Acanthamoeba*. These results suggest a potential novel therapeutic application of chloroquine as an anti-*Acanthamoeba* drug. Our findings also suggest that targeting autophagy could be a therapeutic strategy against *Acanthamoeba* infection.

*Acanthamoeba* are free-living opportunistic amoebae that cause life-threatening infections such as granulomatous amoebic encephalitis (GAE) and amoebic keratitis (1). Monotherapy with biguanide or combination therapy with diamidine shows good therapeutic efficacy against *Acanthamoeba* (2, 3). Combination therapy with voriconazole and miltefosine has been recently used to treat GAE (4). Caspofungin and corfugin have also been proven to be effective against *Acanthamoeba* (5, 6). Despite advances in chemotherapy against *Acanthamoeba*, the emergence of resistance to chemotherapeutic drugs and biocides against *Acanthamoeba* infection contributes to the major problem of treatment (7–9).

The life cycle of *Acanthamoeba* consists of a vegetative trophozoite(s) and a dormant cyst stage. Under stress conditions, including the host immune response and chemical treatment, trophozoites can detect the stress and convert to double-walled cysts in a process called encystation (10). Once encystation is initiated, the synthesis of the cyst wall begins and the organisms become highly resistant to stressful environments. The encysted stage renders *Acanthamoeba* resistant to most biocide treatments (11). Thus, chemical agents inhibiting the encystation mechanism could potentiate the therapeutic efficacy of antiamoebic drugs and could be used in conjunction with other therapies.

The expression profile of genes in trophozoites and cysts has been studied, and a subset of genes related to autophagy is highly expressed during *Acanthamoeba* encystation (12). Moreover, recent studies have shown that autophagy has important roles in the *Acanthamoeba* encystation process (13, 14). Autophagy is a conserved catabolic process involving lysosomes wherein various cellular components, including organelles, are degraded by lysosomal hydrolases (15, 16).

Chloroquine has been used to treat amoebic liver abscess, malaria, and inflammatory diseases (17–20). The mechanism of chloroquine in amoebic liver abscess has not been well identified. However, it has been suggested that chloroquine is accumulated in a high concentration in the liver (19). Chloroquine is a weak lysosomotropic base, and its mechanism includes an increase in intralysosomal pH, which prevents fusion of lysosomes and autophagosomes, and disruption of intracellular trafficking of lysosomes (21–24). As autophagy is highly stimulated during *Acanthamoeba* encystation, and chloroquine inhibits autophagy, this led us to speculate that chloroquine could possess a therapeutic role in the treatment of *Acanthamoeba* by blocking the processes in the autophagic pathway.

The aim of the present study was to evaluate the effects of chloroquine against *Acanthamoeba*, targeting the encystation process. Here, we report that chloroquine effectively reduced cell survival during the encystation process and could be used to treat *Acanthamoeba* infection and for biocide purposes. Our results provide a new approach for screening different therapeutic drugs against encystation of *Acanthamoeba* and other protozoan parasites.

**MATERIALS AND METHODS**

*Acanthamoeba castellanii* culture. *A. castellanii* was obtained from the American Type Culture Collection (ATCC 30011; Manassas, VA) and cultured axenically in peptone-yeast extract-glucose (PYG) medium [20 g/liter proteose peptone, 1 g/liter yeast extract, 0.1 M glucose, 4 mM MgSO₄·7H₂O, 0.4 mM CaCl₂, 3.4 mM sodium citrate, 0.05 mM Fe(NH₄)₂(SO₄)₂, and 2.5 mM (each) Na₂HPO₄ and KH₂PO₄] at 25°C. The final pH of the medium was adjusted to 6.5.

**Encystation induction.** Encystation was induced as described by Bowers and Korn (25), with slight modification. In brief, cells from post-logarithmic-growth-phase cultures were collected aseptically, washed...
once with phosphate-buffered saline (PBS), and incubated at 25°C in encystation medium containing 95 mM NaCl, 5 mM KCl, 8 mM MgSO₄, 0.4 mM CaCl₂, 1 mM NaHCO₃, and 20 mM Tris-HCl (pH 9.0) (26).

