Effects of Cytoskeleton-inhibiting Drugs on Leishmania amazonensis Development in Macrophages

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Leishmaniasis is a disease that affects approximately 2 million people yearly around the world. This disease is caused by a parasite called Leishmania, which has two hosts: mammals and sandflies. In mammals, Leishmania enters macrophages by phagocytosis and develop in a specific compartment called the parasitophorous vacuole (PV). The main objective of this study was to examine the impact of vesicular transport in the host cell on Leishmania development within PVs. We hypothesized that blocking the host vesicular transport will subsequently impair the interaction between PVs and host cell organelles that may provide important sources of nutrients and factors for Leishmania development within the PV. To achieve our objective, Raw264.7 macrophages were first exposed to L. amazonensis promastigotes and after 24 hours, some cultures were treated with colchicine, a microtubule-inhibiting drug, or cytochalasin D, an actin-inhibiting drug, for a 48-hour time course. Samples were processed by immuno-fluorescence assays and analyzed by fluorescence microscopy. We found that blocking the host cell cytoskeleton function significantly inhibits the development of L. amazonensis within the PVs in Raw 264.7 mouse macrophages.

INTRODUCTION

Leishmania is a protozoan parasite that is transmitted to mammals during a bite from infected sandflies. Leishmania causes a disease called Leishmaniasis, which occurs in at least 88 countries worldwide [1]. There are three clinical presentations of Leishmaniasis: cutaneous, mucocutaneous, and visceral. Cutaneous Leishmaniasis causes skin lesions on the body. According to the Center for Disease Control, there are an estimated 1.5 million new cases annually of cutaneous Leishmaniasis. Mucocutaneous Leishmaniasis affects the mucous membranes, causing mucosal ulcerations. Visceral Leishmaniasis and kala azar can be lethal if left untreated. In the visceral form, the parasites infect the macrophages of internal organs, like the liver and spleen, and subsequently cause the organ to enlarge [2]. Controlling Leishmaniasis remains a serious public health issue for many countries. There is no vaccine for Leishmaniasis [3]. Additionally, co-infection with HIV/AIDS has led to the spread of Leishmaniasis. The immune deficiency from the HIV causes the increased susceptibility of Leishmania infection, and the parasite accelerates the onset of AIDS in patients [1].

Leishmania has two hosts in its life cycle. The vector of the parasite is a blood sucking sandfly, which harbors the promastigote form of the parasite. When the infected sandfly takes a blood meal from a mammal, Leishmania transfers to its mammalian hosts, such as dogs and humans. Within the mammalian host, Leishmania transforms from the promastigote to the amastigote form within the macrophages [4]. Macrophages are designed to eliminate foreign material from the body through the process of phagocytosis. Phagocytosis begins by the engulfment of the foreign particle to form a phagosome. Host cell endocytic components, such as early endosomes, late endosomes and lysosomes, which contain digestive enzymes and other microbiocidal factors, fuse with the phagosome and lead to the destruction of the foreign material [5].

However, Leishmania parasites are generally not destroyed by the macrophage. These parasites survive and replicate in macrophages within a parasitophorous vacuole (PV) [4]. There are two different types of PVs. The first is the large communal PV, associated with L. amazonensis and L. Mexicana, where many parasites occupy a single vacuole. There is also the tight individual PV, associated with L. major and L. donovani, which has only one parasite per vacuole [6]. Mechanisms by which Leishmania parasites evade the host’s defense and/or obtain nutrients for their development within PVs in macrophages remain to be established. Recent research has suggested that endoplasmic reticulum components may be recruited to Leishmania PVs through the process of vesicular transport [7].

Intracellular transport of vesicles occurs by using microtubules from ER to cis-Golgi and post-Golgi trafficking [8, 9]. The cell cytoskeleton is made of microtubules and actin filaments. Microtubules and actin filaments are both polar structures with associated motor proteins that can transport material along their tubules or filaments. Microtubules are composed of alpha- and beta-tubulin subunits that form a tubulin heterodimer or a microtubule subunit [10]. There are two types of actin subunits. G-actin is the globular subunits of actin that
assemble into filaments, and F-actin is the filament form of actin. Like microtubules, actin has a plus and minus end of the filament, where the plus end grows faster than the minus end. Myosins are the motor protein associated with actin. Microtubules in the mammalian cell enable long distance transport of vesicles and organelles and serve as support of the cell structure. Actin plays a role in short distance transport of vesicles and organelles. It also plays a major role in the cell locomotion and the endocytosis process [10]. A drug that inhibits actin filaments by inhibiting the addition of actin subunits to the barbed end of the filament is Cytochalasin D (CCD) [11, 12]. Its effects are drug are reversible [12]. A drug that binds to beta-tubulin at the C241 and C356 residues with pseudoirreversible kinetics is colchicine [13].

