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Failure of 3-methyladenine to modulate Semliki Forest virus replication in HeLa cells

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ABSTRACT

Alphaviruses comprise approximately 30 viruses that cause a wide range of symptoms, from mild to severe. These viruses are classified into three main groups: aquatic, arthritogenic (affecting the joints), and encephalitic (causing brain inflammation). Infection with arthritogenic alphaviruses typically results in fever, rash, muscle and joint pain that can persist for months or even years. In contrast, encephalitic alphaviruses, such as Eastern equine encephalitis virus (EEEV) and Venezuelan equine encephalitis virus (VEEV), can lead to severe illness and potentially death. Currently, there are no specific antiviral drugs or vaccines available for these viruses. Understanding the cellular mechanisms that facilitate viral replication may provide new therapeutic targets. The role of autophagy in the replication of Semliki Forest virus (SFV) in HeLa cells remains unclear; therefore, it is important to investigate how 3-methyladenine (3-MA), an autophagy inhibitor, influences this process. In this study, I examined the effect of 3-MA on SFV replication in HeLa cells. Cells were infected with SFV and treated with 3-MA at defined time points. Viral titers were quantified using plaque assays and analyzed statistically. The results revealed no significant difference between the control and treated groups, suggesting that 3-MA does not significantly influence SFV proliferation in HeLa cells. This study contributes to the broader understanding of the role of 3-methyladenine in mammalian cells during alphavirus infection and underscores the need for further research in this area.

Keywords: Semliki Forest virus, 3-methyladenine, HeLa cells, alphaviruses.

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INTRODUCTION

Alphaviruses

Alphaviruses are enveloped, single-stranded, positive-sense RNA viruses belonging to the *Togaviridae* family. They represent a continuously evolving and re-emerging global public health threat. These viruses are transmitted by mosquito vectors to vertebrate hosts, causing diseases in both humans and animals (Lounibos and Kramer, 2016). The *Alphavirus* genus comprises several medically significant pathogens, including Ross River virus, Chikungunya virus, Eastern equine encephalitis virus (EEEV), Western equine encephalitis virus (WEEV), Venezuelan equine encephalitis virus (VEEV), and Sindbis virus (SINV) (Kim and Diamond, 2023).

Infection by these viruses can lead to a spectrum of illnesses ranging from mild febrile conditions to severe outcomes such as crippling polyarthralgia, encephalitis, and death (Rangel and Stapleford, 2021). Currently, no alphavirus-specific antiviral therapy has been approved (Mostafavi et al., 2019). Furthermore, vaccine development using classical platforms remains slow compared to contemporary technologies. Traditional platforms often face challenges such as biosafety level 3 (BSL-3) requirements for large-scale virus cultivation for inactivated vaccines, as well as safety concerns that limit the use of live-attenuated vaccines in pregnant or immunocompromised individuals (Kim and Reyes-Sandoval, 2023).

Semliki Forest virus

Semliki Forest virus (SFV), a member of the *Alphavirus* genus within the *Togaviridae* family, is primarily

transmitted by *Aedes africanus* and *Aedes aegypti* mosquitoes in sub-Saharan Africa (Mathiot et al., 1990). SFV has long served as a safe model for studying the molecular biology of RNA virus replication due to its well-characterized genome and infection cycle (Atkins et al., 1999).

HeLa cells

HeLa was the first human cell line to be cultured, and it is now the most extensively used human cell line in biological research (Landry et al., 2013). The cell line was derived from a cervical cancer tumour in a patient named Henrietta Lacks, who died of the disease in 1951 (Lucey et al., 2009). HeLa cells can divide indefinitely in a laboratory cell culture plate if basic cell survival parameters are met. These cells have also been used to investigate viruses such as oropouche, papillomavirus, and canine distemper (Lyapun et al., 2019). Jonas Salk utilised HeLa cells to test the first polio vaccine in the 1950s. They were shown to be easily infected with poliomyelitis, which causes infected cells to die. This made HeLa cells ideal for polio vaccine testing since results were easily acquired (Soleymani and Abedi Kiasari, 2012). Their high proliferative potential, simplicity of transfection, and sensitivity to viral infection make them a suitable model for examining virus-host interactions. HeLa cells have been frequently employed in studies of alphavirus replication, particularly Semliki Forest Virus (SFV), owing to their reliable response and well-defined cellular environment (Nubgan, 2025).

