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Chloroquine inhibits endosomal viral RNA release and autophagy-dependent viral replication and effectively prevents maternal to fetal transmission of Zika virus

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ABSTRACT

Zika virus (ZIKV) infection can cause neonatal microcephaly and neurological disorders. Currently, there is no designated drug for treating ZIKV infection and preventing neonatal microcephaly. In this study, we evaluated the effect of chloroquine, an anti-malaria drug, in ZIKV infected cells and mouse models. Chloroquine significantly inhibited ZIKV infection in multiple mammalian cell lines. Chloroquine treatment significantly improved the survival of ZIKV-infected 1-day old suckling SCID Beige mice and reduced viremia in adult SCID Beige mice. Importantly, chloroquine protected the fetus from maternal infection by reducing placenta to fetus viral transmission. We found that chloroquine exerts at least two mechanisms in protecting against ZIKV infection: 1) inhibiting endosomal disassembly of the internalized virus and thus reducing the release of viral RNA to the cytoplasm for replication; 2) inhibiting ZIKV RNA replication through blocking ZIKV induced autophagy. Our study suggests that chloroquine treatment warrants to be considered as a mitigation strategy for treating ZIKV infection and preventing ZIKV-associated microcephaly in pregnant women.

1. Introduction

Zika virus (ZIKV), belonging to the Flaviviridae family, is an enveloped and single-stranded RNA mosquito-borne virus and close to dengue virus (Baud et al., 2017). ZIKV was firstly isolated from rhesus macaque in 1947 and from a human in 1952 in Uganda (Dick et al., 1952). Historically, the clinical syndrome of ZIKV infection is mild, characterized by self-limiting fever, headache, and rash. During the recent North American outbreak, it was found that ZIKV infection resulted in congenital malformations, a broad range of neurological disorders and occasional mortality in adults (Baud et al., 2017; Miner and Diamond, 2017). The major concern is that ZIKV infection in pregnant women links to severe microcephaly in the fetus (Mlakar et al., 2016). The proportion of brain abnormality or microcephaly in ZIKV-infected mothers is approximately 20-fold higher than in normal mothers (Cragan et al., 2017). Currently, there is no designated medicine that could reduce the risk of ZIKV induced fetal microcephaly in pregnant women. Therefore, one quick solution would be to search for old drugs for potential new benefits in mitigating the risk of ZIKV infection before new anti-ZIKV drugs become available.

Chloroquine has been used for treating malarial since 1947 (Kitchen et al., 2006; Pullman et al., 1948). Chloroquine is easily distributed to different tissues and is able to cross the placental barrier (Law et al., 2008) and blood-brain barrier (Adelusi and Salako, 1982). Chloroquine is reported to possess antiviral effects on several different viruses, including Dengue virus (DENV) (Farias et al., 2015), human immunodeficiency virus (HIV) (Savarino et al., 2001), Hepatitis C virus (HCV) (Mizui et al., 2010), influenza virus (Di Trani et al., 2007), and Ebola virus (Sweiti et al., 2017). Chloroquine has been reported to have an inhibitory effect on ZIKV infection in the cultured cells (Delvecchio

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et al., 2016). Recently, chloroquine was reported to protect adult immunocompetent and interferon receptor defective A129 mice from ZIKV infection and protect fetal mice from ZIKV-induced brain defects (Gorshkov et al., 2018). In this study, we established new ZIKV infection models using immunocompromised SCID Beige mice. SCID Beige mice are deficient in T, B, and NK cells and thus are more suitable for evaluating the net effect of a potential anti-ZIKV drug. We assessed the therapeutic effects of chloroquine in immunocompromised adult and neonatal mice, and in a maternal to fetus virus transmission model. We further investigated the possible mechanisms of chloroquine in inhibiting ZIKV infection.

2. Material and methods

2.1. Cell culture

African green monkey kidney (Vero) cells, human hepatoma (Huh-7) cells, and mouse embryonic fibroblasts (MEF) were kindly provided by Stem Cell Bank, Chinese Academy of Sciences. Atg5-deficient MEF cells were purchased from Shanghai Biomodel Organism Science & Technology Development Co., Ltd. These cells were cultured in DMEM medium (Life Technology) supplemented with 10% fetal bovine serum (FBS; GIBICO), 100 U/mL penicillin, and 100 μg/mL streptomycin (Life Technology) at 37 °C in 5% CO_2.

2.2. Virus propagation and titration

ZIKV GZ02 (GenBank accession no. KX056898.1) strain was isolated from the urine of an infected child returning from Venezuela. The urine from the infected patient was intracranially inoculated to one-day-old Kunming suckling mice. Ten days post inoculation, the supernatant of homogenized brain was used for propagating the virus in Vero cells. Vero cells were infected at a multiplicity of infection (MOI) of 0.015 ($TCID_{50}$ per cell) at 37 °C for 1 h. Next, unabsorbed virus particles were removed by washing with pre-warmed phosphate-buffered saline (PBS; GIBICO), and the cells were cultured at 70% confluency for an additional 5–7 days in medium with 2% FBS. After each period, the supernatants were collected and centrifuged at 1,500 × *g* at 4 °C for 20 min to remove cellular debris. The virus was stored at −80 °C for further studies.

Monolayers of Vero cells in 96-well plates were exposed to different dilutions of the supernatant containing virus for 2 h at 37 °C. Next, the cells were washed twice with PBS, and DMEM containing 2% FBS was added to the cells. After 8 days at 37 °C, cell viability was determined by CellTiter-Glo® Luminescent Cell Viability Assay (Promega). The virus titers were then calculated by GraphPad Prism v.7.

2.3. Chloroquine treatment at different stage

Cells were infected with ZIKV (MOI = 1) at 4 °C for 1 h and then washed twice with cold PBS. Chloroquine (Sigma Aldrich) in culture medium was added at an early stage (0, 1, 2 h post infection), maintained at 37 °C for 2 h and then replaced with fresh medium, or late stage (6, 12 h post infection) and maintained to the end of experiments. Twenty-four hours post infection, the total RNA was isolated and quantified by Quantitative RT-PCR.

2.4. Early Endosome Isolation

Vero cells were pretreated with chloroquine or PBS 2 h before infection and then were infected with ZIKV at an MOI of 1 at 4 °C for 1 h and then washed twice by cold PBS. Early endosomes were isolated by using the Minute™ Endosome Isolation kit (Invent) according to the instructions. The separated components were resuspended in RLT (Qiagen) for RNA extraction or RIPA lysis buffer (Beyotime Biotechnology) for immunoblotting. The purity of the obtained early endosome fragments was proved by detecting the early endosome-specific (EEA1, Cell Signaling) and nucleus-specific (LaminB1, Cell Signaling) proteins.