The objective of the current study was to assess the impact of vesicular transport on Leishmania development in macrophages. We hypothesized that blocking the actin or microtubule-dependent vesicular transport within the cell will deprive Leishmania of nutrients and other factors vital for its development in macrophages. To carry out the study, either colchicine or Cytochalasin D drugs were used inhibit respectively the microtubule and actin compartments in mouse macrophages infected with L. amazonensis. The effects of the drugs on Leishmania development were examined by assessing the parasite load in infected macrophages over time at varying concentrations of the above cytoskeleton-inhibiting drugs.

The results of the study showed that the parasite load (number of parasites per PV) is reduced to half after 48-hour post drug treatment of infected macrophages with L. amazonensis. This result suggested that the host cell’s cytoskeleton plays an important role in Leishmania replication or survival within the parasitophorous vacuole.

**MATERIALS AND METHODS**

**Cell Culture**

**Macrophages.** Raw 264.7 murine macrophages were obtained from American Type Culture Collection and cultured in RPMI-1640 medium supplemented with 10% FBS and 1% PennStep.

Cells were split every three days under a sterile bio safety hood, when the culture flask is approximately 80% confluent. The cells were scraped off the bottom of a tissue flask using a cell scraper and mixed by pipeting up and down with a serological pipet. One mL of cells was then added to a sterile tissue culture flask using a serological pipet. Nine mL of pre-warmed (to 37°C) complete RPMI medium was added to the new flask. This fresh culture flask was incubated in a Forma Scientific CO2 Water Jacked Incubator at 37.0 °C and 5.5% CO2 atmosphere. The rest of the cells were aspirated out into a waste bottle containing the bleach and the old flask discarded into a biological waste bin.

**Parasite.** L. amazonensis promastigotes were grown at 23 °C in complete medium (Schneider’s Drosophila medium) supplemented with 20% heat inactivated fetal bovine serum (FBS) and 10-µg/ml gentamicin. The parasites were split every 5 days in a 1:100 (0.5 mL parasites and 4.5 mL of fresh complete medium) and a 1:1000 dilution (0.05 mL of parasites and 4.95 mL of fresh complete medium). The parasites are non-adherent and swim in the medium.

**Infections**

**Plating Macrophages.** The adhered macrophage cells were scraped from the tissue flask under the hood using a sterile cell scraper. One 50 uL sample of macrophages was then transferred to a 96 well plate. From the 50 uL, a 10 uL sample of macrophages were transferred to a separate well. 10 uL of trypan blue dye was added to this 10uL macrophage sample and then mixed by pipetting the mixture up and down. The mixture of macrophages and dye was allowed to sit for 5 minutes at room temperature to allow the dye to diffuse into the cell. 10 uL of the mixture was then transferred to a hemocytometer for counting on light microscope. Living cells had excreted the dye and were observed as being without dye. Dead cells retained the blue dye and were not counted. From the counts an approximation was calculated to estimate the concentration of live macrophages in the original tissue flask. Four sterilized Fisherbrand Microscope Cover glasses (also called coverslips) were added to each of the wells in a tissue culture six well plate under a biosafety hood. The coverslips were transferred from their container to the plate using a sterilized Pasteur pipet that was attached to a vacuum pump, which maintained the coverslips sterilized integrity. 2 mL of macrophages from the tissue flask of known concentration were then added to the wells. The cells were then incubated overnight at 37 °C to allow the macrophages to adhere to the coverslips.