**Chloroquine treatment.** Chloroquine diphosphate salt was purchased from the Sigma-Aldrich Co. (St. Louis, MO). The stock solution of (27). To determine the effect of chloroquine on trophozoites, A. castellanii trophozoites (4 × 10⁶ cells/well) were inoculated in a 6-well plate containing 3 ml PYG media. The next day, cells were washed once with PBS and treated with PYG media containing 25, 50, and 100 μM concentrations of chloroquine. The cells were incubated for 24 h at 25°C and observed for amoebicidal activity using trypan blue (0.2% final concentration) staining (28). To observe the effect of chloroquine during the encystation process, trophozoites were washed once with PBS and incubated in encystation media containing chloroquine (25, 50, 60, 70, 80, 90, and 100 μM) at 25°C. To determine the effect of chloroquine on mature cysts, trophozoites were incubated on a non-nutrient 1.5% agar plate for 7 days at 25°C, to allow the transformation of trophozoites into the cyst stage. After this incubation, each plate was flooded with 20 ml PBS, and the cells were collected and then centrifuged at 1,500 for 15 min and treated with sodium dodecyl sulfate (SDS) (0.5% final concentration) for 10 min. The SDS solubilizes trophozoites and the immature cyst. The mature cyst(s) is resistant to 0.5% SDS, and it remains intact (29). Mature cysts were washed five times with PBS to remove the residual SDS, and 5 × 10⁵ mature cysts were inoculated into a 6-well plate containing encystation media with 25, 50, and 100 μM chloroquine. The cells were incubated for 24 h, and cell viability was calculated using trypan blue staining.

**Flow cytometric analysis of lysosomes.** Lysosomal accumulation inside the cells was determined by flow cytometry after cells were stained with LysoTracker Red DND-99 (Molecular Probes/Invitrogen, Carlsbad, CA). Briefly, 4 × 10⁶ trophozoites were cultured in a 6-well plate. After 24 h of incubation at 25°C, cells were treated with encystation medium containing chloroquine (100 μM) for 4 h. Since long incubations with chloroquine cause significant intracellular changes and cell death, an incubation time of 4 h was selected for treatment to observe internal lysosomal accumulation. The cells were centrifuged at 1,000 × g for 5 min, washed twice with 1 ml PBS in an Eppendorf tube, resuspended in PBS, and stained with LysoTracker Red DND-99 at a final concentration of 2.5 μM. The mixture was incubated in the dark for 30 min at 25°C. Stained cells were washed once with PBS and transferred to a Falcon fluorescence-activated cell sorter (FACS) tube and analyzed on a BD FACSCanto II flow cytometer. The data were analyzed with BD FACSDiva software 6.0 (BD Biosciences, San Diego, CA).

**Confocal microscopy.** LysoTracker Red DND-99 was used at a final concentration of 2.5 μM to assess lysosomal number and morphology. Cultured trophozoites (4 × 10⁶) were treated with encystation medium containing chloroquine (100 μM) for 4 h. The cells were centrifuged at 1,000 × g for 5 min, washed twice with 1 ml PBS in an Eppendorf tube, resuspended in PBS, and stained with LysoTracker Red DND-99. The mixture was incubated in the dark for 30 min at 25°C. The stained cells were washed twice with PBS and observed using a Zeiss 510 laser scanning confocal microscope (Oberkochen, Germany). Colocalization images were obtained with LSM software (Carl Zeiss, Germany).

**Long-lived-protein-degradation assay.** Degradation of long-lived protein was measured as described previously, with a slight modification (30). Briefly, the cells were plated at 10⁶ cells/well in 24-well plates containing PYG media for 24 h. The next day, the cells were labeled with a radioactive medium containing [¹⁴C]leucine (0.5 μCi/ml) (PerkinElmer, Waltham, MA) for 24 h. The radioactive medium was removed, and the cells were incubated with PYG medium containing 2 mM unlabeled leucine for 2 h to wash out the short-lived radiolabeled proteins, which are primarily degraded by the proteasome. The cells were washed once with PBS and treated with encystation media containing chloroquine (25 μM and 50 μM) for 4 h. The culture supernatants were obtained, and a 1/10 volume of 100% trichloroacetic acid (TCA) was added to each supernatant to measure radioactivity. The samples were centrifuged at 15,000 × g for 10 min, and acid-soluble radioactivity was measured by the use of a liquid scintillation counter (LSC) (model LSC6500; Beckman, Fullerton, CA). The cells were washed with PBS, and 1 ml cold 10% TCA was added to each culture well for 5 min to fix the cellular proteins and to measure radioactivity. The TCA was removed, and the fixed cells were solubilized with 1 ml of 1 N NaOH. Radioactivity in the solubilized protein was determined by the use of an LSC. The rate of degradation of long-lived radioactive proteins was calculated according to a procedure published previously (31).

