UC San Diego
UC San Diego Electronic Theses and Dissertations

Title

Mechanism of Action of Methotrexate Against Zika Virus

Permalink

https://escholarship.org/uc/item/4ch0p661

Author

Beck, Sungjun

Publication Date

2018

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA SAN DIEGO

Mechanism of Action of Methotrexate Against Zika Virus

A Thesis submitted in partial satisfaction of the requirements for the degree

Master of Science

in

Biology

by

Sungjun Beck

Committee in charge:

Jair Lage de Siqueira-Neto, Chair
Michael David, Co-Chair
Stephanie Mel

The Thesis of Sungjun Beck is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

_____

_____

Co-Chair

_____

Chair

University of California San Diego

2018

DEDICATION

I would like to thank all of professors from University of California, San Diego, who had taught me; truly, each lecture helped me to build the foundation in biological sciences. Specially, the knowledge I gained from the following classes, Dr. Li-fan Lu's Immunology, Dr. Cindy Gustafson-Brown's Virology, and Dr. Matthew Daugherty's Human Pathogenic Viruses, inspired me with the field of host-pathogen interactions. Encouragement and unconditional love from my family: Younglim Lee 이영림, Hanick Beck 백한익, Youngjun Beck 백영준, and Mak-nae 막내, have been the pure source of motivation to complete my Master's thesis. Not to mention, because of your full support, Edie Zhang, I could stay strong during my Biology BSMS program.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

In respect of his passion for education and mentorship, I would like to thank my mentor, Dr. Jair Lage de Sequeira Neto. Because of his guidance and care, I could grow not only as an independent researcher but also a humble human. I appreciate his training for critical thinking and data analysis.

I would like to also acknowledge Dr. David Smith for his lessons in writing a manuscript. Through his lessons, I have learned effective strategies of how to design a conclusion and future directions of the project.

I appreciate the technical training I received from John and Alex, specifically the hands-on experience in Dr. Deborah Spectre's Lab. Additionally, I would like to thank Michelli and Thomas who helped me sharpen my thesis defense and encouraged me throughout this project. The ending is always the most difficult part.

This thesis in full is currently being prepared for submission for publication of the material. Sungjun Beck, the thesis author was the primary investigator and author of this material. This thesis in full is coauthored with Jean A. Bernatchez, Zhe Zhu, Michelli F. De Oliveira, and David M. Smith. The corresponding author is Jair Lage de Siqueira-Neto.

ABSTRACT OF THE THESIS

Mechanism of Action of Methotrexate Against Zika Virus

by

Sungjun Beck

Master of Science in Biology

University of California San Diego, 2018

Professor Jair Lage de Siqueira-Neto, Chair
Professor Michael David, Co-Chair

Zika Virus (ZIKV), which is associated with microcephaly and Guillain-Barré syndrome, brought a serious public health impact to most of Latin America countries in 2015 and 2016 during its endemic outbreak. Still, there is no effective antiviral drug or vaccine for ZIKV infection, addressing urgency to develop therapeutic solutions for ZIKV infection. Previously, using high throughput screening (HTS) technology, several potential hit molecules against ZIKV have been discovered, and Methotrexate (MTX) was identified as one of them. While MTX has been clinically used as a chemotherapy and anti-rheumatoid reagent, in this study, I investigated the mechanism of action of MTX against Zika virus (ZIKV) *in vitro* using Vero cells and human neural stem cells (hNSCs). In plaque assays, the antiviral effects of MTX against ZIKV were studied, showing a ten-fold decrease of the virus titer. To understand the antiviral mechanism of action of

MTX, we focused on MTX's target enzyme, dihydrofolate reductase (DHFR), and its pathways. To confirm that the antiviral effect of MTX is caused from antagonism of DHFR, a downstream metabolite of DHFR pathway, Leucovorin, was used as together with MTX as co-treatment for ZIKV-infected host cells. Surprisingly, addition of Leucovorin rescued the ZIKV replication during MTX treatment, supporting the idea that the antiviral effect of MTX might be facilitated through inhibition of DHFR. The antagonism of DHFR leads to a decrease of the *de novo* synthesis of purines and pyrimidines. Additional antiviral mechanism of MTX were studied using GAT (Glycine, Adenosine, and Thymidine) medium in which adenosine alone showed the rescue effect on ZIKV replication from MTX treatment.

INTRODUCTION

Flaviviruses, such as Dengue virus, (DENV), yellow fever virus (YFV), West Nile virus (WNV), Japanese encephalitis virus (JEV), and Zika virus (ZIKV) are well characterized as human pathogenic viruses transmitted by *Aedes* spp. mosquitoes as one of the main vectors. Key symptoms of an infection with these flaviviruses include hemorrhagic fever (DENV), jaundice (YFV), and encephalitis (WNV, JEV, and ZIKV) (Gould and Solomon 2008). Recently, ZIKV, an emerging flavivirus, has become a long-term global public health threat; the recent outbreak of the virus in Latin America has brought substantial economic burden to the affected countries (Colon-Gonzalez, Peres et al. 2017). Known for its unique neurotropism, ZIKV caused a substantial number of clinical cases of ZIKV-induced congenital neurological disorders, mainly microcephaly to fetuses, during 2015 and 2016 in Brazil (de Oliveira, de Franca et al. 2017). Reports during the ZIKV outbreak also showed a high correlation of viral infection to another neural disorder, Guillain–Barré Syndrome, in adults in affected areas (Parra, Lizarazo et al. 2016). Despite the threat the virus poses to public health, there are currently no specific antivirals or vaccines to treat ZIKV infection, highlighting the importance of studying the viral replication of ZIKV to inform the development of therapeutic solutions.

High-throughput screening (HTS) of various compound libraries against ZIKV have been published, identifying a number of hit molecules against ZIKV (Xu, Lee et al. 2016, Adcock, Chu et al. 2017, Rausch, Hackett et al. 2017, Bernatchez, Yang et al. 2018). We also performed a drug library screen and identified anti-Zika activity for methotrexate (MTX). MTX has been clinically used to treat distinct types of pathologies including leukemia, psoriatic arthritis, and rheumatoid arthritis (Black, O'Brien et al. 1964, Hryniuk and Bertino 1969, Willkens, Watson et al. 1980). As a chemotherapy agent, the main function of MTX is to antagonize dihydrofolate reductase (DHFR)

to decrease *de novo* synthesis of purines and pyrimidines (Hryniuk, Brox et al. 1975, Jolivet, Cowan et al. 1983), thereby inhibiting the viability of highly replicating cells. Nevertheless, MTX has been reported to interfere with diverse cellular mechanisms such as oxidative stress or cellular differentiation via methylation (Elango, Dayalan et al. 2014, Sramek, Neradil et al. 2016). Interestingly, DHFR inhibitors have also been investigated for their efficacy in infectious diseases. Due to its formation of a specific dimer with thymidylate synthase (TS) (Ivanetich and Santi 1990), protozoan DHFR has been studied as a drug target to treat parasitic diseases (Renslo and McKerrow 2006). As antiviral agents, MTX and the antimetabolite floxuridine have been shown to decrease the replication of DENV, a close cousin to ZIKV, through inhibition of host DHFR and TS (Fischer, Smith et al. 2013).

