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Ivermectin Inhibits Growth of *Chlamydia trachomatis* in Epithelial Cells

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Citation	Pettengill, Matthew A., Verissa W. Lam, Ikechukwu Ollawa, Camila Marques-da-Silva, and David M. Ojcius. 2012. Ivermectin inhibits growth of <i>Chlamydia trachomatis</i> in epithelial cells. PLoS ONE 7(10): e48456.
Published Version	doi:10.1371/journal.pone.0048456
Accessed	February 19, 2015 11:53:53 AM EST
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plasma concentrations from such dosage reach around 60 nM [13]. Ivermectin has also been used and well-tolerated at 500 µg/kg body weight via topical administration in veterinary applications [14], which could potentially be an attractive mode of use if it is found to be effective against intracellular bacterial pathogens which primarily infect readily accessible mucosal surfaces. Ivermectin targets glutamate-gated chloride channels in nerve and muscle cells and gamma-aminobutyric acid (GABA) related chloride channels of invertebrates [5], as well as mammalian GABA receptors [15].

Ivermectin has also been reported to interact with the purinergic receptor, P2X₄ [16], which can be stimulated by low (micromolar) concentrations of ATP; and we have observed that stimulation of *C. trachomatis*-infected epithelial cells with micromolar concentrations of ATP leads to chlamydial growth inhibition [17]. However, addition of apyrase (2.5 U/ml) to cells immediately prior to ATP addition prevents ATP-mediated chlamydial growth inhibition (unpublished data), but does not modify the impact of ivermectin on chlamydial growth (1 or 2.5 U/ml apyrase, 5 µM ivermectin, N = 3; data not shown). These results suggest that ivermectin does not inhibit infection through P2X₄ ligation.

We have here demonstrated that ivermectin inhibits *C. trachomatis* infection in epithelial cells. While the concentrations of ivermectin necessary for this inhibitory action *in vitro* are higher than what is achieved distal to absorption sites in current human therapy, topical application may allow therapeutic use of ivermectin against sexually-transmitted infection, or against eye infection with ocular strains of *C. trachomatis*. Additionally, as the target of this particular activity in human cells has yet to be identified, other avermectins or structurally modified avermectin molecules may have greater potency. As it seems that ivermectin mediates this response through interaction with a host cell target, potential efficacy against other obligate intracellular bacteria or parasites is worthy of exploration.

Materials and Methods

Cells and Materials

The epithelial cell line HeLa 229 (American Type Culture Collection, Manassas, VA) was used to model infection with the LGV/L2 strain of *C. trachomatis* (obtained from Dr. Roger Rank, University of Arkansas, Little Rock, AR). HeLa cells were cultured in a humidified incubator at 37°C with 5% CO₂ in Dulbecco's modified Eagle medium (DMEM:F-12, Invitrogen) supplemented with 10% heat-inactivated fetal calf serum. Apyrase and Hoechst were purchased from Sigma (St. Louis, MO), and antibodies against the *C. trachomatis* genus antibodies were from Argene (Sherley, NY).

Preparation and Use of Ivermectin

A concentrated stock of ivermectin (Sigma, St. Louis, MO) was solubilized in ethanol, and dilutions were also prepared in ethanol. Control wells received the same concentration of ethanol (0.2%) as treated wells, which did not influence chlamydial infection relative to control wells without ethanol (data not shown).

Chlamydial Infection and Analysis

HeLa cells growing at 70% confluence in tissue culture plates (Costar) were infected at a multiplicity of infection (MOI) of 1.0, with treatments at the indicated times [18]. Infection was assayed by fluorescence microscopy and qPCR for *C. trachomatis* 16s rRNA, using protocols and primers previously described [18]. To analyze inclusion size and morphology by fluorescent microscopy, HeLa cells were grown on glass coverslips, and after the indicated experimental conditions were fixed with ice cold methanol for 10 minutes. Cells were stained with *C. trachomatis* genus antibodies from Argene (North Massapequa, NY) and Hoechst (Sigma), and observed on a widefield fluorescence microscope (Leica, Deerfield, IL).

Statistical analysis was performed using GraphPad Prism version 5.0b for Mac (GraphPad Software, San Diego, CA).