3-Methyladenine

3-Methyladenine (3-MA) was the first and remains one of the most widely used autophagy inhibitors. It functions as a phosphatidylinositol 3-kinase (PI3K) inhibitor, preventing class III PI3K interaction with multiple ATG proteins and thereby blocking autophagosome formation during the initiation and maturation stages of autophagy (Tseng et al., 2011).

However, 3-MA acts non-selectively by inhibiting both PIK3CB/PIK3C1 and PI3CK3, leading to pleiotropic cellular effects. Interestingly, in nutrient-rich conditions, 3-MA has been shown to promote autophagy by increasing autophagic marker accumulation and enhancing LC3-I to LC3-II conversion. In addition, 3-MA may influence other cellular processes, including glycogen metabolism, endocytosis, proteolysis, and mitochondrial permeability transition, in an autophagy-independent manner (Caro et al., 1988).

Increasing threat to global health

Over the past century, alphaviruses and flaviviruses have posed significant threats to both human and animal health, causing recurrent outbreaks worldwide. Human alphavirus infections typically manifest as arthritogenic or encephalitic diseases, associated with considerable morbidity and, in severe cases, mortality (Azar et al., 2020).

Globally, an estimated 390 million infections occur annually, with approximately 96 million developing clinical symptoms and resulting in about 25,000 deaths. Since 2007, outbreaks of Zika virus, a related flavivirus, have been reported across Africa, Asia, the Americas, and the Pacific, with infection linked to increased incidence of Guillain–Barré syndrome (Pierson and Diamond, 2020). Given the high human and economic costs, research into novel antiviral targets and therapies remains critical for controlling outbreaks and reducing morbidity and mortality.

Hypothesis and aim

Hypothesis (H_1): The addition of the autophagy inhibitor 3-methyladenine (3-MA) to HeLa cells infected with Semliki Forest virus will affect viral replication and proliferation.

Null Hypothesis (H_0): The addition of 3-methyladenine to HeLa cells infected with Semliki Forest virus will not affect viral replication and proliferation.

The aim of this study is to determine whether the inhibitory action of 3-methyladenine alters SFV proliferation in HeLa cells. This understanding may contribute to the development of new antiviral strategies against alphaviruses and flaviviruses.

To achieve this aim, HeLa cells were cultured and infected with SFV. The infected and uninfected cells were examined using electron microscopy to compare their morphology. Following infection, 3-methyladenine was added to the cultures, and samples were harvested at four different time points. Plaque assays were then performed to evaluate the effect of 3-methyladenine on viral growth.

MATERIALS AND METHODS

HeLa cell culture

HeLa cells, complete growth medium, trypsin solution (10×), and sterile phosphate-buffered saline (PBS) were provided by the laboratory teaching staff. Semliki Forest virus (SFV) was kindly supplied by Dr. Sareen Galbraith. The composition of the cell culture growth medium was provided by laboratory staff (Table 1).

To passage cells, the growth medium was aspirated into a waste container, and 5 mL of sterile PBS was immediately added to prevent the monolayer from drying.

Table 1. Composition of the cell culture growth medium.

Component	Volume (mL)	Percentage (%)
Earle's Minimum Essential Medium (1×)	465	93
Heat-inactivated Fetal Calf Serum	25	5
MEM Eagle Non-essential Amino Acids (10 mM)	5	1
Penicillin (10,000 U/mL) / Streptomycin (10 mg/mL)	5	1

The PBS was gently rinsed over the cell layer and discarded. This washing step was repeated twice.