2.5. Flow cytometry

Vero cells were plated 12 h before conducting the experiment. Cells were infected with ZIKV (MOI = 1) at 4 °C for 1 h and washed twice by cold PBS. Then the medium contains chloroquine or PBS was added and incubated for another 24 h. Infected cells were collected by trypsin, fixed and permeabilized on ice for 20 min. Subsequently, cells were stained with 4G2 antibody (EMD Millipore Corp.) at 2 μg/ml on ice for 30 min. The percentage of E-positive cells was measured using BD FACScCanto II.

2.6. Animals

SCID Beige mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. The animals were kept at a constant temperature (25 °C) with free access to chow and water in a 12 h light/dark cycle. Pregnant mice were observed daily until delivery to accurately determine the postnatal day. The Animal Welfare Committee of Guangzhou Institute of Biomedicine and Health (GIBH.CAS) approved IACUC protocol 2017031 for the experiments in this study.

2.7. ZIKV infection in neonatal and adult mice and treatment

6-week old female SCID Beige mice were subcutaneously inoculated in the footpad with ZIKV strain GZ02 (1.5×10^5 $TCID_{50}$/mouse). Mice were administrated with 50 mg/kg chloroquine or vehicle intraperitoneally 4 h post infection and once daily for the following 5 days. Viremia was measured at 1, 3 and 5 days post infection as shown. Blood was collected by retro orbital bleeding and placed on EDTA-containing tubes for plasma separation. For neonatal studies, 1-day-old SCID Beige mice were infected intraperitoneally with 1.5×10^3 $TCID_{50}$ of virus, unless otherwise mentioned. Chloroquine was administered at 50 mg/kg/day intraperitoneally. Treatment started 4 h post infection. In the case, treatment was conducted for 15 days. For comparisons, mock-infected and mock-treated groups of animals were used as controls. Animals were monitored daily for survival and weight gain. Samples (plasma, spleen and brain) were collected and kept in RNA later buffer (Qiagen) to avoid RNA degradation.

2.8. ZIKV infection in pregnant mice and treatment

10-week old SCID Beige female mice were mated and the pregnant female mice were inoculated with ZIKV strain GZ02 (7.5×10^5 $TCID_{50}$/mouse) through subcutaneous route in the footpad at day E9.5. Pregnant mice were administrated with 50 mg/kg chloroquine or vehicle intraperitoneally 4 h post infection and once daily for the following 5 days. At day E14.5, pregnant mice were euthanatized. E14.5 fetuses, placentas, maternal blood and maternal spleen were harvested.

2.9. RNA extraction and virus RNA quantitation

Total RNA from cultured cells, tissues or plasma was extracted using RNeasy lipid tissue mini kit (Qiagen), according to manufacturer's instructions. Quantitative RT-PCR was performed using one-step QuantiTect SYBR Green RT-PCR Kit (Qiagen) in a CFX96 detection system (Bio-Rad). Primers and cycling conditions were described by us previously (Li et al., 2017b).

2.10. Statistical analysis

Mean and standard error (SEM) were calculated for each assay. All

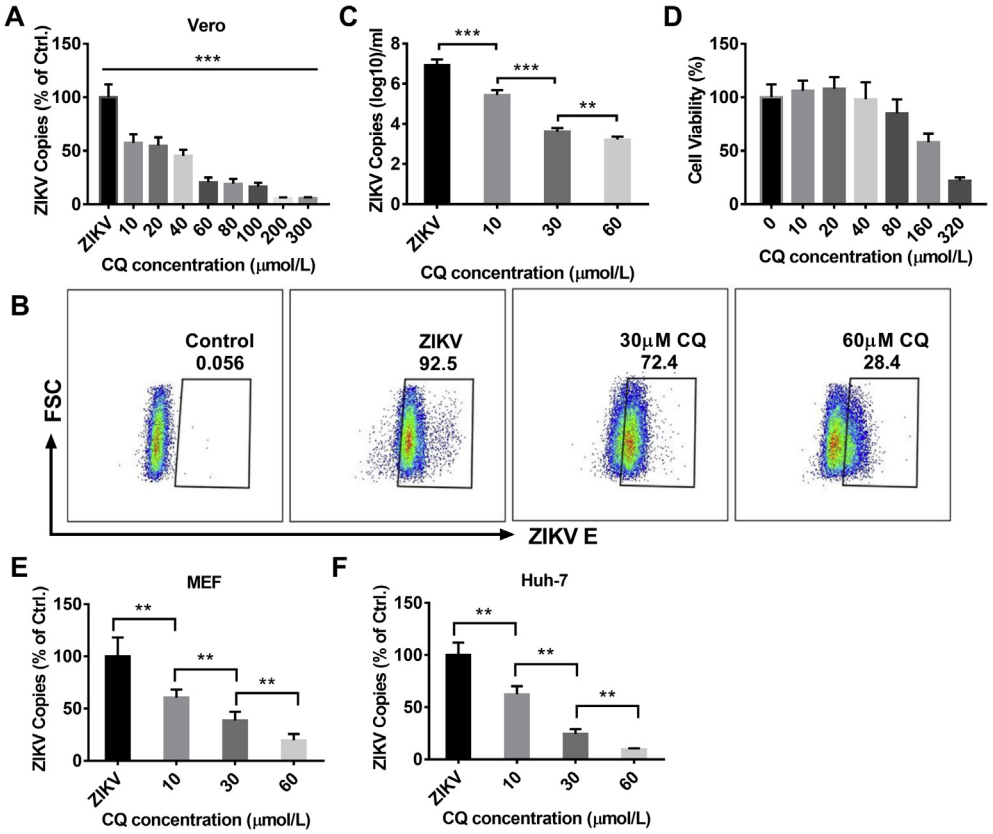


Fig. 1. Chloroquine inhibits ZIKV infection in different cell types. (**A**) Dose-dependent inhibition of ZIKV infection by chloroquine in Vero cells. Cells were infected at a multiplicity of infection (MOI) of 1 at 4 °C for 1 h and were treated with chloroquine at different concentration for 24 h. The copy number of ZIKV in cells was determined by quantitative RT-PCR. ZIKV infected cells with vehicle were used as control. (**B**) The percentage of ZIKV positive cells analyzed by flow-cytometry in ZIKV infected cells with treatment of chloroquine. ZIKV-infected Vero cells were treated with chloroquine for 48 h. The frequency of infected cells was analyzed by flow-cytometry staining ZIKV envelope protein with 4G2 antibody. (**C**) Zika progeny virus's production in Vero cells treated with chloroquine. Progeny viruses in cell culture supernatants were quantified at 72 h post infection. (**D**) Cell toxicity of chloroquine at different concentrations. Vero cells were exposed to chloroquine at different concentrations for 48 h. Cell viability was determined by Cell Counting Kit-8 (CCK-8). Cells treated with vehicle were used as control. (**E-F**) Dose-dependent inhibition of ZIKV infection by chloroquine in MEF cells (**E**) and Huh-7 cells (**F**). ZIKV infected MEF and Huh-7 cells were treated with chloroquine and subjected to quantitative RT-PCR.

analysis was performed on GraphPad Prism v.7 (GraphPad Software). Statistical significance was assessed by unpaired student *t*-test, multiple comparison was performed by one-way analysis of variance (One-way ANOVA) (*P < 0.05, **P < 0.01, ***P < 0.001).