**Statistical analysis.** The data were expressed as means ± standard deviations (SD). Statistical analysis was performed by using Student’s (two-tailed) t-test. A P value of <0.05 was considered statistically significant.

**RESULTS**

**Chloroquine increases cell death during encystation.** Previous reports of cyst-specific gene expression profiles suggest that autophagy genes are highly expressed and have crucial roles during encystation of A. castellanii (13, 14, 32). Accordingly, we speculated that inhibiting the autophagic process could block encystation of Acanthamoeba, ultimately leading to decreased cell viability. Thus, we have selected chloroquine as a candidate drug, as it is a well-established antimalarial drug and has autophagy-inhibitory activity that operates by blocking autophagosome-lysosome fusion (33). After 24 h of incubation of trophozoites in chloroquine-containing encystation media, we measured cell viability using trypan blue staining. Extensive loss of cell viability was observed at 80, 90, and 100 μM chloroquine (5% viability with 80 μM chloroquine) (Fig. 1A), suggesting that chloroquine was able to induce a dose-dependent decrease in cell viability during the Acanthamoeba encystation stage. To check the relative cytotoxicity of chloroquine, we measured the cytotoxic effects on mammalian cells. As Acanthamoeba causes granulomatous amoebic encephalitis, we selected SH-SYSY, a human neuroblastoma cell line, for the evaluation of mammalian cell cytotoxicity. The cytotoxicity of chloroquine was found to be significantly lower in SH-SYSY cells than in the encysting Acanthamoeba (see Fig. S4 in supplemental material). The 50% inhibitory concentrations (IC₅₀) for SH-SYSY and the encysting Acanthamoeba were 116 μM and 65 μM, respectively (data not shown).

It has been demonstrated that trypan blue can enter the viable cells through a toxin-induced increase in membrane permeability (34). To test whether the chloroquine-induced trypan blue staining was due to the membrane permeabilization rather than to cell death, we performed trypan blue staining after further incubation of chloroquine-treated cells in PYG media alone. We treated trophozoites (4 × 10⁶ cells/well) with encystation media containing chloroquine (25 μM to 100 μM) for 24 h. After this incubation at 25°C, the cells were centrifuged at 1,500 × g for 10 min and were washed three times with PBS to remove extracellular chloroquine. Finally, the cells were resuspended in PYG media (3 ml/well) and incubated in 6-well plates for 24 h at 25°C under the assumption that further incubation in chloroquine-free PYG media could cause the Acanthamoeba to recover from membrane permeabilization. We found that the proportion of trypan blue-stained cells was significantly increased even after the incubation of chloroquine-treated cells in chloroquine-free PYG media (86% trypan...
Cytotoxic Effect of Chloroquine on Acanthamoeba

Chloroquine decreased cell viability during encystation. Chloroquine was used to treat encysting cells (A), trophozoites (B), and mature cysts (C) as described in Materials and Methods. After 24 h of incubation, cell viability was determined by trypan blue staining. Data are means ± standard deviations (SDs) of the results from three separate experiments. *, P < 0.05; **, P < 0.005 (compared with untreated control cells).

![Graphs showing cell viability effects of chloroquine on encysting cells, trophozoites, and mature cysts](http://aac.asm.org/)

- **Encysting cell**
  - Control: 9 ± 1
  - Chloroquine: 25, 50, 60, 70, 80, 90, 100 µM
- **Trophozoite**
  - Control: 25 ± 2
  - Chloroquine: 25, 50, 70, 80, 100 µM
- **Mature cyst**
  - Control: 6 ± 1
  - Chloroquine: 25, 50, 70, 80, 100 µM

blue-stained cells at 60 µM chloroquine) (Fig. 2A). These data suggest that the chloroquine-induced staining of trypan blue was due to cell death rather than to an increase in cell membrane permeability. Moreover, further incubation in PYG media decreased cell viability beyond the rate shown in Fig. 1A (i.e., the rate decreased from 84.6% to 13.9% at 60 µM concentration). This result could have been due to a longer incubation time along with accumulated chloroquine inside the cells. In parallel, we observed cellular detachment and a significant number of damaged cells starting at the 60 µM concentration of chloroquine treatment even after incubation in chloroquine-free PYG media (Fig. 2B). Taken together, these results suggest that chloroquine decreases cell viability during encystation of *A. castellanii*.