A working trypsin solution was prepared by diluting 1 mL of trypsin stock in 9 mL of sterile PBS. Then, 1 mL of this diluted trypsin solution was added to the flask, evenly covering the cell sheet. The flask was incubated at 37 °C in a 5% CO_2 incubator for 1–2 minutes to allow enzymatic detachment of the cells. Care was taken to avoid overexposure, as prolonged trypsinization can damage cells.

Once cells began to detach, the flask was gently tapped to obtain a uniform cell suspension. Immediately after detachment, 9 mL of complete growth medium was added to neutralize the trypsin. The cell suspension was pipetted up and down to achieve a single-cell suspension. The suspension was examined microscopically to confirm proper detachment before reseeding.

Appropriate volumes of the cell suspension were transferred into new T75 flasks or six-well plates. Growth medium was added to reach the required volume, and cultures were incubated at 37 °C with 5% CO_2.

Infection of HeLa cells with Semliki Forest virus

The growth medium was removed from confluent HeLa cell cultures and discarded. A mixture of 500 µL PBS and 500 µL of SFV inoculum was added to each flask. The flask was incubated at 37 °C and 5% CO_2, with gentle rocking every 15 minutes to ensure even distribution of the virus.

After incubation, the inoculum was removed and 10 mL of fresh growth medium was added. The cells were then incubated under the same conditions until harvest for plaque assay. Typically, cytopathic effects (CPE) became apparent within 18–24 hours, at which point most of the monolayer detached.

The culture medium was transferred to 15 mL centrifuge tubes and centrifuged at maximum speed for 5 minutes at 4 °C to pellet cell debris. The supernatant containing the virus was aliquoted on ice without disturbing the pellet and stored at −80 °C. Repeated freeze–thaw cycles were avoided to preserve viral integrity.

Plaque assay

HeLa cells were seeded in six-well plates as described above. Difco Noble Agarose (25 mL aliquots) was prepared by dissolving 3.6 g of agar powder in 200 mL of distilled water in a 500 mL sterile bottle, followed by autoclaving. The molten agar was aliquoted into 50 mL centrifuge tubes and stored at room temperature.

Overlay medium composition:

330 mL sterile distilled water
100 mL 10× Minimum Essential Medium (Eagle's) without L-glutamine or $NaHCO_3$
10 mL Penicillin/Streptomycin solution
10 mL L-Glutamine
50 mL Fetal Bovine Serum

The overlay medium was melted in a microwave and maintained at 45 °C in a water bath. Equal volumes of molten agarose and overlay medium were equilibrated at 37 °C prior to mixing.

Ten-fold serial dilutions of the virus (10^{-1} to 10^{-6}) were prepared by transferring 100 µL of virus into 900 µL of PBS sequentially. The growth medium was aspirated from the six-well plates, and 200 µL of PBS was added to the first well as an uninfected control. Subsequently, 200 µL of each viral dilution was added to the remaining wells. The plates were incubated at 37 °C and 5% CO_2 for 1 hour, with rocking every 15 minutes.

Following incubation, equal parts (25 mL each) of molten agarose and overlay medium were mixed, and 3 mL of the mixture was carefully added to each well to avoid disturbing the monolayer. The plates were allowed to cool at room temperature for the agar to solidify, then incubated for 48 hours at 37 °C and 5% CO_2.

After incubation, 1 mL of 4% neutral paraformaldehyde was added to each well to fix the cells for 30 minutes in a fume hood. The agar overlays were removed and disposed of, and the wells were stained with crystal violet for 5 minutes. The plates were rinsed under running water, air-dried, and plaques were counted to determine viral titers.

Adding 3-methyladenine

HeLa cells were cultured in six-well plates as described above. Three wells were designated as controls (C1, C2, C3) and three as test wells (T1, T2, T3). Cells were passaged the day before the experiment to ensure active growth.