3. Results

3.1. Chloroquine inhibits ZIKV infection in different cell lines

We first assessed the effect of chloroquine on ZIKV infection in Vero cells which was originated from African Green monkey kidney. Chloroquine treatments decreased the copy number of ZIKV-RNA in a dose-dependent manner in Vero cells (Fig. 1A). Flow cytometry analysis also showed a reduction of ZIKV E protein expression in ZIKV-infected cells treated with chloroquine (Fig. 1B). Moreover, chloroquine significant decreased viral particles in the medium of ZIKV infected Vero cells. Compared to about 50% reduction of viral RNA copies in cells, exposure of infected Vero cells to 30 μM chloroquine caused approximately 1000-fold reduction of viral RNA copies in the culture media (Fig. 1C). To confirm that this viral inhibition is independent of chloroquine cytotoxicity on cells, uninfected Vero cells were treated with chloroquine for 2 days. Chloroquine did not significantly impact cell viability at a concentration up to 80 μM (Fig. 1D). Therefore, chloroquine might not only inhibit the infectivity of ZIKV, but also reduce the viral production. Considering that several FDA-approved drugs such as daptomycin and sertraline have been reported to show inhibition on ZIKV infection in one cell line but failed to show inhibition in other cell lines (Barrows et al., 2016), we thus investigated if chloroquine could inhibit ZIKV infection in two additional cell lines including MEFs and Huh-7 cells. Chloroquine dramatically reduced ZIKV infectivity in these cells (Fig. 1E and F). These data supported that chloroquine could effectively inhibit ZIKV infection in different cell types in a dose-dependent manner.

3.2. Chloroquine protects against ZIKV infection in immunocompromised neonatal and adult SCID Beige mice

To evaluate the protective effects of chloroquine *in vivo*, we first established a lethal ZIKV infection model in 1-day old neonatal SCID Beige mice. Intraperitoneal infection with 1.5×10^3 $TCID_{50}$ of GZ02 ZIKV resulted in severe mortality around two weeks (Fig. S1). Treatment with 50 mg/kg chloroquine daily significantly protected infected suckling mice from ZIKV-induced mortality. All vehicle-treated ZIKV-infected mice died within 2 weeks after infection, whereas 80% of chloroquine-treated ZIKV-infected animals survived (Fig. 2A). Remarkably, chloroquine treatment resulted in decreased viral load in neonatal brains and spleens (Fig. 2B and C). We also evaluated the protective effects of chloroquine in adult SCID Beige mice. 6-week old SCID Beige mice were subcutaneously inoculated in the footpad with 1.5×10^5 $TCID_{50}$ ZIKV per mouse and were intraperitoneally administrated with 50 mg/kg chloroquine or vehicle once daily for 5 days. Viremia was significantly reduced in chloroquine-treated mice (Fig. 2D–F). These data demonstrated that chloroquine could protect ZIKV-infected immune compromised SCID Beige neonatal and adult mice.

3.3. Chloroquine prevents maternal to fetal ZIKV transmission in a pregnant SCID Beige mouse model

To determine if chloroquine has any effect on maternal to fetal ZIKV transmission, pregnant female SCID Beige mice were firstly infected with ZIKV at 7.5×10^5 $TCID_{50}$ per mouse at E9.5. Chloroquine was administrated with 50 mg/kg intraperitoneally 4 h post infection and once daily for the following 5 days. Compared to PBS treatment group, chloroquine treatment significantly reduced the viral loads in the blood and spleens of ZIKV infected pregnant mice (Fig. 3A and B). Most importantly, chloroquine treatment dramatically reduced ZIKV infection in the placentas (Fig. 3C) and fetal brains (Fig. 3D). Chloroquine

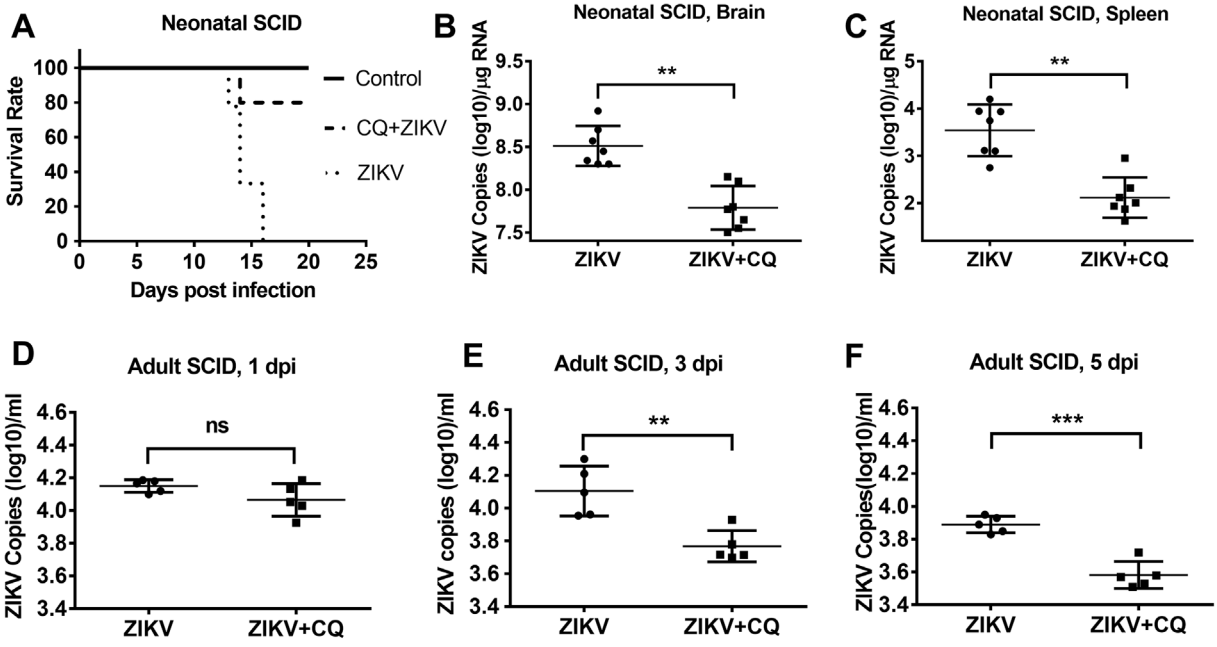
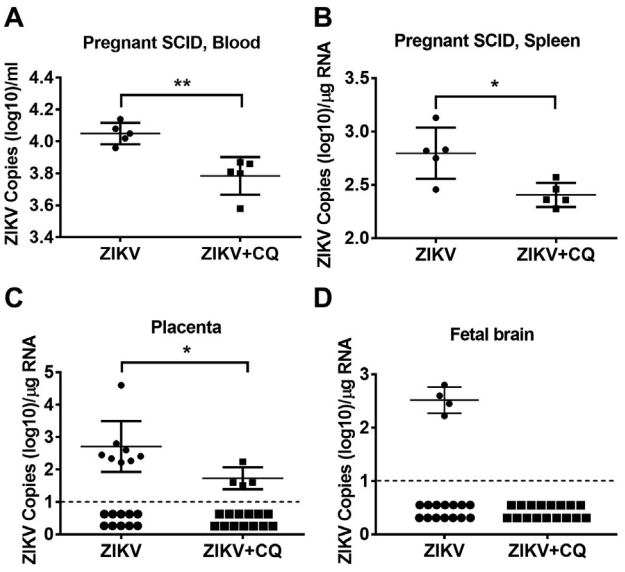