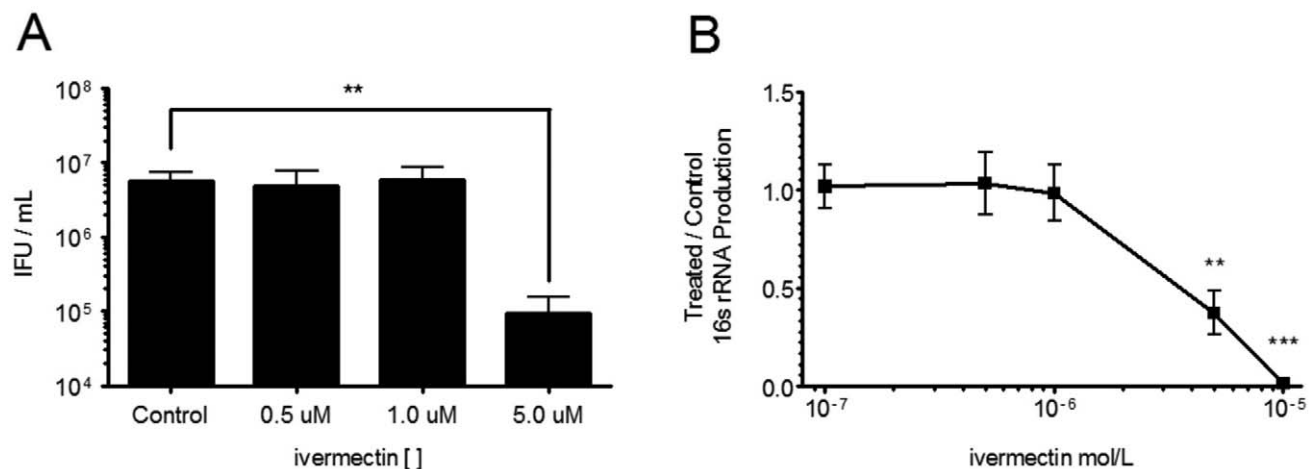


Figure 1. Ivermectin inhibits chlamydial infection of epithelial cells. (A) HeLa cells were infected with *C. trachomatis* serovar L2 at an MOI of 1, followed by treatment with ivermectin at the indicated concentrations at 1 hour post infection (hpi). Samples were harvested at 24 hpi for quantification of reinfectious yield (IFU/ml) on new HeLa cell monolayers utilizing fluorescent microscopy and anti-*C. trachomatis* antibodies. The values show means plus standard deviation of three independent experiments. (n = 3, **, P = 0.0059). (B) Total RNA was harvested at 24 hpi for quantification of chlamydial 16s rRNA. The values shown are relative to control values for each experiment, and are means and standard deviations of 3 independent experiments (n = 3, **, P = 0.0034, ***, P < 0.001, compared to 100 nM condition). Two-tailed unpaired t tests were performed using GraphPad Prism version 5.0b for Mac. doi:10.1371/journal.pone.0048456.g001