A 2.5 mM working solution of 3-methyladenine (3-MA) was prepared. The culture medium was aspirated from each well, and 3 mL of fresh medium containing 3-MA was added to the test wells, while 3 mL of medium without 3-MA was added to the control wells. The plate was incubated for 4 hours at 37 °C and 5% CO_2.

Following incubation, the medium was removed, and cells were infected with SFV according to the procedure in Section 2.2. Samples were harvested at 2, 24, and 45.5 hours post-infection (HPI). After each collection, 1 mL of fresh medium with 3-MA was added to the test wells, and 1 mL of medium without 3-MA was added to the control wells.

Samples were collected into pre-labeled microcentrifuge tubes corresponding to the initials, sample type, and time point. For each well, 1 mL of culture was removed, and 330 µL was transferred into each of three tubes. The removed volume was replaced with fresh medium. This procedure was repeated for all wells and time points, resulting in 24 total samples. All samples were stored at −80 °C until analysis by plaque assay.

Statistical analysis

Plaque assay data were analyzed using the t-test in SPSS software to obtain p-values and determine statistical significance between experimental groups. Analysis of variance (ANOVA) with multiple comparisons was used to evaluate differences in viral titers across the various time points post-infection.

RESULTS

Successful culture of HeLa cells

The HeLa cells were healthy and grew effectively, receiving adequate nutrients from the growth medium and thriving in an optimal culture environment that supported their proliferation and viability (Figure 1).

Virus growth

Following SFV infection, the morphology of HeLa cells showed marked alterations in both size and shape when compared to uninfected cells, demonstrating the cytopathic effects of the virus on HeLa cells (Figure 2).

Plaque assay

As an initial approach, HeLa cells infected with SFV were analyzed using a plaque assay. Infected cells exhibited distinct plaque formation (Figure 3). The plaque assay was used to quantify viral titers and assess the extent of infection across different virus dilutions.

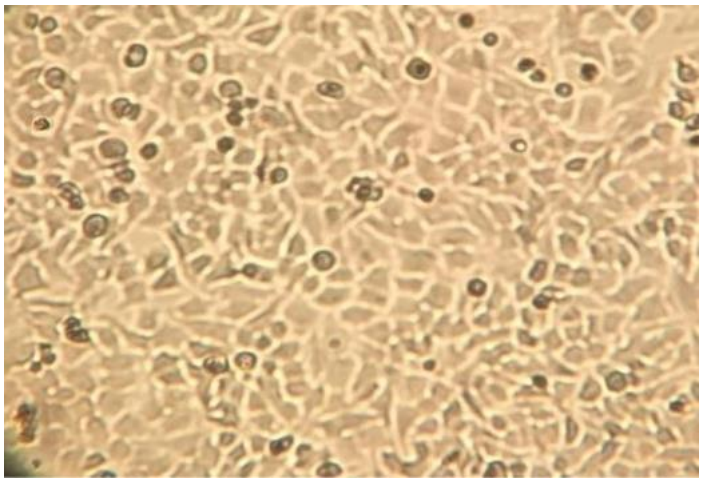

Figure 1. Healthy Hela cells in cell culture. The morphology of Hela cells under a cell culture microscope showed a polygonal or spindle shape. Magnification 200X.

The wells were labeled as follows:

Control (C): The bottom of the well appeared uniformly purple, indicating healthy HeLa cells with no viral infection, as no virus was added.

10^{-3} dilution: The well showed minimal purple coloration with widespread cell death, indicating a high viral concentration that infected nearly all HeLa cells.

10^{-4} dilution: The well displayed scattered purple areas, showing that most HeLa cells were infected.

