Chloroquine has cytotoxic effect in encysting *Acanthamoeba* through modulation of autophagy

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ABSTRACT

Encystation of Acanthamoeba castellanii is associated with resistance to chemotherapeutic agents. Blocking the encystation 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 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.
INTRODUCTION

Acanthamoeba is a free-living opportunistic amoeba that causes 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). Voriconazole and miltefosine combination therapy has been recently used to treat GAE (4). Caspofungin and corifungin 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 vegetative trophozoite(s) and dormant cyst stages. Under stress conditions, including the host immune response and chemical treatment, trophozoites can detect 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 anti-amoebic drugs and 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 hydrolase (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 screening different therapeutic drugs against encystation of *Acanthamoeba* and other protozoan parasites.
MATERIALS AND METHODS

Acanthamoeba culture. *A. castellanii* was obtained from the American Type Culture Collection (ATCC 30011; Manassas, VA, USA) and cultured axenically in peptone-yeast-glucose (PYG) medium (20 g/liter proteose peptone, 1 g/liter yeast extract, 0.1 M glucose, 4 mM MgSO₄, 0.4 mM CaCl₂, 3.4 mM sodium citrate, 0.05 mM Fe(NH₄)₂(SO₄)₂, and 2.5 mM each of 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 Sigma-Aldrich Co., St. Louis, MO, 63103 USA. The stock solution of drug (0.05 M) was prepared in sterile distilled water and stored at -20°C till use. We used 25-100 μM concentrations of chloroquine in our experiment because it has been determined that chloroquine concentration in tissue can be achieved as high as 100 μM after normal doses (27). To determine the effect of chloroquine on trophozoites, *A. castellanii* trophozoites (4×10⁵ 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).
observe the effect of chloroquine during 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. For the effect of chloroquine on mature cysts, trophozoites were incubated on non-nutrient 1.5% agar plate for 7 days at 25°C to allow the transformation of trophozoites to cyst stage. After this incubation, each plate was flooded with 20 ml PBS, and the cells were collected, then centrifuged at 1500×g for 15 min, and treated with sodium dodecyl sulphate (SDS) (0.5% final concentration) for 10 min. The SDS solubilizes trophozoites and immature cyst. Mature cyst(s) are resistant to 0.5% SDS and they remain intact (29). Mature cysts were washed five times with PBS to remove the residual SDS, and 5×10⁴ mature cysts were inoculated in 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, USA). Briefly, 4×10⁵ trophozoites were cultured in a 6-well plate. After 24 h incubation at 25°C, cells were treated with encystation medium containing chloroquine (100 μM) for 4 h. Long incubations with chloroquine causes significant intracellular changes and cell death, 4 h incubation time were selected for treatment to observe internal lysosomal accumulation. The cells were centrifuged at 1000×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 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, USA).

Confocal microscopy. LysoTracker Red DND-99 at a final concentration of 2.5 μM was used to assess lysosomal number and morphology. Cultured trophozoites (4×10^5) were treated with encystation medium containing chloroquine (100 μM) for 4 h. The cells were centrifuged at 1000×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 Zeiss Laser Scanning Confocal Microscope 510 (Oberkochen, Germany). Co-localization images were obtained with the 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 10^5 cells/well in 24-well plates containing PYG media for 24 h. The next day, the cells were labeled with a radioactive medium containing [^{14}C]-leucine (0.5 μCi/ml) (Perkin Elmer, Waltham, MA, USA) 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 one-tenth volume of 100% trichloroacetic acid (TCA) was added to each supernatant to measure radioactivity. The samples were centrifuged at 15000×g for 10 min, and acid soluble
radioactivity was measured by liquid scintillation counter (LSC) (Beckman LSC6500; Fullerton, CA, USA). 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 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 mean ± S.D. Statistical analysis was performed by using Student’s t-test (two-tailed). 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 it is a well-established anti-malarial drug and has inhibitory activity on autophagy by blocking autophagosome-lysosome fusion (33). After 24 h 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 a human neuroblastoma cell lineage SH-SY5Y for the evaluation of mammalian cell cytotoxicity. The cytotoxicity of chloroquine was found to be significantly lower in SH-SY5Y cells than the encysting *Acanthamoeba* (see Fig. S4 in supplemental material). IC50s for SH-SY5Y and the encysting *Acanthamoeba* were 116 μM and 65 μM, respectively (data not shown).

It has been demonstrated that trypan blue can enter inside the viable cells through toxin-induced increase of membrane permeability (34). To test whether the chloroquine-induced trypan blue staining was due to the membrane permeabilization rather than cell death, we...
performed trypan blue staining after further incubation of chloroquine-treated cells in PYG media alone. We treated trophozoites (4×10^5 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 1500×g for 10 min, and 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, assuming that further incubation in chloroquine-free PYG media could recover the *Acanthamoeba* from membrane permeabilization. We found that the trypan blue stained cells were significantly increased even after the incubation of chloroquine-treated cells in chloroquine-free PYG media (86% trypan blue-stained cells at 60 μM chloroquine) (Fig. 2A). This data suggest that chloroquine-induced staining of trypan blue was due to the cell death rather than the increase of cell membrane permeability. Moreover, further incubation in PYG media decreased the rate of cell viability more than that of Fig. 1A (decreased from 84.6 % to 13.9% at 60 μM concentration). It could be due to longer incubation time along with accumulated chloroquine inside the cells. In parallel, we observed the cellular detachment and significant number of damaged cells starting at 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 cell 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 cell 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 in heat-inactivated *Escherichia coli* agar plate instead of PYG media. However, chloroquine did not change the viability of *Acanthamoeba* trophozoites cultured in heat-inactivated *E. coli* agar plate (see Fig. S3 in the supplemental material). Next, we tested the effect of chloroquine on mature cysts. Mature cysts were prepared as described in Materials and Methods, and treated with different concentrations of chloroquine in encystation media. As shown in Fig. 1C, no significant death of mature cysts was observed during chloroquine treatment (98% viable with 100 μM chloroquine). Again, we evaluated cytotoxicity assay for mature cyst which was prepared in encystation medium. As expected, chloroquine did not show any cytotoxic effect on 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 on encysting cells, we tested the effects of chloroquine in another strain of *Acanthamoeba* (ATCC 50492). Chloroquine showed significant cytotoxic effect at concentrations of 70, 80, 90, and 100 μM specifically on encysting stage, whereas trophozoites and mature cysts remained unaffected (see Fig. S2 in the supplemental material).