**Co-incubation of Parasites and Macrophages.** The parasite stock was diluted to a 1:20 ratio in 2% paraformaldehyde (PFA) solution; the aliquot was incubated at room temperature (RT) for 15 minutes to immobilize the parasites. A hemocytometer was used to quantify the parasite sample. The volume of parasite stock corresponding to 10 times the amount of macrophages was aseptically added to a centrifuge tube. The parasites were then centrifuged at 2000 rpm for 5 minutes. Under the biosafety hood, the supernatant was then discarded and the parasite pellet was resuspended in RPMI complete medium. The appropriate volume of the parasite solution was then added to each well of the six-well plate. The plate was gently swirled by hand to ensure even distribution of parasites, which was confirmed under a light microscope. The plate containing the mixture of parasites and
macrophages was placed at 34 °C in an incubator supplemented with 5.5% CO₂ atmosphere.

**Drug Treatment**

After a 24-hour infection of Raw 264.7 cells with *L. amazonensis*, colchicine was added at concentrations of 1 μM and 10 μM, as previously described [14, 15]. Cytochalasin D was added at concentrations of 1 μg/mL and 5 μg/mL, as previously described by Parsa et al. (2006). Two μL of dimethyl sulfoxide (DMSO) was added to infected Raw 264.7 cells in 2 mL of RPMI 1640 complete medium. Treated cells were incubated at 34 °C. Time points were taken by removing a coverslip and fixing in 2% PFA at 0 hr drug treatment (24 hr infection), 12 hr drug treatment (36 hr infection), 24 hr drug treatment (48 hr infection), and 48 hr drug treatment (72 hr infection).

**Immuno-Fluorescence Assays (IFA)**

The infections were processed by IFA analysis. The cells on coverslips were fixed in 2% paraformaldehyde (PFA). The PFA was aspirated, and then the cells were washed twice in 1x PBS (phosphate buffered saline) and quenched in 50mM of ammonium chloride/ 1xPBS solution for 5 minutes. The cells were then washed twice in 1xPBS, and permeabilized with a blocking buffer of 2% milk and 0.5% saponin in 1xPBS for 30 minutes. The coverslips were then placed on 37uL of a primary antibody mixture for 30 minutes to 1hr in darkness. The primary antibody mixture was composed of 2% ID4B (rat) and blocking buffer for the coverslips of DMSO, 1 ug/mL of CCD and 5 ug/mL of CCD. The primary antibody mixture for DMSO, 1μM colchicine, and 10 uM colchicine was comprised of 2% ID4B IgG (from rat), 2.5% beta-tubulin (from mouse), and blocking buffer. After the primary antibody was added, the coverslips were then washed three times with a binding buffer (2% milk, 0.05% saponin, 1xPBS). The coverslips were then mounted onto 37uL of a secondary antibody mixture for 1hr in darkness. A secondary antibody mixture for the coverslips of DMSO, 1 ug/mL of CCD, and 5 ug/mL of CCD was of AlexaFluor 488 chicken anti-rat IgG (1:200), 4',6-diamidino-2-phenylindole (DAPI) (1.2:1000), 2.5% phalloidin, and blocking buffer. A secondary antibody mixture for the coverslips of DMSO, 1μM colchicine, and 10 uM colchicine was composed of AlexaFluor 488 chicken anti-rat IgG (1:200), DAPI (1.2:1000), AlexaFluor 568 goat anti-mouse IgG (1:100), and blocking buffer. After the secondary antibody treatment, the coverslips were washed with binding buffer three times, followed by a wash with 1xPB three times. The coverslips were mounted onto slides with Fluoro-Gel with Tris Buffer (Electron Microscopy Sciences). The slides were then stored at 4 °C for future analysis under fluorescence microscope.

**Fluorescence Microscopy**

Preparations of microscope slides were examined using a Zeiss Axiosvert 200M fluorescence microscope outfitted with DAPI, fluorescein isothiocyanate (FITC), and tetramethyl rhodamine isothiocyanate (TRITC) filters. The images were observed at a 100x oil immersion objective. Pictures were taken with a Zeiss AxioCam MRm camera attached to the microscope and processed using the AxioVision Rel 4.7 software. A Z-stack of images with a slice distance of 0.250-0.700 um between focal planes was collected and then merged to create the final image.

**Data Collection**

Samples were collected at 24 hrs of infection and 12 hrs, 24 hrs, and 48 hrs after drug treatment. Non-treated samples were used as control. The samples, both treated and non-treated, were evaluated by counting at least 60 infected macrophages. The number of parasites within PVs was also counted. Parasite load was obtained by dividing the number of parasites within PVs by the number of infected cells. The infection ratio was calculated as number of infected macrophages divided by the total number of macrophages counted multiplied by 100.