Fig. 2. Chloroquine protects against ZIKV infection in adult and neonatal SCID Beige immunodeficient mice. (**A**) Survival of ZIKV-infected suckling SCID Beige mice treated with chloroquine. 1-day old suckling SCID Beige mice were intraperitoneally infected with ZIKV strain GZ02 (1.5×10^3 $TCID_{50}$/mouse). 50 mg/kg chloroquine or vehicle was administrated to mice intraperitoneally 4 h post infection and once daily for another 5 days. Survival of ZIKV-infected suckling SCID Beige mice were followed until 20 days post infection. (**B–C**) Fifteen days after infection, suckling mice were euthanatized. The viral loads in the brains (**B**) and spleens (**C**) were evaluated using quantitative RT-PCR. n = 7–10, representation from 3 independent experiments. (**D**) Viremia was measured at 1 day post infection. 6-week old SCID Beige mice were subcutaneously inoculated in the footpad with ZIKV strain GZ02 (1.5×10^5 $TCID_{50}$/mouse). Mice were administrated with 50 mg/kg chloroquine or vehicle intraperitoneally at 4 h post infection and once daily for 5 days. n = 5, representation from 3 independent experiments. (**E**) Viremia was measured at 3 days post infection. (**F**) Viremia was measured at 5 days post infection.

treatment showed no apparent adverse effect on pregnant mice and fetuses (Figs. S2A and B). As a result, chloroquine treatment of ZIKV infected pregnant mice had apparent healthy fetuses and placentas, whereas ZIKV infected pregnant mice with PBS had stillbirth and deformed placentas (Fig. 3E). These data demonstrated that chloroquine can effectively inhibit ZIKV infection in pregnant mice and reduce maternal to fetal ZIKV transmission, which resulted in lower risk of microcephaly and fetus defects.

3.4. Chloroquine blocks the release of viral RNA from the endosome into the cytoplasm

We next investigated the stage of the ZIKV life cycle that may be affected by chloroquine. Although we observed that chloroquine has no effect on the binding of ZIKV to the cell membrane (Fig. S3), a transient treatment of chloroquine during the early stage of infection could significantly inhibit ZIKV infection (Fig. 4A). Chloroquine is a weakly basic compound and is able to increase pH in endosome. We speculated that chloroquine prevented the disassembly of viral particles to release viral RNA from endosome to cytoplasm for replication. To test this assumption, endosomes were isolated from ZIKV infected Vero cells and



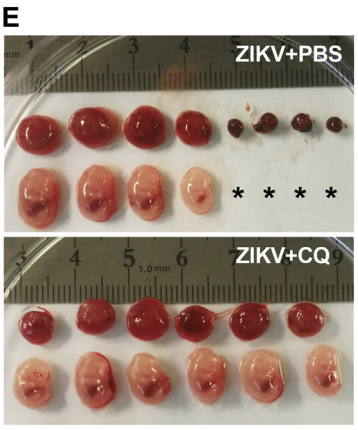


Fig. 3. Chloroquine prevents maternal to fetal ZIKV transmission in mice. (A–B) 10-week old female pregnant SCID Beige mice were inoculated with ZIKV strain GZ02 (7.5×10^5 $TCID_{50}$/mouse) through subcutaneous route in the footpad at day E9.5. Pregnant mice were administrated with 50 mg/kg chloroquine or vehicle intraperitoneally 4 h post infection and once daily for the following 5 days. At day E14.5, pregnant mice were euthanatized. Viral loads of blood (**A**) and spleens (**B**) of pregnant mice were determined by quantitative RT-PCR. (**C**) Viral loads in the placentas of pregnant mice. (**D**) Viral loads of ZIKV in fetal brain. (**E**) Photographs of fetuses and placenta from ZIKV-infected and chloroquine-treated pregnant mice. Highly deformed fetuses and placentas are indicated by asterisks (*).