Here, I demonstrate the antiviral activity of MTX against ZIKV and probe the mechanism of action of MTX using downstream metabolites of the DHFR pathway, leucovorin and adenosine. ZIKV replication was readily rescued from MTX inhibition with excess amounts of leucovorin and adenosine, supporting an antiviral mechanism for MTX through the antagonism of DHFR and a concomitant decrease in the host adenosine triphosphate (ATP) pool. This work provides the basis for further studies examining the DHFR pathway as a potential host-directed target for ZIKV therapeutic intervention.

Specific Aims

In this study, I have investigated the antiviral mechanism of MTX against ZIKV *in vitro*, using two mammalian cell lines, Vero cells and human neural stem cells (hNSCs). To test antiviral activity of MTX against ZIKV, immunofluorescence imaging was used to observe the difference in the level of ZIKV envelope protein synthesis from MTX treatment. Then, to study the kinetics

of viral replication, I used a standard plaque assay to observe the fluctuation of ZIKV titers at three time points, 1 h post infection (PI), 48 h PI, and 96 h PI, in the presence of MTX. To study the efficacy of MTX against ZIKV, I also performed a standard plaque assay to observe the reduction of ZIKV titers at four different concentrations of MTX: 50.0 μM, 6.25 μM, 0.781 μM, and 0.0977 μM, in a dose-response assessment.

Thorough cytotoxicity tests of MTX were performed using two different cell viability assays: first, the Cell TiterGlo® (CTG) reagent to measure the level of adenosine triphosphate (ATP) as a luminescent readout of cell viability biomarker, and second, the Cell TiterFluor™ (CTF) reagent to measure viable cellular protease activity as a fluorescent readout of cell viability biomarker. Then, the cytotoxic concentration 50 (CC_{50}) was calculated. Finally, through bright-field image analysis and directly counting the number of cells using trypan blue staining, the CC_{50} values obtained from the two reagents, CTG and CTF, were evaluated.

To study if the mechanism of action of MTX is through inhibition of its target enzyme, dihydrofolate reductase (DHFR), ZIKV infection was co-treated with MTX and Leucovorin, a downstream metabolite of DHFR pathway. Then, ZIKV titer was measured to confirm if leucovorin could rescue ZIKV replication from MTX. As a control, folic acid, a natural substrate of DHFR, was co-treated with MTX to the ZIKV-infected host cells. Then, to investigate if GAT (Glycine, Adenosine, and Thymidine) medium could rescue ZIKV replication from MTX treatment, I co-treated ZIKV-infected Vero cells with MTX in GAT medium; then, the ZIKV titer was measured. After observing that GAT medium can rescue ZIKV replication from MTX treatment in Vero cells, I separately investigated each component of GAT medium on ZIKV-infected Vero cells during MTX treatment, discovering adenosine alone can rescue ZIKV replication from MTX treatment.

In summary, I discovered that 5 μM of MTX can reduce ZIKV titer up to hundred-fold at 48 h PI, but it could not continuously suppress the ZIKV replication after 48 h PI. The toxicity of MTX varied depending on which cell viability assay reagent was used. CTG reagent indicated high toxicity of MTX, CC_{50} in nanomolar range; however, CTF reagent indicated relatively low toxicity of MTX ($CC_{50} > 100$ μM). I discovered that the antiviral mechanism of MTX was through the antagonism of DHFR. Moreover, GAT medium also rescued ZIKV replication from MTX treatment. Interestingly, adenosine alone rescued ZIKV replication from MTX treatment in Vero cells. This can be explained by, as a positive single-stranded RNA (+ssRNA) virus, ZIKV does not incorporate thymidine triphosphate but ATP into its genome. MTX readily inhibits *de novo* synthesis of purine and pyrimidine synthesis, thereby ATP as well. Here, exogeneous adenosine was probably converted into ATP, rescuing ZIKV replication in the infected Vero cells during MTX treatment.

METHODS

Cells and ZIKV Culture

Vero cells (ATCC CCL-81) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) with high glucose (4.5g/L), 10% fetal calf serum (FCS) (Sigma), and 1% Penicillin-Streptomycin (PS) (Sigma-Aldrich). Human neural stem cells (hNSCs) (Y40050) were purchased from Clontech and cultured in Neurobasal-A medium without phenol red (Thermo Fisher) with the addition of B27 supplement (1:100, Thermo Fisher, #12587010), N2 supplement (1:200, Invitrogen, #17502-048), 20 ng/ml FGF (R&D Systems 4114-TC-01M), 20 ng/ml EGF (R&D Systems 236-EG-01M), GlutaMax (Thermo Fisher, #35050061), and sodium pyruvate. Both Vero and hNSCs were cultured in T75 flasks (Corning). Two strains of ZIKV from Puerto Rico (PRV) (PRVABC59, NR-50240) and Panama (HPAN) (H/PAN/2016/BEI-259634, NR-50210), were cultured on Vero cells in DMEM with high glucose (4.5g/L), 1% fetal bovine serum (FBS) (Sigma), and 1% PS (Sigma-Aldrich). When 100% confluency of Vero cells was reached, the initial medium was removed, and the cells were infected with ZIKV at multiplicity of infection (MOI) 0.5 Plaque Forming Unit (PFU)/cell. At 24 h post-infection (PI), the initial medium was removed, and fresh medium was added. At 48 h PI, the medium was collected and spun down at 1500 rpm for 10 minutes. The supernatant was collected, 1% DMSO was added, and the sample was stored at -80°C. The titer of the virus stock was measured by plaque assay.

Plaque Assay

Vero cells were seeded at a density of 30,000 cells/well on a 96-wells plate (Corning) and incubated at 37°C and 5% CO_2 for 24 h before infection. Each ZIKV sample was diluted by ten-fold serial dilution, added to Vero cells at least in duplicates then incubated for 1 h. After

incubation, media with virus dilutions were removed from the cells, and ZIKV-infected Vero cells were covered with an overlay consisting of DMEM with 0.35% UltraPure agarose (Life Sciences). The plates were further incubated for 72 h and fixed with 37% formaldehyde overnight. After fixation, the agarose overlay was aspirated, and fixed cell monolayer was stained with 0.25% crystal violet. For each sample, the titer of ZIKV was reported in PFU/mL.
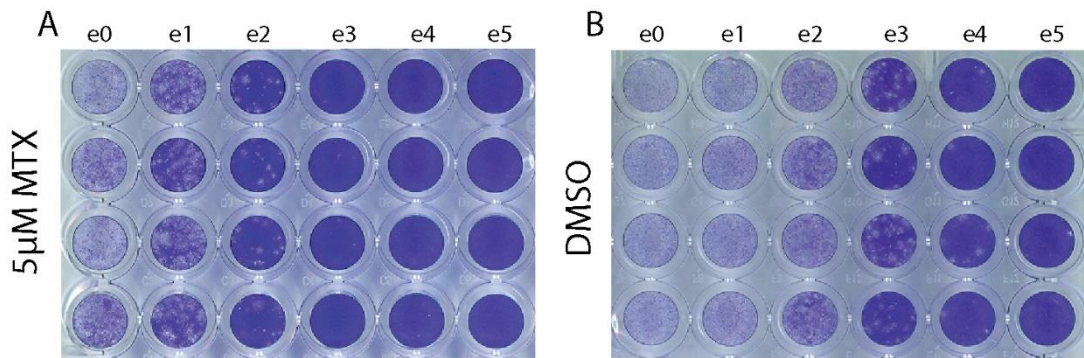

Figure 1. Image of plaque assay on a 96-wells plate. ZIKV inoculum is seriously diluted in ten-fold; total six diluents are prepared (maximum dilution $1:10^5$, i.e., e5). Here, the plaque assay image (HPAN MOI 0.2, 48 h PI) of **A.** 5μM of MTX treatment to ZIKV-infected Vero cells and **B.** DMSO treatment to ZIKV-infected Vero cells were shown. Each white dot represents a single virion, an infectious virus particle, of ZIKV.