**RESULTS**

**I: Infection Rate of Raw264.7 Macrophages by *L. amazonensis* Parasites**

Raw 264.7 macrophages were co-incubated with *L. amazonensis* promastigotes, and after 24 hours, an infection rate of 51.6% was obtained. This indicated that about half of our total macrophages internalized the *Leishmania* parasite before the drug treatment. A representative sample of an infected Raw 264.7 macrophage is shown in Figure 1.
II: Effect of Cytoskeleton-Inhibiting Drugs on L. amazonensis Development in Macrophages

To determine the impact of vesicular transport on L. amazonensis development, cytoskeleton-inhibiting drugs (cytochalasin D and colchicine) were used in our experiments.

II-A: Effect of Cytochalasin D on L. amazonensis Development in Macrophages

After 24 hours of infection and no drugs, the parasite load for DMSO, 1 ug/mL CCD, and 5 ug/mL CCD were varied slightly with values of 1.25, 1.20, and 1.22, respectively. After 24 hours of drug treatment, the parasite load began to vary between the samples. DMSO samples had increased to 1.64, 1 ug/mL CCD had increased to 1.5, and the parasite load for 5 ug/mL CCD was 1.26. After 48 hours of drug treatment, the parasite load of DMSO had reached 2.0, indicating that the parasites had replicated within the PV. The parasite load of 1 ug/mL CCD dropped to 1.24, and for 5 ug/mL CCD the parasite load dropped to 1.12.

Figure 2 and Figure 3 show representative images of the macrophages at the four time points in the experiment. Figure 2 shows the effect of 1 ug/mL of CCD and Figure 3 shows the effect of 5 ug/mL of CCD. In the images, the parasites are not dividing within the PV after 48 hours like the untreated infected macrophages in Figure 1. Figure 4, a graph of the parasite load, shows that over time the parasites do not divide within the PV when treated with 1 ug/mL of CCD or 5 ug/mL of CCD. Figure 4 shows that when the cell is not treated the parasites are able to divide, as there are two parasites per PV.

Figure 1: Images of Raw264.7 infected at (A) 24 h and (B) 72 h. Lysosomal associated membrane protein-1 (Lamp-1), in green, shows lysosomes and PVs. The nuclei of both parasite and macrophage are stained in blue (DAPI). Solid arrows point to parasites. Dashed arrows point to the PVs containing the parasite(s).
Figure 2: Images of infected cells treated with 1 ug/mL CCD. Staining of F-actin (red) and Lamp-1 (green) with phalloidin and ID4B, respectively, in L. amazonensis infected Raw 264.7 murine macrophages treated with 1 ug/mL cytochalasin D. Solid arrows point to parasite. DAPI (blue) stains dsDNA of macrophage and parasite. (A) 0-hr drug treatment (B) 12-hr drug treatment (C) 24-hr drug treatment (D) 48-hr drug treatment.

Figure 3: Images of infected cells treated with 5 ug/mL CCD. Staining of F-actin and Lamp-1 with phalloidin and ID4B, respectively, in L. amazonensis infected Raw 264.7 murine macrophages treated with 5 ug/mL cytochalasin D. Arrow points to parasite. (A) 0-hr drug treatment (B) 12-hr drug treatment (C) 24-hr drug treatment (D) 48-hr drug treatment.
II-B. Effect of Colchicine on L. amazonensis Development in Macrophages

Over the course of the experiment, 1 uM colchicine and 10 uM colchicine never had parasite loads above 1.4. Prior to the addition of drugs, the parasite load was 1.25, 1.26, and 1.38 for the infections that would receive DMSO, 1 uM colchicine, and 10 uM colchicine, respectively. Throughout the 48 hours of drug treatment, the parasite load of 1 uM colchicine and 10 uM colchicine remained fairly constant to end with parasite load values of 1.33 and 1.28, respectively, as seen in Figure 5. However, the treatment of DMSO had doubled to reach a parasite load value of 2.0, indicating the replication of parasites within the PV.