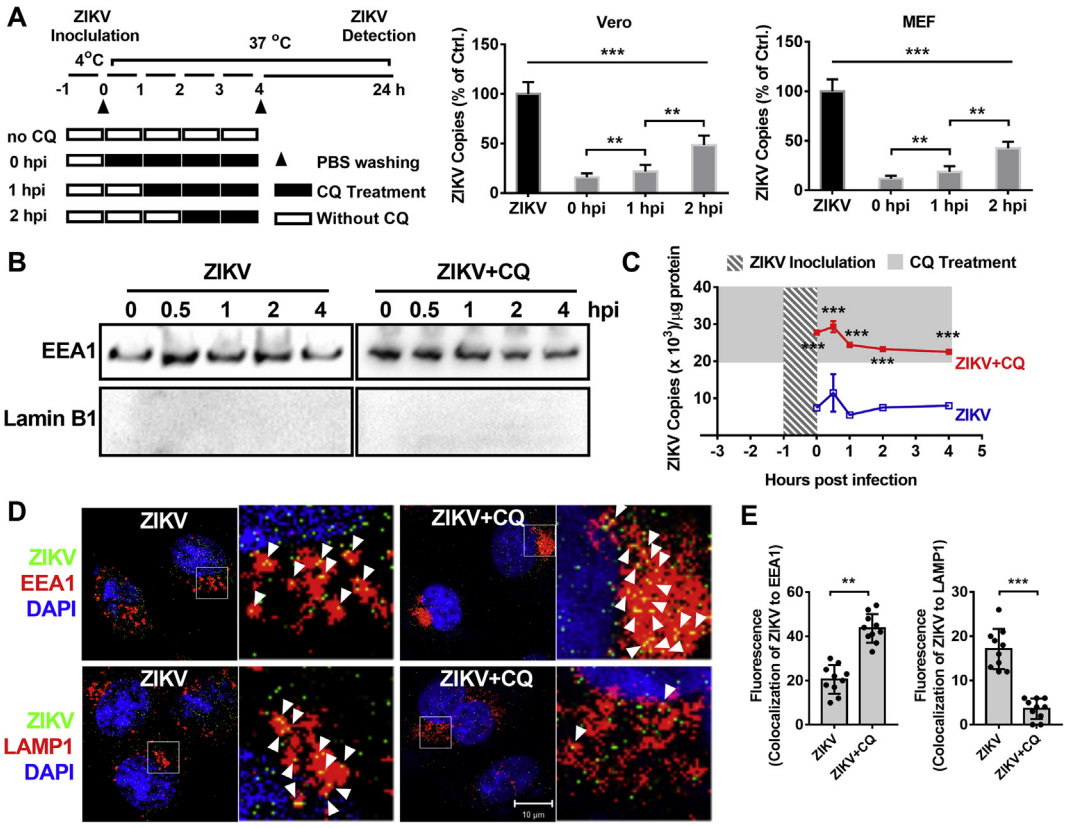


Fig. 4. Chloroquine blocks the release of ZIKV RNA from endosome to cytoplasm. (**A**) Cellular ZIKV RNA copy in Vero cells and MEFs treated with chloroquine at early stage after infection. Vero cells and MEFs were infected with ZIKV (MOI = 1) at 4 °C for 1 h and washed twice by cold PBS. Chloroquine at 60 μM was added at early stage (0, 1, 2 h post infection) and changed to fresh medium at 4 h post infection. 24 h post infection, the total RNA was isolated and quantified by quantitative RT-PCR. Cells infected with ZIKV were used as control. (**B**) Immunoblot analysis for endosome marker EAA1 and nucleus marker Lamin B1. (**C**) Level of ZIKV RNA in purified endosomes from ZIKV-infected Vero cells with or without chloroquine treatment. Vero cells were pretreated with 60 μM chloroquine for 2 h and then infected with ZIKV for 1 h. Endosomes were isolated and viral RNA was quantified by quantitative RT-PCR. The amount of endosomal viral RNA was normalized to total endosomal proteins. (**D**) Co-localization of ZIKV RNA and endosomes in Vero cells. Vero cells were pretreated with 60 μM chloroquine for 2 h and then infected with EU labeled ZIKV (green). The EU labeled ZIKV was incubated with cells for 2 h, then fixed and stained by cy3-labeled antibodies (red) against EEA1 for early endosome or LAMP1 for late endosome. Diamidino-2-phenylindole (DAPI) was used for staining nucleus (blue). The co-localization of ZIKV RNA and EEA1 or LAMP1 are indicated by white arrows. (**E**) Quantification of co-localization determined by confocal fluorescence microscopy. Ten randomly selected regions were imaged and the data were analyzed using ImageJ software.

verified by early endosome marker EAA1 and nucleus marker Lamin B1 (Fig. 4B). We measured the viral RNA copy numbers in purified endosomes from ZIKV infected cells with or without chloroquine treatment and the amount of endosomal viral RNA was normalized to total endosomal proteins. The amount of viral RNA in the endosome in ZIKV infected cells had a transient increase at 0.5 h and then decreased rapidly after infection. However, the amount of viral RNA in the endosome of chloroquine pretreated cells was approximately 4-folds higher than that of the untreated group and remained at higher level for at least for 4 h (Fig. 4C). We further demonstrated that chloroquine could block ZIKV endosomal disassembly using immunofluorescence. Cells were stained using antibodies against EEA1 for early endosomes or Lysosomal-Associated Membrane Protein 1 (LAMP1) for late endosomes and lysosomes, respectively. Co-localization of virus particles with each compartment can be used to assess progression through the endocytic network. At 2 h post infection of untreated cells, ZIKV particles were both localized with EEA1 and LAMP1 staining (Fig. 4D left). In contrast, in cells treated with chloroquine, most particles were associated with EEA1 and very few virus particles were co-localized with LAMP1 (Fig. 4D right). Decreased transfer of ZIKV from early endosome to late endosome or lysosome was confirmed by quantification based on immunofluorescence (Fig. 4E). These findings indicated that chloroquine could block the release of ZIKV RNA from the endosome into the cytoplasm through inhibiting disassembly of the internalized virus.

3.5. Chloroquine inhibits ZIKV replication through blocking ZIKV-induced autophagy

To assess whether chloroquine inhibits ZIKV replication by blocking autophagy flux, we treated ZIKV infected cells with chloroquine at a later stage of infection. Chloroquine given after the endosomal release of ZIKV RNA could also decrease cellular viral RNA copies (Fig. 5A). Infection with ZIKV efficiently induced LC3-I to LC3-II conversion, a hallmark of autophagy initiation (Fig. 5B). Rapamycin, an autophagy inducer, promoted ZIKV replication and progeny virus production (Fig. 5C and D). Furthermore, we assessed ZIKV replication using Atg5 deficient MEFs, in which autophagy is completely defective due to the loss of the *Atg5* gene. ZIKV RNA replication was abolished in Atg5 deficient MEFs as compared to wild-type MEFs (Fig. 5C). Deficiency of autophagy also abrogated viral production in Atg5 deficient MEFs (Fig. 5D). We next observed that the level of p62, an autophagy substrate, decreased due to ZIKV-mediated autophagosome maturation. Chloroquine could reverse the ZIKV-induced p62 decrease (Fig. 5B), indicating that chloroquine can block ZIKV-induced autophagic flux. Importantly, chloroquine could achieve the same inhibition effect on ZIKV production in wild-type MEFs as the abrogation of ZIKV replication in Atg5 deficient MEFs (Fig. 5C and D). There was no difference of ZIKV entry to cells between wild-type MEFs and Atg5-deficinet and MEFs (Fig. S4). These results indicated that ZIKV replication is