Immunofluorescence Imaging

Vero cells were seeded at a density of 30,000 cells/well in an opaque black 96-wells plate (Corning) and incubated at 37°C and 5% CO_2 for 24 h before infection. Then, Vero cells were infected with ZIKV at MOI of 0.2. The infected Vero cells were incubated for 48 h then fixed with 4% formaldehyde in phosphate buffered saline (PBS). After fixation, the cells were permeabilized with 0.2% TritonX-100 in PBS for 5 min, then blocked with 1% bovine serum albumin (BSA) (Amresco) in PBS for 30 min at room temperature. After blocking, the cells were incubated with diluted primary antibody, anti-flavivirus group antigen primary antibody, clone D1-4G2-4-15

(Millipore), in PBS with 1% BSA and 0.1% TritonX-100 at 4°C overnight. The cells were washed with PBS three times and treated with diluted secondary antibody, anti-mouse Alexa Fluor 594, in PBS with 1% BSA for 1 h at room temperature while covered with aluminum foil. The cells were then washed with PBS three times and stained with SYTOX Green (Thermo Fisher) to visualize nuclei. Immunofluorescence images of cells were acquired using a ZEISS Fluorescence Microscope (Carl Zeiss, Jena, Germany, Axio Vert A1), and images were taken using Zen2 Software. The images were processed with ImageJ (https://imagej.nih.gov/ij/).

Cytotoxicity and Efficacy Study of MTX

The cytotoxic concentration 50 (CC_{50}) of MTX was determined using two reagents, CellTiter Glo® (CTG) (Promega), measuring ATP levels as a readout for cell viability, and CellTiter Fluor™ (CTF) (Promega), measuring live-cell protease activity, as per manufacturer's protocol. To validate the CC_{50} value from the two cell viability assays, a direct cell number count was performed using trypan-blue staining after MTX treatment. The efficacy of MTX against ZIKV was studied by reduction of ZIKV titer using four different MTX concentrations, 50.0 µM, 6.25 µM, 0.781 µM, and 0.0977 µM, in dose-response manner.

Rescue of ZIKV Replication after MTX Treatment by Leucovorin

To test the rescue effect of leucovorin on ZIKV replication with MTX treatment, Vero cells and hNSCs were seeded in a 96-wells plate (Corning) at a cell density of 30,000 and 10,000 cells/well, respectively. The Vero cells and hNSCs were then infected with ZIKV at MOI 0.2 and 0.1, respectively, and simultaneously treated with 5 µM MTX, or a combination of 5 µM MTX

with 50 µM folic acid or leucovorin. The cells were further incubated for 48 h at 37°C and 5% CO_2. After incubation, culture supernatant was collected to measure the virus titer. The cells were then treated under the same conditions as described above but without ZIKV infection to assess the effect of leucovorin on countering the cytotoxic effects of MTX on the cells. Then, the cell viability was measured using CTG (Promega) reagent.

Rescue Effect of GAT Medium on ZIKV Replication and Cell Viability from MTX

To test the rescue effect of GAT medium (Glycine, Adenosine, and Thymidine) on MTX-mediated suppression of ZIKV replication, Vero cells were seeded in a 96-wells plate at a cell density of 10,000 cells/well. GAT medium was prepared as described previously (Rosenblatt, Whitehead et al. 1982), in MEM (Gibco) with 10% FCS (Sigma) and 1% PS (Sigma-Aldrich) with a final concentration of 0.67 mM glycine, 37.5 µM adenosine and 41.3 µM thymidine. Vero cells were infected with ZIKV at a MOI 0.2 and simultaneously treated with 5 µM MTX in GAT medium. After incubation for 48 h at 37°C and 5% CO2, culture supernatant was collected to measure the virus titer. Cells were treated under the same conditions as described above but without ZIKV infection as a cell viability control. Cell viability was measured using CTG reagent (Promega). To investigate the rescue effect of individual components of GAT medium, Vero cells were infected with ZIKV as described above. Then, a combination of 5 µM MTX with 0.67 mM glycine, 37.5 µM adenosine, 41.3 µM thymidine, and both adenosine and thymidine were simultaneously added to the infected Vero cells. The treated Vero cells were further incubated for 48 h, and supernatant from each sample was collected to measure the virus titer. The same conditions were also applied to Vero cells without ZIKV infection to study which component of

GAT medium could protect cells from MTX-induced cytotoxicity, measure by cell viability using both CTG (Promega) and CTF (Promega) reagents.

RESULTS

Inhibition of ZIKV Replication with MTX

To validate the antiviral effect of MTX, immunofluorescence imaging and plaque assay were performed to investigate inhibition of ZIKV replication after MTX treatment. Through immunofluorescent detection, reduced ZIKV (HPAN MOI 0.2) envelope protein synthesis in Vero cells was observed from 5 μM MTX treatment at 48 h PI (Figure 2A). We next tested the replication kinetics of ZIKV (HPAN and PRV; both MOI 0.2) in Vero cells by plaque assay at three different time points, 1 h, 48 h, and 96 h PI (Figure 2B, C). Upon 5 μM MTX treatment, the replication of two ZIKV strains had greatest reduction (approximately ten-fold decrease in ZIKV titer) at 48 h PI, which is consistent with the immunofluorescence images. However, the ZIKV replication resumed at 96 h PI, compared to DMSO control, indicating MTX could not continuously suppress ZIKV virion formation after 48 h PI. In the case of efficacy of MTX against ZIKV, four different MTX concentrations, 50.0 μM, 6.25 μM, 0.781 μM, and 0.0977 μM, were incubated with ZIKV-infected Vero cells (HPAN MOI 0.2) (Figure 2D). At PI 48 h, the ZIKV titer was decreased about ten-fold after 6.25 μM MTX treatment.