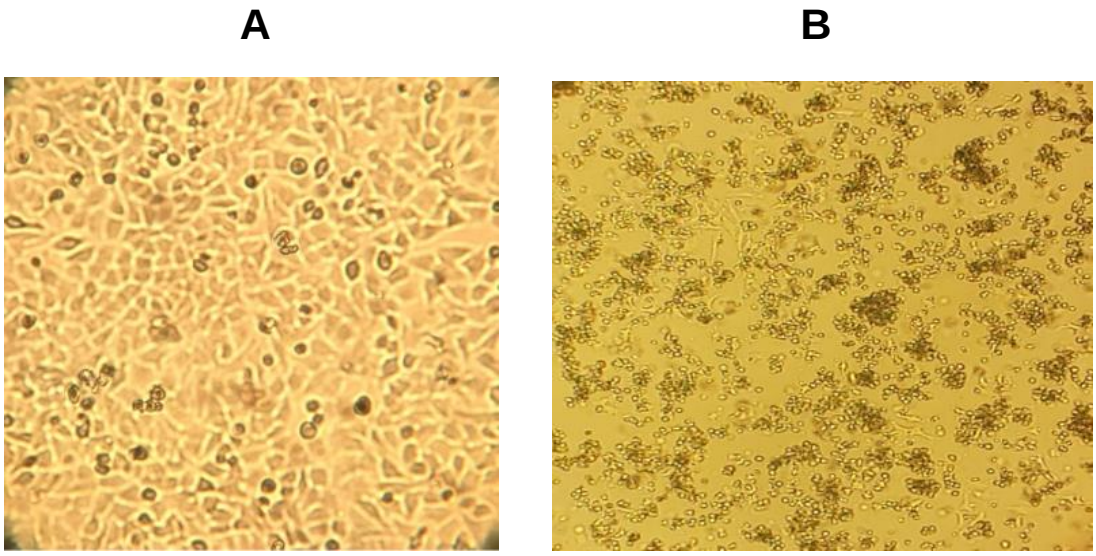

Figure 2. Hella cells post-infection with SFV. A. uninfected cells which show spindle shape. B. infected cells which show cell trimming, shrinking, and detachment from the substrate. Both photos magnification 200X.

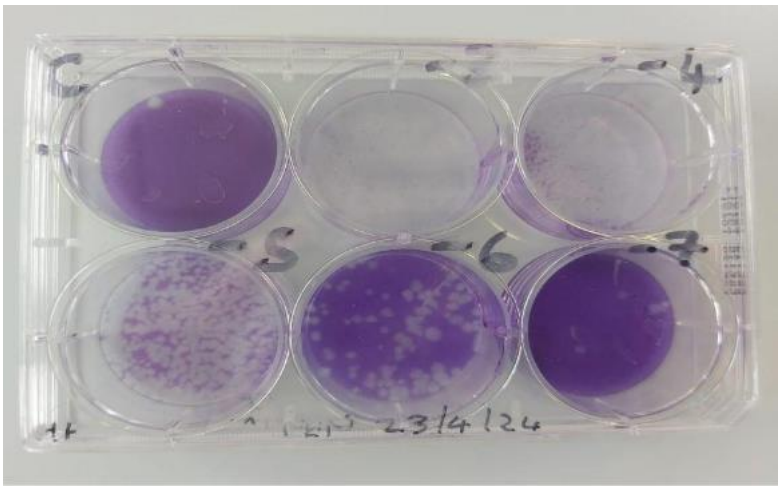

Figure 3. Plaque assay for virus dilutions titer.

10^{-5} dilution: Plaques became more distinct and easily visible, indicating reduced viral infection due to dilution.
10^{-6} dilution: The plaques appeared clearer and countable, corresponding to a smaller number of infected HeLa cells.
10^{-7} dilution: Few plaques were visible, and the bottom of the well remained largely purple, suggesting that only a few HeLa cells were infected (Figure 3).

The calculated viral titer from the plaque assay was 2.6×10^8 PFU/mL.