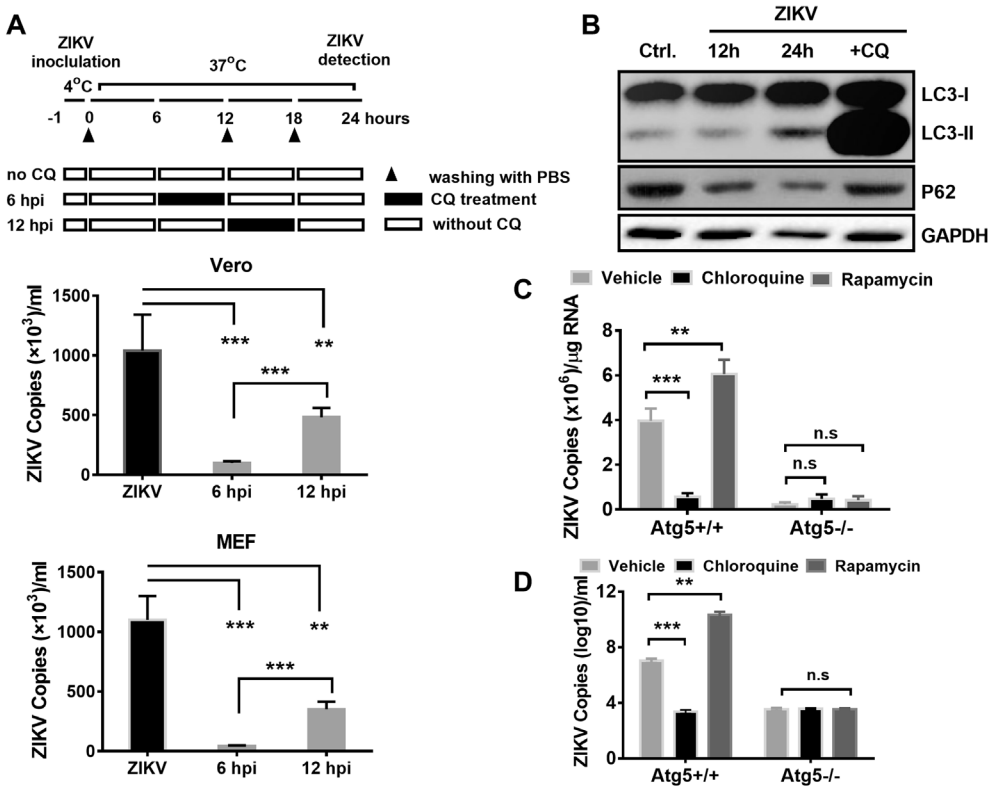


Fig. 5. Chloroquine blocks ZIKV-induced autophagy and autophagy-dependent replication. (**A**) Progeny virus in the cultured media of ZIKV infected Vero cells and MEFs treated with chloroquine at later stage after infection. Cells were infected with ZIKV (MOI = 1) at 4 °C for 1 h and washed twice by cold PBS. Chloroquine at 60 μM was added at later stage (6, 12 h post infection), maintained in culture medium for 6 h, and then fresh media was replaced. The total RNA was purified and ZIKV RNA copy number was quantified by quantitative RT-PCR at 24 h post infection. (**B**) Immunoblot analysis of autophagy markers LC3 and p62. MEFs were infected with ZIKV (MOI = 2) and treated with 60 μM chloroquine. Increasing LC3-II/LC3-I ratio indicates the initiation of autophagy, whereas p62, an autophagy substrate, decreases with the increasing autophagy. Uninfected MEFs were used as the control. (**C**) ZIKV replication in MEFs and Atg5-deficient MEFs treated with chloroquine and Rapamycin. MEFs and Atg5-deficient MEFs were infected with ZIKV (MOI = 1) and exposed to 60 μM chloroquine or 5 μM Rapamycin. ZIKV RNA levels were measured by quantitative RT-PCR at 24 h post infection. (**D**) Progeny ZIKV production of MEFs and Atg5-deficient MEFs treated with chloroquine or Rapamycin. Progeny viruses were quantified in the cultured media at 72 h post infection. All data were shown as mean ± SEM from 3 independent experiments.

associated with ZIKV induced autophagy and chloroquine inhibits autophagy-dependent ZIKV replication.

4. Discussion

Chloroquine is a widely used anti-malarial drug and has been exploited as a non-specific antiviral agent. Previous reports showed that chloroquine inhibited ZIKV infection in cultured cells and protected ZIKV-induced mortality and microcephaly in mice (Delvecchio et al., 2016; Gorshkov et al., 2018; Li et al., 2017a; Shiryaev et al., 2017). In this study, we performed a more in-depth evaluation of the anti-ZIKV effect of chloroquine in diverse cell types and in mouse models, especially in a new maternal to fetal ZIKV transmission model using immunocompromised SCID Beige mice. We demonstrated that chloroquine could reduce ZIKV viremia in adult SCID Beige mice, improved the survival of ZIKV-infected neonatal SCID Beige mice, and prevented maternal to fetal ZIKV transmission. We further demonstrated that chloroquine inhibited ZIKV infection by at least two mechanisms: impeding viral RNA release from endosome and reducing autophagy-dependent viral replication.

ZIKV is an enveloped virus and similar to other *flavivirus*. Virions bind to cell-surface attachment molecules and receptors and are internalized through endocytosis (Hsieh et al., 2014). After internalization, in the low pH of the endosome, viral glycoproteins mediate fusion of viral and cellular membranes, allowing disassembly of the virions and release of RNA into the cytoplasm (Perera et al., 2008). Our study shows that internalized Zika virions progresses from early endosome to late endosome and delivers the viral RNA into cytoplasm. Chloroquine impedes the release of ZIKV RNA from the endosome and further demonstrates the inhibition activity of chloroquine on *flavivirus* infection. It has been documented that chloroquine also exerted antiviral effects at an early step of other enveloped virus infection including Influenza virus and Ebola virus (Di Trani et al., 2007; Madrid et al., 2013).

Autophagy is thought to prepare for the turnover and recycling of cellular components, this process has been shown to promote the replication of Zika virus (Hamel et al., 2015; Liang et al., 2016). Inhibition of autophagy by chloroquine has been proposed as a possible mechanism of its anti-ZIKV infection (Gorshkov et al., 2018). We confirmed that ZIKV infection induced autophagy is associated with ZIKV replication. ZIKV replication and progeny virus production were reduced in Atg5-deficient MEF cells. We further demonstrated that chloroquine inhibited autophagy dependent viral replication. Chloroquine treatment can reduce ZIKV replication and progeny virus production in wildtype MEF cells to the same level as in Atg5-deficient MEF cells. It remains yet to be explored how autophagy regulates ZIKV infection. One possible mechanism is that autophagy promote ZIKV replication. It has been reported that ZIKV can induce autophagy in skin fibroblasts, human fetal neural stem cells and human trophoblasts, and ZIKV replication was promoted by autophagy (Hamel et al., 2015; Liang et al., 2016). On the other hand, secretory autophagy is a non-degradative pathway where autophagosomes fuse with the plasma membrane and release single membrane-bound vesicles to the extracellular environment (Ponpuak et al., 2015). It is known that secretory autophagy pathway helps positive-sense RNA viruses including poliovirus, Coxsackievirus and enterovirus 71 to exit cells (Bird et al., 2014; Chen et al., 2015; Robinson et al., 2014). Recent report shows the importance of autophagy in ZIKV infection in a placental trophoblast Atg16l1 deficient mice model. Atg16l1 deficiency does not affect systemic ZIKV infection, but is sufficient to restrict ZIKV infection in the placenta and its vertical transmission (Cao et al., 2017). Based on these findings, we raise the possibility that autophagy has a potentially facilitative rather than a degradative role in the ZIKV infection, specifically in its vertical transmission from placental trophoblasts to fetal cells.