Table 1. CC_{50} values of MTX in Vero cells and hNSCs

CC_{50}(μM) Values of MTX		Vero cells	hNSCs
Assay Reagent	CTG	0.104	0.0163
	CTF	>100	>100

CTG: CellTiter Glo® measures ATP as a luminescent readout of cell viability biomarker.
CTF: CellTiter Fluor™ measures live protease activity as a fluorescent readout of cell viability biomarker.
95% Cl(μM) of CC_{50} value of MTX in Vero cells and hNSCs is 0.0931 - 0.118 and 0.0148 - 0.0181, respectively.
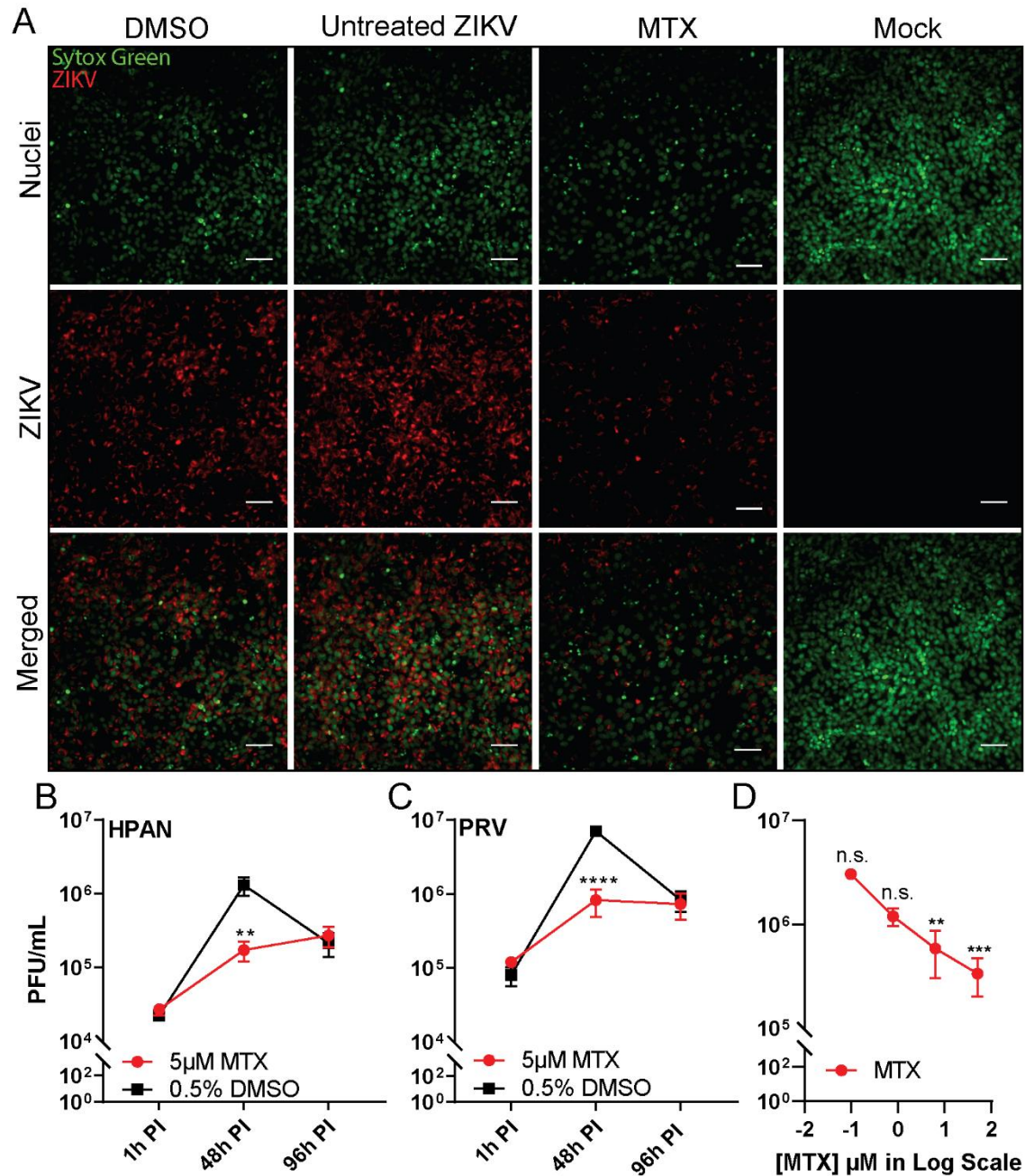
Figure 2. Inhibition of ZIKV replication in Vero Cells after MTX treatment. ZIKV-infected Vero cells were treated with 5 µM MTX or 0.5% of DMSO as a negative control. **A.** Immunofluorescence images (20X) of ZIKV-infected (HPAN MOI 0.2) Vero cells were acquired to analyze the level of ZIKV-Envelope protein after 5µM MTX treatment at 48 h PI. SYTOX Green was used to stain nuclei of Vero cells. Scale bars represent 5 µm. The virus titer of two ZIKV strains, **B.** HPAN MOI 0.2 and **C.** PRV MOI 0.2 were measured after 5 µM MTX treatment at three different time points, 1 h PI, 48 h PI, and 96 h PI. **D.** ZIKV titer after MTX treatment in dose-response manner, 50 µM, 6.25 µM, 0.781 µM, and 0.0977 µM, from ZIKV-infected (HPAN MOI 0.2) Vero cells at 48 h PI. At least two independent replicates were performed. For B and C, Two-way ANOVA, followed by Sidak's multiple comparisons test, were used for statistical analysis. For **D**, One-way ANOVA, followed by Tukey's multiple comparisons test, were used for statistical analysis; average viral titer at each MTX concentration was compared to that of untreated ZIKV at 48 h PI as a control. Error bars represent standard error of the mean (SEM). ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$, n.s. not significant

11

Cell Cytotoxicity and Antiviral Efficacy of MTX

Given the known toxicity of MTX associated with its use in treating other disease models such as cancer (Howard, McCormick et al. 2016), I surveyed the cytotoxicity of MTX to Vero cells and hNSCs. Using both CTG and CTF reagents, cell viability assays were performed and the CC_{50} of MTX was calculated in the two host cells (Table 1). Using CTG reagent, the CC_{50} values for MTX in Vero cells and hNSCs were 0.104 µM and 0.0163 µM, respectively, suggesting a high level of toxicity for MTX. Surprisingly, using CTF reagent, the CC_{50} values for MTX in Vero cells and hNSCs were both >100 µM. To resolve this discrepancy, Vero cells were directly counted after 0.5 µM of MTX treatment (Figure 3A, B). The number of viable Vero cells after 0.5 µM MTX treatment did not decrease compared to controls, but rather the cells appeared to grow more slowly in the presence of MTX. This supports the notion that lower cellular nucleotide metabolism caused by MTX treatment would account for the low CC_{50} value in the CTG assay, which measures ATP as a readout for cell viability.
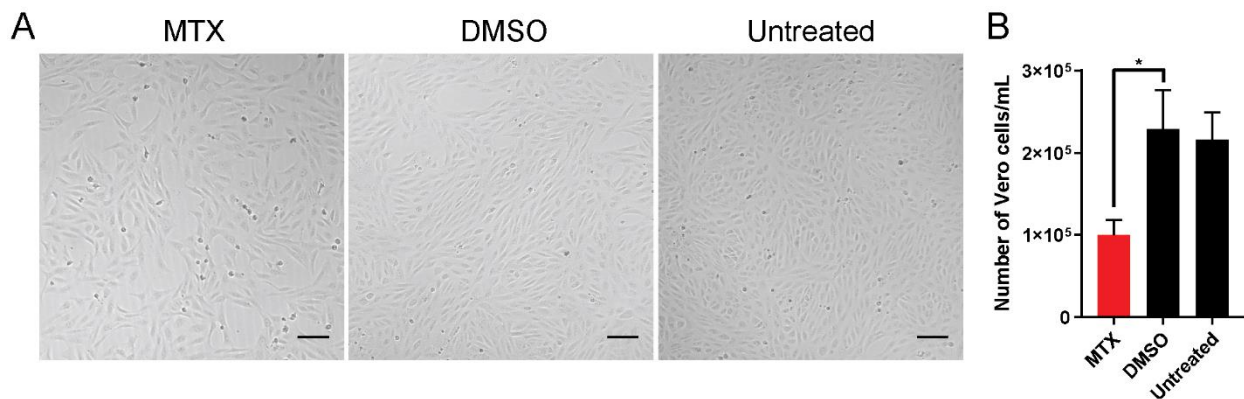