For quantitative comparison, three biological replicates were analyzed for both control (C1, C2, C3) and treatment (T1, T2, T3) groups. Viral titers were expressed in plaque-forming units per milliliter (PFU/mL), and values are presented as mean ± standard deviation (SD) at each time point, measured in hours post-infection (HPI) (Table 2).

At 18.5 HPI: The mean viral titer of the control group (4.3×10^7 PFU/mL) was slightly higher than that of the 3-MA treatment group (4.1×10^7 PFU/mL) (Figure 4). Variability was lower in the control group (8.1×10^6 PFU/mL) compared to the treatment group (1.4×10^7 PFU/mL). Statistical analysis (t-test) yielded a p-value of 0.871, which is greater than 0.05, indicating no significant difference between the groups.

At 24 HPI: The mean titers of the control (2.7×10^7 PFU/mL) and treatment (2.8×10^7 PFU/mL) groups were nearly identical (Figure 4). The control group exhibited

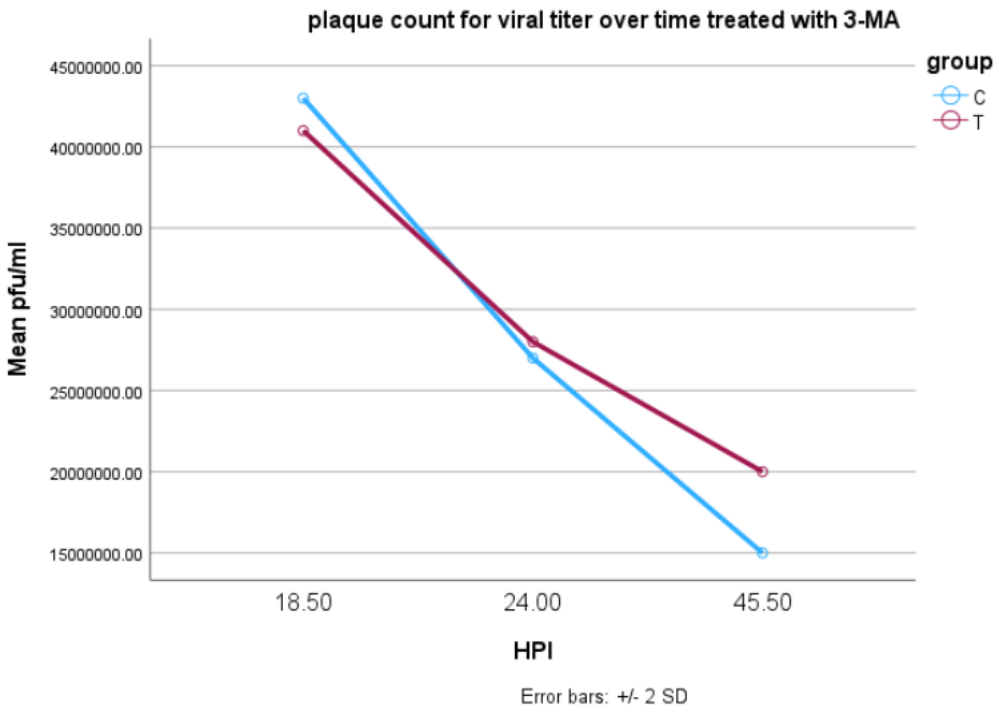

Figure 4. A line graph presenting plaque-forming unit pfu/ml over the different time points 18.5, 24, 45.5 HPI, comparing control (blue) and 3-MA-treated (red) HeLa cells by using t-test.

higher variability (3.7 × 10^6 PFU/mL) compared to the treatment group (1.6 × 10^6 PFU/mL). The t-test produced a p-value of 0.704, also greater than 0.05, indicating no significant difference between the groups.