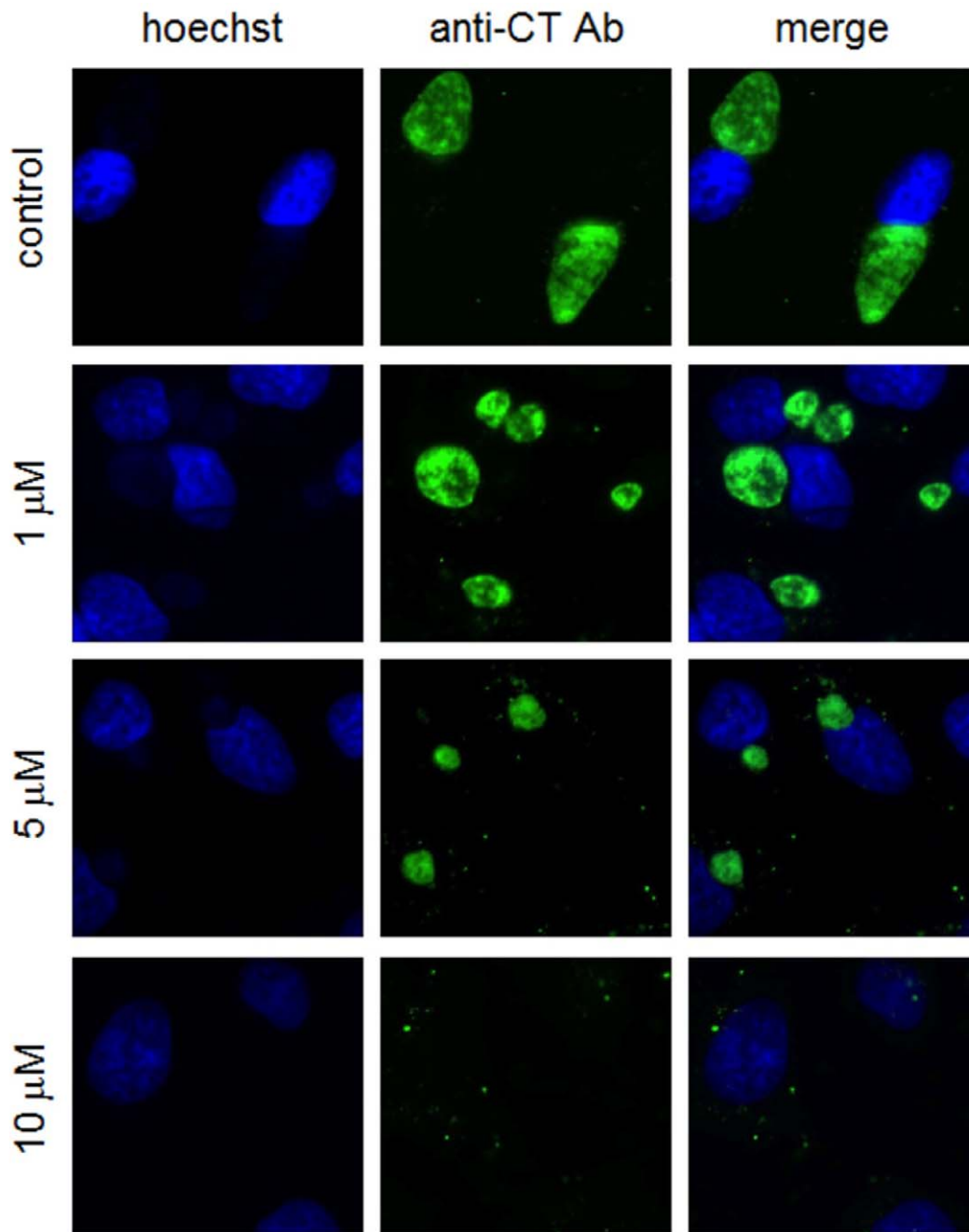


Figure 2. Ivermectin inhibits the development of chlamydial inclusions in epithelial cells. HeLa cells grown on glass coverslips were infected with *C. trachomatis* serovar L2 at an MOI of 1, followed by treatment with ivermectin at the indicated concentrations at 1 hour post infection (hpi). At 24 hpi, cells were fixed with ice cold methanol for 10 minutes, followed by staining with *C. trachomatis* genus antibodies (Argene) and Hoechst (Sigma), and observed on a widefield fluorescence microscope (Leica).
doi:10.1371/journal.pone.0048456.g002

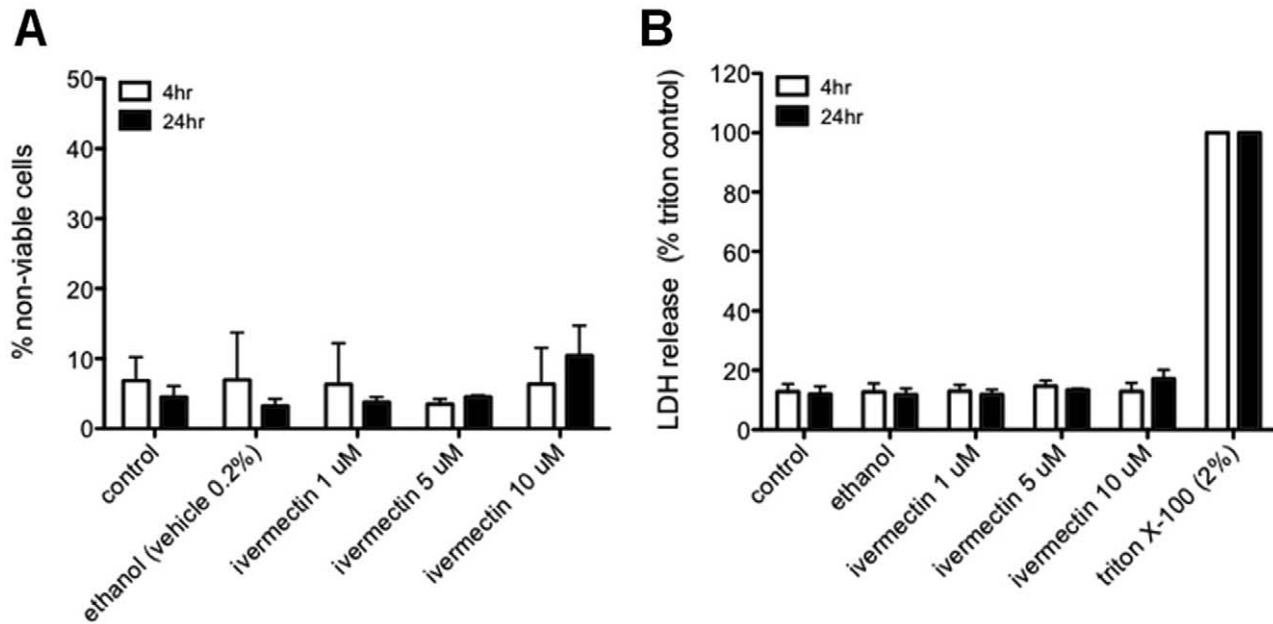


Figure 3. Ivermectin is not cytotoxic to epithelial cells. (A) HeLa cells were cultured in the presence of the indicated concentrations of ivermectin or vehicle (0.2% ethanol), supernatants were then centrifuged at 500 g for 5 minutes, and adherent cells were removed with trypsin/EDTA and gently recombined with supernatant pellets. Cell viability was determined by trypan blue exclusion, evaluated using a hemocytometer. (B) Supernatants from the cultures described in panel A, and from wells treated with 2% Triton X-100 30 minutes prior to collection time points, were combined and evaluated for LDH activity using a Roche Cytotoxicity Detection Kit per the manufacturer's instructions. Shown are means plus standard deviations for 3 independent experiments. doi:10.1371/journal.pone.0048456.g003

Author Contributions

Conceived and designed the experiments: MAP DMO. Performed the experiments: MAP VWL IO CM. Analyzed the data: MAP CM.

Contributed reagents/materials/analysis tools: DMO. Wrote the paper: MAP DMO.

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