It has been reported earlier that chloroquine has a protective effect on fetus received embryonic intracerebral ZIKV infection in immunocompetent ICR mice. Chloroquine reduced burden of ZIKV and its associated apoptosis in fetal brain (Li et al., 2017a). However, embryonic intracranial infection of ZIKV does not mimic maternal to fetal transmission and requires highly technical performance to avoid brain damage caused by the injection. A recent paper reported that chloroquine treatment attenuated vertical transmission of ZIKV and reduced viral load in fetal brain in interferon signaling competent SJL mice (Shiryaev et al., 2017). Previous reports show that chloroquine treatment inhibits protective immune response against *Plasmodium yoelii* infection via suppressing the activation of T helper cells, macrophages, and B cells in mice (Qin et al., 2014). Chloroquine also depresses the percentage of lymphocyte subpopulation and their proliferative response in rhesus monkeys (Prasad et al., 1987). Another finding indicates that chloroquine modulates cytokine release by promoting Th17 immunity (Said et al., 2014). We thus established a novel ZIKV infection model in immune-deficient SCID Beige mice to assess the anti-ZIKV effect of chloroquine. SCID Beige is a strain of double-mutant mice with impaired T and B cell development and diminished natural killer (NK) cell activity (Bosma and Carroll, 1991; Roder and Duwe, 1979). These mice are highly susceptible to viral and bacterial infection (Percy and Barta, 1993; Winslow et al., 1998) and have minimal immunological influences on drug evaluation. Our study further demonstrated that chloroquine could protect fetuses from maternal transmission in pregnant mouse mice in the absence of ZIKV specific adaptive immune response. Notably, chloroquine and its derivatives such as hydroxychloroquine is a class C drug that is used to treat pregnant women with malaria and autoimmune diseases (Sperber et al., 2009). It has been documented that chloroquine does not increase the fetal defects or other adverse events at various stages of pregnancy for the prevention of malaria (Villegas et al., 2007). Taken together, these studies provided valuable information for supporting the use of chloroquine against ZIKV infection in pregnant women.

In summary, chloroquine effectively protected ZIKV-infected mice from mortality and microcephaly. Chloroquine inhibited ZIKV infection by impeding ZIKV release from endosome to cytoplasm and by blocking autophagy-dependent replication. Chloroquine should be considered as a drug for treating ZIKV infection especially for minimizing the risk of ZIKV induced microcephaly in pregnant women.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.104547.

References

Adelusi, S.A., Salako, L.A., 1982. Tissue and blood concentrations of chloroquine following chronic administration in the rat. J. Pharm. Pharmacol. 34, 733–735.
Barrows, N.J., Campos, R.K., Powell, S.T., Prasanth, K.R., Schott-Lerner, G., Soto-Acosta, R., Galarza-Munoz, G., McGrath, E.L., Urrabaz-Garza, R., Gao, J., Wu, P., Menon, R., Saade, G., Fernandez-Salas, I., Rossi, S.L., Vasilakis, N., Routh, A., Bradrick, S.S., Garcia-Blanco, M.A., 2016. A screen of FDA-approved drugs for inhibitors of Zika virus infection. Cell Host Microbe 20, 259–270.
Baud, D., Gubler, D.J., Schaub, B., Lanteri, M.C., Musso, D., 2017. An update on Zika virus infection. Lancet 390, 2099–2109.
Bird, S.W., Maynard, N.D., Covert, M.W., Kirkegaard, K., 2014. Nonlytic viral spread enhanced by autophagy components. Proc. Natl. Acad. Sci. U. S. A. 111, 13081–13086.
Bosma, M.J., Carroll, A.M., 1991. The SCID mouse mutant: definition, characterization, and potential uses. Annu. Rev. Immunol. 9, 323–350.
Cao, B., Parnell, L.A., Diamond, M.S., Mysorekar, I.U., 2017. Inhibition of autophagy limits vertical transmission of Zika virus in pregnant mice. J. Exp. Med. 214, 2303–2313.
Chen, Y.H., Du, W., Hagemeijer, M.C., Takvorian, P.M., Pau, C., Cali, A., Brantner, C.A., Stempinski, E.S., Connelly, P.S., Ma, H.C., Jiang, P., Wimmer, E., Altan-Bonnet, G., Altan-Bonnet, N., 2015. Phosphatidylserine vesicles enable efficient en bloc transmission of enteroviruses. Cell 160, 619–630.
Cragan, J.D., Mai, C.T., Petersen, E.E., Liberman, R.F., Forestieri, N.E., Stevens, A.C., Delaney, A., Dawson, A.L., Ellington, S.R., Shapiro-Mendoza, C.K., Dunn, J.E., Higgins, C.A., Meyer, R.E., Williams, T., Polen, K.N., Newsome, K., Reynolds, M., Isenburg, J., Gilboa, S.M., Meaney-Delman, D.M., Moore, C.A., Boyle, C.A., Honein, M.A., 2017. Baseline prevalence of birth defects associated with congenital Zika virus infection - Massachusetts, North Carolina, and Atlanta, Georgia, 2013-2014. MMWR Morb. Mortal. Wkly. Rep. 66, 219–222.
Delvecchio, R., Higa, L.M., Pezzuto, P., Valadao, A.L., Garcez, P.P., Monteiro, F.L., Loiola, E.C., Dias, A.A., Silva, F.J., Aliota, M.T., Caine, E.A., Osorio, J.E., Bellio, M., O'Connor, D.H., Rehen, S., de Aguiar, R.S., Savarino, A., Campanati, L., Tanuri, A., 2016. Chloroquine, an Endocytosis Blocking Agent, Inhibits Zika Virus Infection in Different Cell Models. Viruses 8.
Di Trani, L., Savarino, A., Campitelli, L., Norelli, S., Puzelli, S., D'Ostilio, D., Vignolo, E., Donatelli, I., Cassone, A., 2007. Different pH requirements are associated with divergent inhibitory effects of chloroquine on human and avian influenza A viruses. Virol. J. 4, 39.
Dick, G.W., Kitchen, S.F., Haddow, A.J., 1952. Zika virus. I. Isolations and serological specificity. Trans. R. Soc. Trop. Med. Hyg. 46, 509–520.
Farias, K.J., Machado, P.R., Muniz, J.A., Imbeloni, A.A., da Fonseca, B.A., 2015. Antiviral activity of chloroquine against dengue virus type 2 replication in Aotus monkeys. Viral Immunol. 28, 161–169.
Gorshkov, K., Shiryaev, S.A., Fertel, S., Lin, Y.W., Huang, C.T., Pinto, A., Farhy, C., Strongin, A.Y., Zheng, W., Terskikh, A.V., 2018. Zika virus: origins, pathological action, and treatment strategies. Front. Microbiol. 9, 3252.
Hamel, R., Dejarnac, O., Wichit, S., Ekchariyawat, P., Neyret, A., Luplertlop, N., Perera-Lecoin, M., Surasombatpattana, P., Talignani, L., Thomas, F., Cao-Lormeau, V.M., Choumet, V., Briant, L., Despres, P., Amara, A., Yssel, H., Misse, D., 2015. Biology of Zika virus infection in human skin cells. J. Virol. 89, 8880–8896.
Hsieh, S.C., Wu, Y.C., Zou, G., Nerurkar, V.R., Shi, P.Y., Wang, W.K., 2014. Highly conserved residues in the helical domain of dengue virus type 1 precursor membrane protein are involved in assembly, precursor membrane (prM) protein cleavage, and entry. J. Biol. Chem. 289, 33149–33160.
Kitchen, L.W., Vaughn, D.W., Skillman, D.R., 2006. Role of US military research programs in the development of US Food and Drug Administration–approved antimalarial drugs. Clin. Infect. Dis. 43, 67–71.
Law, I., Ilett, K.F., Hackett, L.P., Page-Sharp, M., Baiwog, F., Gomorrai, S., Mueller, I., Karunajeewa, H.A., Davis, T.M., 2008. Transfer of chloroquine and desethylchloroquine across the placenta and into milk in Melanesian mothers. Br. J. Clin. Pharmacol. 65, 674–679.
Li, C., Zhu, X., Ji, X., Quanquin, N., Deng, Y.Q., Tian, M., Aliyari, R., Zuo, X., Yuan, L., Afridi, S.K., Li, X.F., Jung, J.U., Nielsen-Saines, K., Qin, F.X., Qin, C.F., Xu, Z., Cheng, G., 2017a. Chloroquine, a FDA-approved drug, prevents Zika virus infection and its associated congenital microcephaly in mice. EBioMedicine 24, 189–194.
Li, F., Wang, P.R., Qu, L.B., Yi, C.H., Zhang, F.C., Tang, X.P., Zhang, L.G., Chen, L., 2017b. AXL is not essential for Zika virus infection in the mouse brain. Emerg. Microb. Infect. 6, e16.
Liang, Q., Luo, Z., Zeng, J., Chen, W., Foo, S.S., Lee, S.A., Ge, J., Wang, S., Goldman, S.A., Zlokovic, B.V., Zhao, Z., Jung, J.U., 2016. Zika virus NS4A and NS4B proteins deregulate Akt-mTOR signaling in human fetal neural stem cells to inhibit neurogenesis and induce autophagy. Cell Stem Cell 19, 663–671.
Madrid, P.B., Chopra, S., Manger, I.D., Gilfillan, L., Keepers, T.R., Shurtleff, A.C., Green, C.E., Iyer, L.V., Dilks, H.H., Davey, R.A., Kolokoltsov, A.A., Carrion Jr., R., Patterson, J.L., Bavari, S., Panchal, R.G., Warren, T.K., Wells, J.B., Moos, W.H., Burke, R.L., Tanga, M.J., 2013. A systematic screen of FDA-approved drugs for inhibitors of biological threat agents. PLoS One 8, e60579.
Miner, J.J., Diamond, M.S., 2017. Zika virus pathogenesis and tissue tropism. Cell Host Microbe 21, 134–142.
Mizui, T., Yamashina, S., Tanida, I., Takei, Y., Ueno, T., Sakamoto, N., Ikejima, K., Kitamura, T., Enomoto, N., Sakai, T., Kominami, E., Watanabe, S., 2010. Inhibition of hepatitis C virus replication by chloroquine targeting virus-associated autophagy. J. Gastroenterol. 45, 195–203.
Mlakar, J., Korva, M., Tul, N., Popovic, M., Poljsak-Prijatelj, M., Mraz, J., Kolenc, M., Resman Rus, K., Vesnaver Vipotnik, T., Fabjan Vodusek, V., Vizjak, A., Pizem, J., Petrovec, M., Avsic Zupanc, T., 2016. Zika virus associated with microcephaly. N. Engl. J. Med. 374, 951–958.
Percy, D.H., Barta, J.R., 1993. Spontaneous and experimental infections in scid and scid/beige mice. Lab. Anim. Sci. 43, 127–132.
Perera, R., Khaliq, M., Kuhn, R.J., 2008. Closing the door on flaviviruses: entry as a target for antiviral drug design. Antivir. Res. 80, 11–22.
Ponpuak, M., Mandell, M.A., Kimura, T., Chauhan, S., Cleyrat, C., Deretic, V., 2015. Secretory autophagy. Curr. Opin. Cell. Biol. 35, 106–116.
Prasad, R.N., Mahajan, R.C., Ganguly, N.K., 1987. Effect of chloroquine on cellular immune-responses of normal and P-Knowlesi-Infected rhesus-monkeys. Immunol. Cell Biol. 65, 211–216.
Pullman, T.N., Craige Jr., B., et al., 1948. Comparison of chloroquine, quinacrine (atabrine) and quinine in the treatment of acute attacks of sporozoite-induced vivax