Figure 3. Determination of cytotoxicity of MTX and efficacy of MTX against ZIKV. To determine the cytotoxicity of MTX, the number of Vero cells after MTX treatment was directly counted by trypan-blue staining. Then, the number of healthy cells were counted under a light microscope with a hemocytometer. **A.** 10X bright-field images of Vero cells with 0.5 µM MTX treatment. Scale bars represent 5µm. **B.** The cell density of Vero cells after 0.5 µM MTX treatment after incubation for 48 h at 37°C and 5% CO_2. At least two independent replicates were performed. One-way ANOVA, followed by Tukey's multiple comparisons test, were used for statistical analysis. The error bars represent the standard error of the mean (SEM). * $P \leq 0.05$

Relationship between MTX, Folate, Leucovorin and ZIKV Replication

To understand the mechanism of action for the antiviral effect of MTX, I proceeded to probe the dihydrofolate reductase (DHFR) pathway. DHFR, which reduces dihydrofolate (DHF) to tetrahydrofolate (THF), is a key enzyme for *de novo* synthesis of purines and thymidylate, thereby playing a critical role in cellular growth. As an antifolate, MTX has been reported as a competitive inhibitor of DHFR and shares a similar chemical structure to folate, the natural substrate of DHFR. Through MTX-mediated antagonism of DHFR, folate metabolism is inhibited, which in turn decreases cellular replication. However, addition of leucovorin (folinic acid), a downstream metabolite of the DHFR pathway, can readily rescue the antagonistic effect of MTX in cancer cells (Bernard, Etienne et al. 1991).

To understand the metabolic relationship between MTX, folate, and leucovorin in a viral infection context we tested different competition scenarios. First, Vero cells were infected with two ZIKV strains, HPAN and PRV (both MOI 0.2) and treated with three different conditions: MTX, MTX with folic acid, and MTX with leucovorin. At 48 h PI, while the combination treatment of MTX with folic acid could not rescue the ZIKV replication, the combination treatment of MTX with leucovorin rescued the ZIKV replication, compared to DMSO control (Figure 4A, B). To study the effect of these three different conditions to Vero cells, the same experiment was performed without ZIKV infection, and using CTG reagent, the cell viability of the Vero cells from each condition was measured (Figure 4D). Interestingly, 50 µM leucovorin alone could rescue the cell viability of Vero cells after MTX treatment. Furthermore, the same rescue effect to ZIKV replication and cell viability of leucovorin from MTX treatment was observed in hNSCs (Figure 4C, E). The results suggest that the antiviral effect of MTX against ZIKV is through antagonizing DHFR.
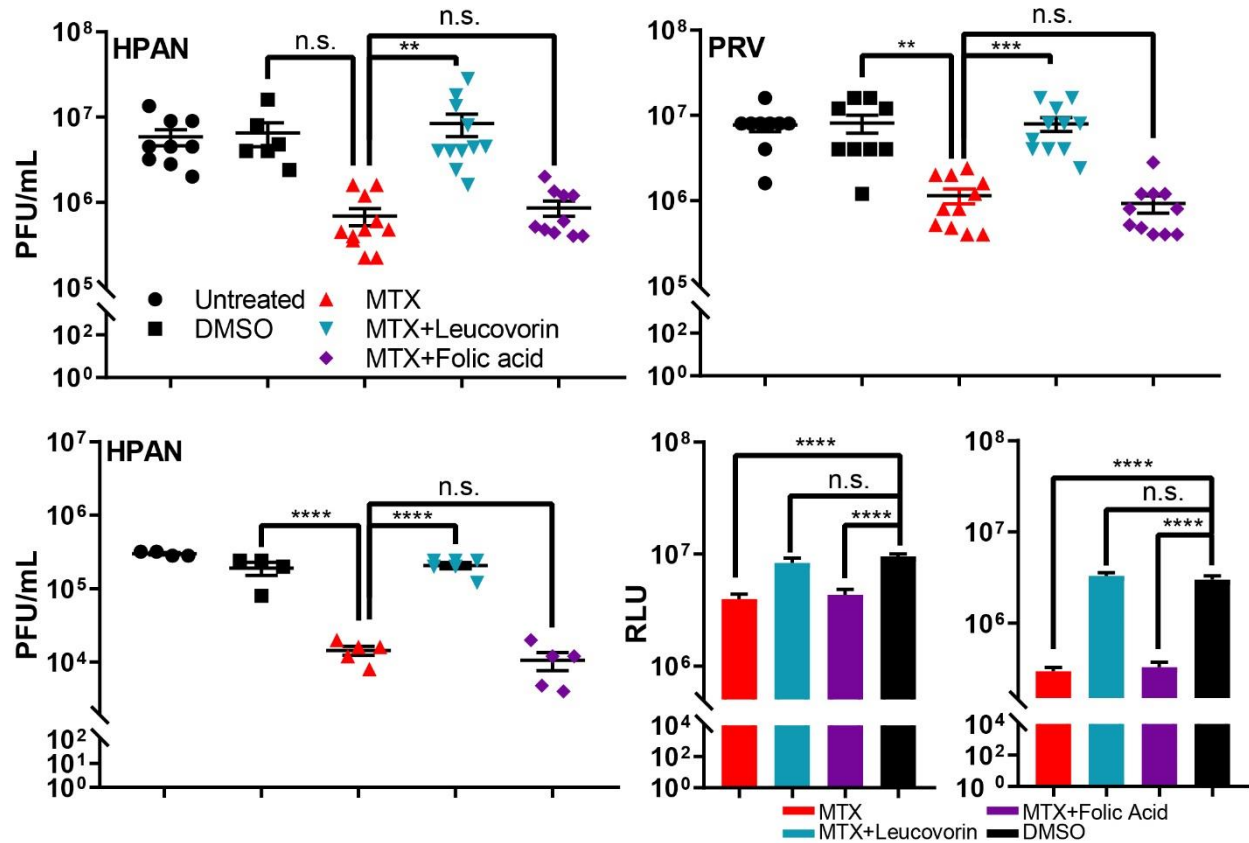
Figure 4. Rescue effect of leucovorin on the cell viability and ZIKV replication during MTX treatment. Two host cell lines, Vero and hNSCs, were used to examine the mechanism of action of MTX against ZIKV replication through the DHFR pathway. Virus titers of the two ZIKV strains, HPAN MOI 0.2 **A.** and PRV MOI 0.2 **B.** on Vero cells was measured by standard plaque assay. In the case of HPAN infection in Vero cells, the ZIKV titer of DMSO control showed slightly less significant difference to that of 5μM MTX treatment (P=0.11). **C.** Virus titer of HPAN MOI 0.1 on hNSCs was measured by standard plaque assay. **D.** The cytotoxicity of 5 μM MTX and co-treatment of MTX with 50 μM folic acid or leucovorin on Vero cells was studied by CTG reagent. **E.** The cytotoxicity of 5 μM MTX and co-treatment of MTX with 50μM folic acid or leucovorin on hNSCs was cells was studied by CTG reagent. RLU relative luminescence unit. At least two independent replicates were performed. One-way ANOVA, followed by Tukey's multiple comparisons test, were used for statistical analysis. The error bars represent the standard error of the mean (SEM). ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$, n.s. not significant