**Chloroquine accumulates 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 cell 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 than 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 and an increased number of lysosomes inside the chloroquine-treated encysting cells (Fig. 3B). However, the intact and a small number of lysosomes were observed in control cells, which agreed with our flow cytometry data. These results together suggest that chloroquine could 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 formation of the autophagosomal 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 to evaluate autophagic flux is to measure 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). High concentration of chloroquine caused rapid cell death of encysting cells and released cellular contents to the supernatant which triggered false increase in autophagic flux assay (data not shown). Thus, we used 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.
Encystation of *Acanthamoeba* has been associated with the increased resistant to medication and disinfectants (41). The blocking of *Acanthamoeba* encystation might have beneficial effects on the treatment of *Acanthamoeba* infection. Several researchers have identified important regulating factors of encystation process including serine proteinase, protein kinase C, and cysteine protease and showed that 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 infected nervous system and disseminated diseases died (1). Chloroquine may be an effective anti-amoebic drug targeting the *Acanthamoeba* encystation process and could improve the efficacy of the anti-amoebic drugs when used in combination.

Chloroquine is a widely used anti-malarial 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 induce cell death by inhibiting autophagy. As expected, we found that
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
1C). Therefore, our results demonstrate that chloroquine has a specific effect during the
*Acanthamoeba* encystation process.

We showed an accumulation of lysosomes and decreased autophagic flux after chloroquine
treatment (Fig. 3 and 4) to determine whether inhibiting autophagy is the main mechanism
by which of chloroquine was able to induce cell death during encystation. Autophagic flux
reflects autophagosome formation, engulfment of cytoplasmic substances, lysosomal fusion,
and lysosomal degradation (40). Our data of confocal microscopy, flow cytometry, and
long-lived protein degradation assay reflect impaired function of lysosomes/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
suggests that chloroquine might act as anti-amoebic drug by inhibiting autophagy process
in other amoeba including *Entamoeba histolytica*.

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

This is the first report to show the cytotoxic effect of chloroquine during Acanthamoeba encystation. We suggest that chloroquine has effects in Acanthamoeba as an autophagy inhibitor by altering lysosomes, eventually leading to the cell death of encysting cells. Our results provide a potential novel therapeutic application of chloroquine as an anti-Acanthamoeba drug used in conjunction with other therapies, and suggest that screening of therapeutic drugs against the Acanthamoeba encystation process as well as other protozoan parasites targeting autophagy/lysosome is needed.

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FIGURE LEGENDS

FIG 1 Chloroquine decreased cell viability during encystation. Chloroquine was treated in encysting cells (A), trophozoites (B), and mature cysts (C) as described in Materials and Methods. After 24 h incubation, cell viability was determined by trypan blue staining. Results are means ± standard deviations (SDs) from three separate experiments. *P < 0.05 and **P < 0.005, compared with untreated control cells.

FIG 2 Chloroquine-treated encysting cells did not revive after replacement of PYG media. (A) Chloroquine-treated encysting cells were further incubated in chloroquine-free PYG media for 24 h and cell viability was determined by trypan blue staining. The bar graph shows percentage of cells stained by trypan blue (*P < 0.05 and **P < 0.005, compared with untreated control cells). (B) Morphology of further incubated cells in chloroquine-free PYG media was observed at 20 × magnification using Nikon Eclipse TS100 inverted microscope. The results are representative of three separate experiments. The scale bars represent 100 μM.

FIG 3 Chloroquine increased lysosomal accumulation inside the cell. Trophozoites were treated with encystation media containing chloroquine (100 μM) for 4 h. (A) Cells were harvested and lysosomal accumulation was quantified by flow cytometry after staining with LysoTracker Red DND-99 at a final concentration of 2.5 μM. Shown is a representative flow cytometry image of LysoTracker Red DND-99 stained cells. Bar graph shows the percent change in fluorescence intensity after chloroquine treatment compared to that in the control. The results are mean ± standard deviation from three independent experiments. (B)
Cells were harvested, stained with LysoTracker Red DND-99 at a final concentration of 2.5 μM, and observed under a confocal microscope. Bar = 20 μM.

**FIG 4** Chloroquine inhibits degradation of long-lived proteins. The degradation rate of \(^{14}\text{C}\)-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 mean (%) ± standard deviation from three separate experiments. * indicates a significant difference compared with the trophozoites incubated in PYG media (\(p < 0.005\)). # indicates a significant difference compared with untreated control cells (\(p < 0.05\)).