**Trophozoites and mature cysts are resistant to chloroquine.** We tested the effect of chloroquine in trophozoites and mature cysts to confirm whether chloroquine specifically causes death of encysting cells. First, the effect of chloroquine on the *Acanthamoeba* trophozoite stage was tested. Trophozoites in PYG media were treated with different concentrations of chloroquine over 24 h, and cell viability was measured by trypan blue staining. Chloroquine had no effect on the viability of *Acanthamoeba* trophozoites (Fig. 1B). Moreover, chloroquine did not induce encystation even at a higher concentration (data not shown). These data suggest that chloroquine has no effect on cell viability or induction of encystation of the *Acanthamoeba* trophozoites. We again examined whether chloroquine can change the viability of *Acanthamoeba* trophozoites when the trophozoites are grown by the use of a heat-inactivated *Escherichia coli* agar plate instead of PYG media. However, chloroquine did not change the viability of *Acanthamoeba* trophozoites cultured using a heat-inactivated *E. coli* agar plate (see Fig. S3 in the supplemental material). FIG. 1C, no significant death of mature cysts was observed during chloroquine treatment (98% viability with 100 µM chloroquine).

Again, we evaluated a cytotoxicity assay for the mature cyst which was prepared in encystation medium. As expected, chloroquine did not show any cytotoxic effect on the mature cyst (see Fig. S1 in the supplemental material). From these results, we concluded that chloroquine is effective specifically during encystation and causes cell death in *Acanthamoeba*. To confirm the specific cytotoxicity of chloroquine for encysting cells, we tested the effects of chloroquine in another strain of *Acanthamoeba* (ATCC 50492). Chloroquine at concentrations of 70, 80, 90, and 100 µM showed a significant cytotoxic effect specifically on the encysting stage, whereas trophozoites and mature cysts remained unaffected (see Fig. S2 in the supplemental material).

**Chloroquine treatment results in the accumulation of lysosomes in encysting cells.** Several reports have suggested that chloroquine disrupts lysosomal structure and function, leading to the inhibition of autophagic flux (35–37). As autophagy plays an essential role as a survival mechanism during the encystation process of *Acanthamoeba* (14, 32), we hypothesized that chloroquine-induced death of encysting cells is associated with inhibition of autophagy by lysosome dysregulation. To test this hypothesis, we treated encysting cells with 100 µM chloroquine for 4 h and stained them with LysoTracker Red DND-99. It has also been reported that chloroquine induces accumulation and enlargement of lysosomes, causing lysosomal dysfunction (36, 38). In our experiment, we found that chloroquine elicited the accumulation of lysosomes intracellularly (84% greater accumulation than was seen with the control) (Fig. 3A). To confirm our result, we also stained the encysted cells with LysoTracker Red DND-99 and observed them under a confocal microscope. We found enlarged lysosomes that had increased in number inside the chloroquine-treated encysting cells (Fig. 3B). However, a small number of intact lysosomes were observed in control cells, which agreed with our flow cytometry data. These results together suggest that chloroquine was able to elicit dysfunction of lysosomes in *Acanthamoeba*.

**Chloroquine inhibits long-lived protein degradation in encysting cells.** As chloroquine caused lysosomal dysfunction in *Acanthamoeba*, we tested whether chloroquine could block autophagic flux. Autophagy begins with the formation of the au-
trophagosomal membrane to capture long-lived proteins and abnormal organelles. Long-lived captured proteins are degraded by lysosomal proteases after fusion of autophagosomes with lysosomes. Dysfunction of lysosomes can cause blockage of autophagic flux (39). One of the methods used to evaluate autophagic flux is measurement of the degradation of long-lived proteins (40). Accordingly, we analyzed long-lived-protein degradation rates using an isotope-labeled amino acid ([14C]leucine) (Fig. 4). A high concentration of chloroquine caused rapid death of encysting cells and released cellular contents to the supernatant, which triggered a false increase in the degradation rate determined by the autophagic flux assay (data not shown). Thus, we used a lower concentration of chloroquine (25 and 50 μM) to determine the degradation rates of long-lived proteins. As shown in Fig. 4, exposure of trophozoites to encystation media increased the degradation rate of long-lived proteins, which was consistent with previous reports suggesting that autophagy plays a role in the Acanthamoeba encystation process. The encystation-induced enhancement of long-lived protein degradation was inhibited by chloroquine in a dose-dependent manner. This result suggests that autophagic flux is inhibited by chloroquine during the Acanthamoeba encystation process.