Rescue Effect of GAT Medium on ZIKV Replication and Cell Viability during MTX Treatment

As MTX antagonizes DHFR, I wanted to address which metabolites based on purine or pyrimidine synthesis could affect the ZIKV replication. Previously, the cytotoxicity of MTX was also reversed in human fibroblasts not only by leucovorin, but also GAT medium, which contains glycine, adenosine, and thymidine (Rosenblatt, Whitehead et al. 1982). Therefore, the rescue effect of GAT medium and each of its individual nucleotide base component was studied in Vero cells. As expected, complete GAT medium could rescue both cell viability and ZIKV replication (HPAN MOI 0.2) from MTX treatment. (Figure 5A, B). Then, glycine, adenosine, thymidine, and combination of adenosine and thymidine, were individually co-treated with MTX in the ZIKV infected Vero cells. Interestingly, only adenosine rescued the ZIKV replication from MTX treatment (Figure 5C). To observe if adenosine alone could rescue the cell viability from MTX treatment, we tested cell viability of Vero cells using both, CTG and CTF reagents. While both, glycine and thymidine, could not rescue the cell viability, in the case of adenosine, it rescued both ATP level and live-cell protease activities from MTX treatment in Vero cells (Figure 5D, E). Understanding the substrate of CTF cell viability assay is glycyl-phenylalanyl-aminofluorocoumarin (GF-AFC), which is readily cleaved by a Cathepsin C/Dipeptidyl Peptidase I (Niles, Moravec et al. 2007), it is an interesting finding that exogenous adenosine can rescue the activity of Cathepsin C, that is known to activate other pro-inflammatory serine proteases in various immune cells, from MTX (Methot, Rubin et al. 2007).
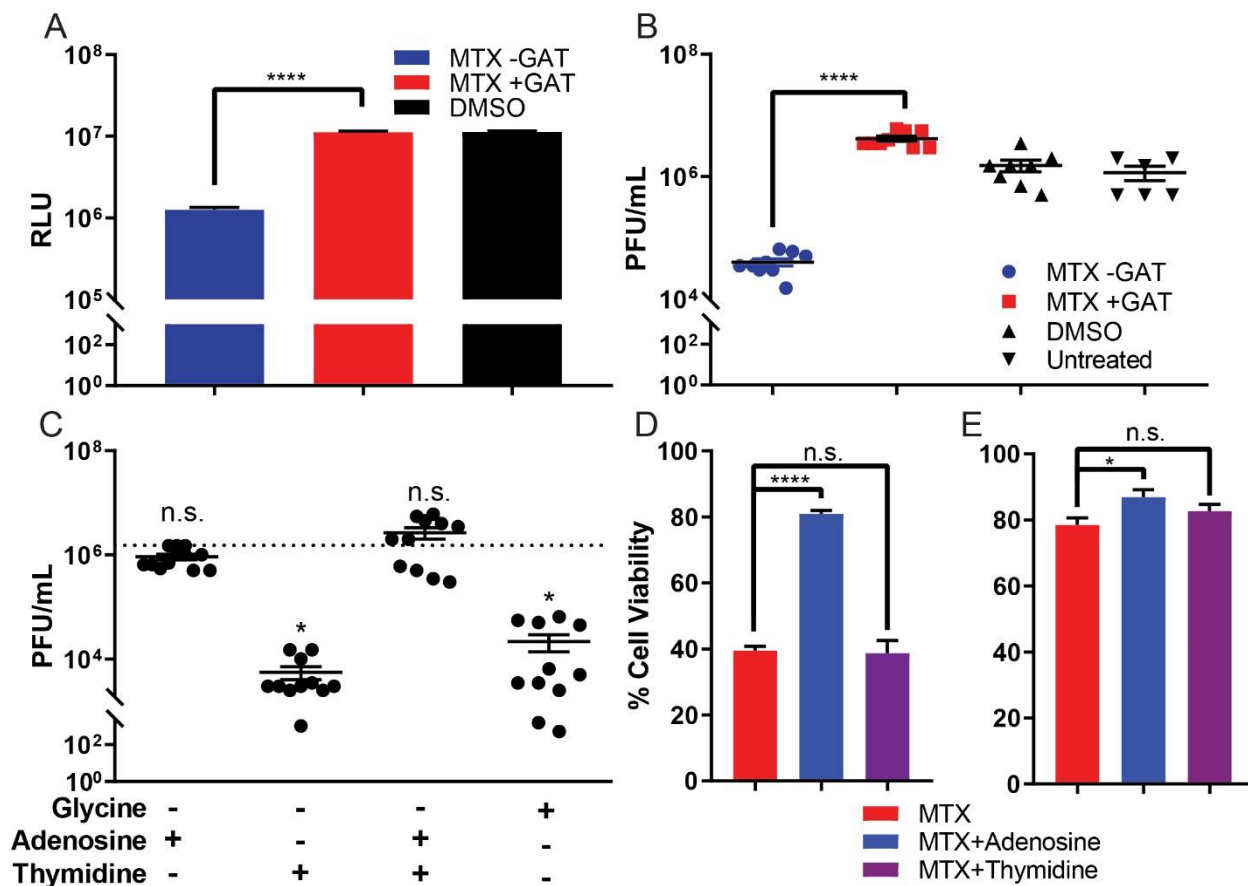
Figure 5. Rescue effect of GAT medium on cell viability and ZIKV replication during MTX treatment. A. Cell viability of Vero cells was studied by CTG reagent after MTX treatment with or without GAT medium. **B.** GAT medium rescued the ZIKV replication from MTX treatment of the ZIKV-infected (HPAN MOI 0.2) Vero cells. **C.** Adenosine alone can save ZIKV replication during MTX treatment of the ZIKV-infected (HPAN MOI 0.2) Vero cells. **D.** Exogenous adenosine rescued cellular ATP levels, but thymidine and glycine could not rescue ATP levels during MTX treatment in Vero cells. **E.** Adenosine also rescued live-protease activity measured by CTF reagent during MTX treatment in Vero cells. RLU relative luminescence unit. At least two independent replicates were performed. The error bars represent the standard error of the mean (SEM). One-way ANOVA, followed by Tukey's multiple comparisons test, were used for statistical analysis. * $P \leq 0.05$, **** $P \leq 0.0001$, n.s. not significant