In Figure 6, the microtubules are inhibited by 1 uM colchicine. The images show that the microtubules are present at 0 hr and 12 hr of drug treatment and have depolymerized by 24 hours of drug treatment, indicating the drug is working to inhibit microtubules. Figure 6 also shows the representative images of the infection over time. The treated infected cells do not experience any parasite replication within the PV.
Figure 5: Effect of colchicine on *L. amazonensis* development in macrophages. Macrophages incubated with *L. amazonensis* and DMSO (solid black), 1 uM colchicine (few dots), and 10 um colchicine (many dots). Data is the mean of two experiments.

Figure 6: Images of infected cells treated with 1 uM Colchicine. Staining of beta-tubulin and Lamp-1 in *L. amazonensis* infected Raw 264.7 murine macrophages treated with 1 uM colchicine. Arrow points to parasite. (A) 0-hr drug treatment (B) 12-hr drug treatment (C) 24-hr drug treatment (D) 48-hr drug treatment.
Figure 7 shows that 10uM of colchicine works to block microtubules between 0 and 12 hr. In Figure 7c, although there are many parasites, the parasites are in separate PVs so no reproduction has occurred. After 48 hours, there is still no parasite development within the PV, as shown in Figure 7d with only one parasite per PV.

**DISCUSSION**

The objective of the study was to determine the effects of cytoskeleton-inhibiting drugs on *Leishmania* development within the PV. The hypothesis was that inhibiting microtubules and actin with drugs would slow the development of *Leishmania* within the PV. The cell cytoskeleton is made of microtubules and actin filaments. Microtubules and actin filaments are both polar structures with associated motor proteins that can transport material along their tubules or filaments.

Microtubules are composed of alpha- and beta-tubulin subunits that form a tubulin heterodimer or a microtubule subunit. Microtubules grow faster at the end with the beta-tubulin subunit, which is called the plus end, compared to the alpha-tubulin subunit, which is called the minus end. Kinesin and dynein are the motor proteins associated with microtubules. The role of microtubules in the mammalian cell is to provide long distance transport of vesicles and organelles. Microtubules also play a role in cell structure and support [10].

Actin is composed of the monomeric molecule G-actin that polymerizes into a filamentous polymer, F-actin [16]. The roles of actin in the cell include cell migration by way of myosin II and short distance vesicular or organelle transport using myosin I, V, and VI [10]. Myosin V has been shown to transport ER vesicles in neurons [17]. Myosin I and myosin VI have been shown to be associated with Golgi-derived vesicles and cytoplasmic vesicles [17].

To evaluate the effects the drugs had on *Leishmania* development, the parasite load and infection rate were analyzed. The main finding of the study was that the parasite development was significantly hindered when microtubule- and actin-inhibiting drugs, colchicine and...
CCD, were used, separately, during a *Leishmania* infection of macrophages. When the cells were not treated with microtubule- or actin-inhibiting drugs, the parasites multiplied within the PVs. The parasite load (number of parasites per PV) for untreated cells reached 2.0 after 48 hours. The parasite load for all treated cells after 48 hours was less than 1.3, demonstrating that the pharmacological inhibition of actin and microtubules impacted the development of *L. amazonensis* within the PV. *L. amazonensis* replicates within a single PV to form a larger PV with many parasites. Because the large PV with many parasites was not seen after 48 hours of drug treatment and was seen after 48 hours without drug treatment, it was concluded that actin and microtubules of the host’s cytoskeleton are necessary components for the development of this parasite.

Some limitations of the study were that the infected cells should have been incubated at 34 °C during the infection, instead of 37 °C. The difference in temperature may have played a role in the parasite load. It may have made it difficult for the parasites to reproduce. The literature recommends that infections using *L. amazonensis* occur in conditions of 34 °C rather than 37 °C [18, 19, 20]. Another limitation of the study was the effect the drugs had on other parts of the cell. For instance, at 10 μM colchicine, the cells’ nucleus began to fragment and it became difficult to distinguish between the parasites and the cell after 24 hours of drug treatment. With DMSO, it became difficult to count the cells because the field of view was overpopulated with macrophages, making it difficult to distinguish one cell’s vacuoles from that of another.

In this study we demonstrated that *L. amazonensis* parasites develop very well in their mammalian host macrophages. However, successful development of *Leishmania* parasites requires a well-functioning cell cytoskeleton.

### REFERENCES