malaria, Chesson strain. J. Clin. Investig. 27, 46–50.
Qin, X., Chen, G., Feng, Y., Zhu, X., Du, Y., Pang, W., Qi, Z., Cao, Y., 2014. Early treatment with chloroquine inhibits the immune response against Plasmodium yoelii infection in mice. Tohoku J. Exp. Med. 234, 271–280.
Robinson, S.M., Tsueng, G., Sin, J., Mangale, V., Rahawi, S., McIntyre, L.L., Williams, W., Kha, N., Cruz, C., Hancock, B.M., Nguyen, D.P., Sayen, M.R., Hilton, B.J., Doran, K.S., Segall, A.M., Wolkowicz, R., Cornell, C.T., Whitton, J.L., Gottlieb, R.A., Feuer, R., 2014. Coxsackievirus B exits the host cell in shed microvesicles displaying autophagosomal markers. PLoS Pathog. 10, e1004045.
Roder, J., Duwe, A., 1979. The beige mutation in the mouse selectively impairs natural killer cell function. Nature 278, 451–453.
Said, A., Bock, S., Lajqi, T., Muller, G., Weindl, G., 2014. Chloroquine promotes IL-17 production by CD4+ T cells via p38-dependent IL-23 release by monocyte-derived Langerhans-like cells. J. Immunol. 193, 6135–6143.
Savarino, A., Gennero, L., Chen, H.C., Serrano, D., Malavasi, F., Boelaert, J.R., Sperber, K., 2001. Anti-HIV effects of chloroquine: mechanisms of inhibition and spectrum of activity. AIDS 15, 2221–2229.
Shiryaev, S.A., Mesci, P., Pinto, A., Fernandes, I., Sheets, N., Shresta, S., Farhy, C., Huang, C.T., Strongin, A.Y., Muotri, A.R., Terskikh, A.V., 2017. Repurposing of the anti-malaria drug chloroquine for Zika Virus treatment and prophylaxis. Sci. Rep. 7, 15771.
Sperber, K., Hom, C., Chao, C.P., Shapiro, D., Ash, J., 2009. Systematic review of hydroxychloroquine use in pregnant patients with autoimmune diseases. Pediatr. Rheumatol. Online J. 7, 9.
Sweiti, H., Ekwunife, O., Jaschinski, T., Lhachimi, S.K., 2017. Repurposed therapeutic agents targeting the Ebola virus: a systematic review. Curr. Ther. Res. Clin. Exp. 84, 10–21.
Villegas, L., McGready, R., Htway, M., Paw, M.K., Pimanpanarak, M., Arunjerdja, R., Viladpai-Nguen, S.J., Greenwood, B., White, N.J., Nosten, F., 2007. Chloroquine prophylaxis against vivax malaria in pregnancy: a randomized, double-blind, placebo-controlled trial. Trop. Med. Int. Health 12, 209–218.
Winslow, G.M., Yager, E., Shilo, K., Collins, D.N., Chu, F.K., 1998. Infection of the laboratory mouse with the intracellular pathogen Ehrlichia chaffeensis. Infect. Immun. 66, 3892–3899.