DISCUSSION

In this study, I validated MTX as an antiviral targeting ZIKV replication. MTX was previously discovered as a potent hit molecule against ZIKV in human brain microvascular endothelial cells, with inhibitory concentration 50 (IC_{50}) of 0.28 μM and host cytotoxicity (CC_{50}) of >10 μM (Rausch, Hackett et al. 2017). Such neurotropism may explain the possible neurological consequences of ZIKV infection, and MTX influences the folate pathway may explain that one of these consequences is a neural tube defect, i.e. microencephaly in infants. MTX is approved by the FDA to treat diseases such as cancer and rheumatoid arthritis. Accordingly, the mechanism of action of MTX against such diseases have been well studied. MTX is primarily known to antagonize DHFR as a competitive inhibitor (Waltham, Holland et al. 1988) and antagonized DHFR causes inhibition of *de novo* synthesis of purines and pyrimidines, which are essential for cell replication (Bokkerink, Deabreu et al. 1988). Hence, MTX shows great cytotoxicity to highly replicating cancer cell lines. Interestingly, with 5 μM MTX treatment, I observed reduced ZIKV replication in infected Vero cells and hNSCs. Although MTX reduced ZIKV titer about ten-fold at 48 h PI, MTX could not continuously suppress the ZIKV replication after 48 h PI. Observing the antiviral effect of MTX against ZIKV, the toxicity of MTX in Vero cells and hNSCs and its efficacy against ZIKV in Vero cells were measured. Understanding that MTX can inhibit purine and pyrimidine metabolism, cells treated with MTX showed significantly decreased viability in terms of ATP level. Accordingly, CC_{50} measurement with CTG reagent also resulted in high cytotoxicity in the two host cells; however, such toxicity could be misleading because the drug mechanism directly interferes with ATP levels, i.e. the readout measurement of the assay. Accordingly, Vero cells with 0.5 μM MTX treatment, exceeding the CC_{50} value obtained from

CTG reagent, did not exhibit clear phenotypic cytotoxicity but rather showed slower cellular replication than the controls.

MTX decreased ZIKV titer in Vero cells and hNSCs by antagonizing DHFR. The inhibited DHFR activity by MTX was readily reversed by simultaneous co-treatment with leucovorin, thereby rescuing ZIKV replication from MTX as well. Further metabolite analysis with GAT medium allowed us to understand adenosine alone could rescue ZIKV replication by increasing ATP level from MTX treatment. Although MTX is known to inhibit TS (Chu, Drake et al. 1990), thymidine alone could not rescue the ZIKV replication. Considering the genome of ZIKV, a single stranded positive sense RNA (+ssRNA), thymidine triphosphate (TTP) would not be incorporated into its genome during the replication. In fact, Fischer *et al* reported inhibition of TS can reduce flavivirus replications but also found DENV replication was not rescued when excess amount of thymidine was co-treated with MTX, suggesting an alternative mechanism such as activation of P53 for general antiviral activity due to low level of thymidine (Fischer, Smith et al. 2013).

Together, our results identify that DHFR may be a promising target for antiviral research against ZIKV and other flaviviruses, like Chikungunya virus that shares similar features to those of ZIKV (Musso, Cao-Lormeau et al. 2015). For example, the mammalian DHFR inhibitor, pemetrexed, which is used to treat non-small cell lung carcinoma (Hanna, Shepherd et al. 2004), should be investigated. However, repurposing MTX as an antiviral against ZIKV infection would not be an ideal option, because the most vulnerable ZIKV infected patients are pregnant females, and MTX has been used as a clinical abortion agent (Tanaka, Hayashi et al. 1982). Furthermore, although excess amount of thymidine could not rescue the cell viability from MTX *in vitro* (Figure 4D, E), such combination should be tested *in vivo*. Considering the number of reports on the rescue effect of thymidine from MTX (Ensminger and Frei 1977, Howell, Ensminger et al. 1978, Jackson

18

1980), perhaps, MTX-thymidine could be an optional medical regime to treat ZIKV infection while MTX inhibits ZIKV replication, and thymidine rescues the host from the cytotoxicity of MTX. In conclusion, this study clarified the antiviral mechanisms of MTX, and clarifies how the DHFR pathway may be targeted for antiviral purposes.

This thesis in full is currently being prepared for submission for publication of the material. Sungjun Beck, the thesis author was the primary investigator and author of this material. This thesis in full is coauthored with Jean A. Bernatchez, Zhe Zhu, Michelli F. De Oliveira, and David M. Smith. The corresponding author is Jair Lage de Siqueira-Neto.

REFERENCES

Adcock, R. S., Y. K. Chu, J. E. Golden and D. H. Chung (2017). "Evaluation of anti-Zika virus activities of broad-spectrum antivirals and NIH clinical collection compounds using a cell-based, high-throughput screen assay." <u>Antiviral Research</u> **138**: 47-56.

Bernard, S., M. C. Etienne, J. L. Fischel, P. Formento and G. Milano (1991). "Critical factors for the reversal of methotrexate cytotoxicity by folinic acid." <u>Br J Cancer</u> **63**(2): 303-307.

Bernatchez, J. A., Z. Yang, M. Coste, J. Li, S. Beck, Y. Liu, A. E. Clark, Z. Zhu, L. A. Luna, C. D. Sohl, B. W. Purse, R. Li and J. L. Siqueira-Neto (2018). "Development and Validation of a Phenotypic High-Content Imaging Assay for Assessing the Antiviral Activity of Small-Molecule Inhibitors Targeting Zika Virus." <u>Antimicrob Agents Chemotherpy</u> **62**(10): 302927.

Black, R. L., W. M. O'Brien, E. J. Vanscott, R. Auerbach, A. Z. Eisen and J. J. Bunim (1964). "Methotrexate Therapy in Psoriatic Arthritis; Double-Blind Study on 21 Patients." <u>JAMA</u> **189**(10): 743-747.

Bokkerink, J. P. M., R. A. Deabreu, M. A. H. Bakker, T. W. Hulscher, J. M. Vanbaal, E. D. A. M. Schretlen and C. H. M. M. Debruijn (1988). "Effects of Methotrexate on Purine and Pyrimidine Metabolism and Cell-Kinetic Parameters in Human-Malignant Lymphoblasts of Different Lineages." <u>Biochemical Pharmacology</u> **37**(12): 2329-2338.

Chu, E., J. C. Drake, D. Boarman, J. Baram and C. J. Allegra (1990). "Mechanism of thymidylate synthase inhibition by methotrexate in human neoplastic cell lines and normal human myeloid progenitor cells." <u>J Biol Chem</u> **265**(15): 8470-8478.