**DISCUSSION**

Encystation of Acanthamoeba has been associated with increased resistance to medication and disinfectants (41). Blocking of Acanthamoeba encystation might have beneficial effects on the treatment of Acanthamoeba infection. Several researchers have identified important factors regulating the encystation process, including serine proteinase, protein kinase C, and cysteine protease, and have shown that the encystation process could be regulated using chemical inhibitors (42–44). In the present study, we evaluated the efficacy of chloroquine, an autophagy inhibitor, against A. castellanii. We found that chloroquine was significantly cytotoxic during the Acanthamoeba encystation process. It is known that both forms (trophozoites and cysts) of Acanthamoeba are found within the infected tissue (45). Although Acanthamoeba trophozoites are sensitive to most chemotherapeutic agents, long-term infections with cysts are difficult to treat due to chemotherapeutic resistance. Despite various chemotherapeutic agents showing beneficial effects in patients with Acanthamoeba infections (46–49), no effective treatments for GAE or disseminated infections have been established, and most patients with an infected nervous system and disseminated diseases have died (1). Chloroquine may be an effective antiamoebic drug targeting the Acanthamoeba encystation process and could improve the efficacy of the antiamoebic drugs when used in combination with those drugs.

Chloroquine is a widely used antimalarial drug that accumulates in acid organelles such as lysosomes and blocks fusion of autophagosomes with lysosomes. Because it blocks autophagy,
chloroquine has also been used as a treatment for cancer, lupus erythematosus, and rheumatoid arthritis (50). Recent studies suggest that autophagy plays an important role as a survival mechanism during the Acanthamoeba encystation process (13,14). In this sense, we hypothesized that chloroquine treatment might have a specific effect during encystation and might induce cell death by inhibiting autophagy. We found that, as expected, chloroquine effectively killed the cells in a dose-dependent manner during encystation (Fig. 1A).

A previous report suggested that chloroquine has no effect on Acanthamoeba (51). We treated trophozoites and mature cysts with chloroquine at different concentrations, and our results suggest that chloroquine has no effect on trophozoites or mature cysts (Fig. 1B and C). Therefore, our results demonstrate that chloroquine has a specific effect during the Acanthamoeba encystation process.

We performed experiments resulting in an accumulation of lysosomes and decreased autophagic flux after chloroquine treatment (Fig. 3 and 4) to determine whether inhibition of autophagy is the main mechanism by which chloroquine is able to induce cell death during encystation. Autophagic flux reflects autophagosome formation, engulfment of cytoplasmic substances, lysosomal fusion, and lysosomal degradation (40). Our data from confocal microscopy, flow cytometry, and a long-lived-protein-degradation assay reflect impaired function of lysosomes and autophagy, leading to cell death. Although the exact mechanism of treatment is unknown, chloroquine has been used to treat infection caused by Entamoeba histolytica (19, 52–54). Our study results suggest that chloroquine might act as antiamoebic drug by inhibiting the autophagy process in other amoebae, including Entamoeba histolytica.

Most of the histological findings in studies of Acanthamoeba-infected tissue show the presence of trophozoites and cysts, along with inflammatory cells (1, 46), indicating that the host defense is mounted during infection and that Acanthamoeba tends to encyst under adverse immune response conditions. Our results are clinically significant because chloroquine was able to ablate encysting cells at the stage when trophozoites tend to transform into cysts due to adverse conditions.

This is the first report to show the cytotoxic effect of chloro-
Chloroquine inhibits degradation of long-lived proteins. The degradation rate of [14C]-leucine-labeled long-lived proteins was measured. Trophozoites were cultured in PYG media for 24 h at 25°C and further incubated for 4 h in media containing chloroquine as indicated. Data are expressed as percent degradation of cellular protein at 4 h. Percent degradation is expressed as means ± standard deviations of data from three separate experiments. The asterisk (*) indicates a significant difference from the drug-free untreated-cell results (P < 0.005). The octothorpes (#) indicate a significant difference from the untreated-control-cell results (P < 0.05).

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