Colon-Gonzalez, F. J., C. A. Peres, C. S. Sao Bernardo, P. R. Hunter and I. R. Lake (2017). "After the epidemic: Zika virus projections for Latin America and the Caribbean." <u>Plos Neglected Tropical Diseases</u> **11**(11): e0006007.

de Oliveira, W. K., G. V. A. de Franca, E. H. Carmo, B. B. Duncan, R. de Souza Kuchenbecker and M. I. Schmidt (2017). "Infection-related microcephaly after the 2015 and 2016 Zika virus outbreaks in Brazil: a surveillance-based analysis." <u>Lancet</u> **390**(10097): 861-870.

Elango, T., H. Dayalan, P. Gnanaraj, H. Malligarjunan and S. Subramanian (2014). "Impact of methotrexate on oxidative stress and apoptosis markers in psoriatic patients." <u>Clin Exp Med</u> **14**(4): 431-437.

Ensminger, W. D. and E. Frei, 3rd (1977). "The prevention of methotrexate toxicity by thymidine infusions in humans." <u>Cancer Res</u> **37**(6): 1857-1863.

Fischer, M. A., J. L. Smith, D. Shum, D. A. Stein, C. Parkins, B. Bhinder, C. Radu, A. J. Hirsch, H. Djaballah, J. A. Nelson and K. Fruh (2013). "Flaviviruses are sensitive to inhibition of thymidine synthesis pathways." <u>J Virol</u> **87**(17): 9411-9419.

Gould, E. A. and T. Solomon (2008). "Pathogenic flaviviruses." Lancet **371**(9611): 500-509.

Hanna, N., F. A. Shepherd, F. V. Fossella, J. R. Pereira, F. De Marinis, J. von Pawel, U. Gatzemeier, T. C. Tsao, M. Pless, T. Muller, H. L. Lim, C. Desch, K. Szondy, R. Gervais, Shaharyar, C. Manegold, S. Paul, P. Paoletti, L. Einhorn and P. A. Bunn, Jr. (2004). "Randomized phase III trial of pemetrexed versus docetaxel in patients with non-small-cell lung cancer previously treated with chemotherapy." J Clin Oncol **22**(9): 1589-1597.

Howard, S. C., J. McCormick, C. H. Pui, R. K. Buddington and R. D. Harvey (2016). "Preventing and Managing Toxicities of High-Dose Methotrexate." Oncologist **21**(12): 1471-1482.

Howell, S. B., W. D. Ensminger, A. Krishan and E. Frei, 3rd (1978). "Thymidine rescue of high-dose methotrexate in humans." Cancer Res **38**(2): 325-330.

Hryniuk, W. M. and J. R. Bertino (1969). "Treatment of leukemia with large doses of methotrexate and folinic acid: clinical-biochemical correlates." J Clin Invest **48**(11): 2140-2155.

Hryniuk, W. M., L. W. Brox, J. F. Henderson and T. Tamaoki (1975). "Consequences of methotrexate inhibition of purine biosynthesis in L5178Y cells." Cancer Res **35**(6): 1427-1432.

Ivanetich, K. M. and D. V. Santi (1990). "Bifunctional thymidylate synthase-dihydrofolate reductase in protozoa." FASEB J **4**(6): 1591-1597.

Jackson, R. C. (1980). "Modulation of methotrexate toxicity by thymidine: sequence-dependent biochemical effects." Mol Pharmacol **18**(2): 281-286.

Jolivet, J., K. H. Cowan, G. A. Curt, N. J. Clendeninn and B. A. Chabner (1983). "The Pharmacology and Clinical Use of Methotrexate." New England Journal of Medicine **309**(18): 1094-1104.

Methot, N., J. Rubin, D. Guay, C. Beaulieu, D. Ethier, T. J. Reddy, D. Riendeau and M. D. Percival (2007). "Inhibition of the activation of multiple serine proteases with a cathepsin C inhibitor requires sustained exposure to prevent pro-enzyme processing." Journal of Biological Chemistry **282**(29): 20836-20846.

Musso, D., V. M. Cao-Lormeau and D. J. Gubler (2015). "Zika virus: following the path of dengue and chikungunya?" Lancet **386**(9990): 243-244.

Niles, A. L., R. A. Moravec, P. Eric Hesselberth, M. A. Scurria, W. J. Daily and T. L. Riss (2007). "A homogeneous assay to measure live and dead cells in the same sample by detecting different protease markers." Anal Biochem **366**(2): 197-206.

Parra, B., J. Lizarazo, J. A. Jiménez-Arango, A. F. Zea-Vera, G. González-Manrique, J. Vargas, J. A. Angarita, G. Zuñiga, R. Lopez-Gonzalez and C. Beltran (2016). "Guillain–Barré syndrome associated with Zika virus infection in Colombia." N Engl J Med **375**(16): 1513-1523.

Rausch, K., B. A. Hackett, N. L. Weinbren, S. M. Reeder, Y. Sadovsky, C. A. Hunter, D. C. Schultz, C. B. Coyne and S. Cherry (2017). "Screening Bioactives Reveals Nanchangmycin as a Broad Spectrum Antiviral Active against Zika Virus." Cell Reports **18**(3): 804-815.

Renslo, A. R. and J. H. McKerrow (2006). "Drug discovery and development for neglected parasitic diseases." Nat Chem Biol **2**(12): 701-710.

Rosenblatt, D. S., V. M. Whitehead, N. V. Matiaszuk, A. Pottier, M. J. Vuchich and D. Beaulieu (1982). "Differential-Effects of Folinic Acid and Glycine, Adenosine, and Thymidine as Rescue Agents in Methotrexate-Treated Human-Cells in Relation to the Accumulation of Methotrexate Polyglutamates." Molecular Pharmacology **21**(3): 718-722.

Sramek, M., J. Neradil, J. Sterba and R. Veselska (2016). "Non-DHFR-mediated effects of methotrexate in osteosarcoma cell lines: epigenetic alterations and enhanced cell differentiation." Cancer Cell Int **16**(1): 14.

Tanaka, T., H. Hayashi, T. Kutsuzawa, S. Fujimoto and K. Ichinoe (1982). "Treatment of interstitial ectopic pregnancy with methotrexate: report of a successful case." Fertil Steril **37**(6): 851-852.

Waltham, M. C., J. W. Holland, S. C. Robinson, D. J. Winzor and P. F. Nixon (1988). "Direct experimental evidence for competitive inhibition of dihydrofolate reductase by methotrexate." Biochem Pharmacol **37**(3): 535-539.

Willkens, R. F., M. A. Watson and C. S. Paxson (1980). "Low dose pulse methotrexate therapy in rheumatoid arthritis." J Rheumatol **7**(4): 501-505.

Xu, M., E. M. Lee, Z. Wen, Y. Cheng, W. K. Huang, X. Qian, J. Tcw, J. Kouznetsova, S. C. Ogden, C. Hammack, F. Jacob, H. N. Nguyen, M. Itkin, C. Hanna, P. Shinn, C. Allen, S. G. Michael, A. Simeonov, W. Huang, K. M. Christian, A. Goate, K. J. Brennand, R. Huang, M. Xia, G. L. Ming, W. Zheng, H. Song and H. Tang (2016). "Identification of small-molecule inhibitors of Zika virus infection and induced neural cell death via a drug repurposing screen." Nat Med **22**(10): 1101-1107.