At 45.5 HPI: The mean viral titer in the control group (1.5 × 10^7 PFU/mL) was lower than in the treatment group (2.0 × 10^7 PFU/mL) (Figure 4). However, the treatment group showed higher variability (2.6 × 10^7 PFU/mL) compared to the control (5.0 × 10^6 PFU/mL). The t-test yielded a p-value of 0.763, again greater than 0.05, indicating no significant difference between the two groups.

A one-way ANOVA test comparing all groups produced a p-value of 0.871, which is greater than 0.05, confirming that there were no statistically significant differences in plaque counts between the control and 3-MA-treated groups.

Overall, these results indicate that treatment with 3-MA does not have a substantial impact on viral replication or plaque formation in SFV-infected HeLa cells.

DISCUSSION

This study found that treatment with 3-MA did not significantly affect the replication of SFV in HeLa cells. At 18.5 hours post-infection (HPI), the control group showed a slightly higher mean viral titer and lower variability than the 3-MA-treated group (Table 2). At 24 HPI, both groups exhibited similar mean values, although the control group had higher variability. At 45.5 HPI, the control group displayed a lower mean, while the treatment group showed higher variability (Figure 4). Statistical analyses using t-tests and ANOVA indicated no significant differences in plaque counts between the control and 3-MA-treated groups, suggesting that 3-MA does not substantially impact SFV replication in HeLa cells.

Our results are consistent with the findings of Chen and Smartt (2021), who reported that 3-MA had no significant effect on dengue virus (DENV) titers in Aedes aegypti-derived cell lines. Although their study focused on a mosquito cell line and a different virus, the comparable outcome suggests that 3-MA's limited influence on autophagy might extend to other alphaviruses, including SFV in mammalian systems. This alignment indicates that 3-MA's modulation of autophagy may be largely virus-independent within the *Alphavirus* genus.

Additionally, the lack of a substantial effect of 3-MA on SFV infection may be related to the virus's intrinsic replication mechanisms, which might not rely heavily on autophagy. The importance of autophagy in viral replication is known to vary greatly among viruses and even across different host cell types infected by the same virus (Chen and Smartt, 2021).

Lee et al. (2008) also observed that while 3-MA reduced LC3-II expression, it did not significantly lower viral titers. 3-MA is known to have a dual role in autophagy: under starvation conditions, it inhibits class III

phosphoinositide 3-kinase (PI3K) and suppresses autophagy, but under normal conditions, it can stimulate autophagic flux. Therefore, experimental conditions, including nutrient availability must be carefully optimized to achieve a consistent inhibitory effect of 3-MA on autophagy (Lee et al., 2008). These findings suggest that inhibition of autophagy alone may not be sufficient to suppress viral replication under certain physiological states.

In a related study, Pei et al. (2014) employed the MTT assay to evaluate whether pharmacological modulation of autophagy using rapamycin or 3-MA influenced classical swine fever virus replication through changes in cell viability. Their results demonstrated that autophagy modulation did not significantly affect cell viability, as supported by statistical analyses showing no substantial differences between treated and untreated cells (Pei et al., 2014).

Previous studies in liver cells from fasting rats also demonstrated that 3-MA inhibits autophagy by targeting a specific enzyme, although the precise molecular mechanism of this inhibition remains unclear (Petiot et al., 2000). Despite this, 3-MA has been shown to suppress autophagy induced by several *Flaviviruses*, including Zika virus (ZIKV), in vitro (Cao et al., 2017). For example, Krejbich-Trotot et al. (2011) reported that pre-treatment with 3-MA two hours before chikungunya virus (ChikV) infection significantly reduced the number of infected cells (Krejbich-Trotot et al., 2011). This differential outcome suggests that some viruses, such as ChikV, rely more heavily on autophagic mechanisms for replication than SFV. Variations in viral dependency on autophagy likely stem from differences in viral life cycles, host cell responses, and the specific autophagic pathways engaged during infection.

Furthermore, the complex interplay between autophagy, cell survival, and viral propagation may help explain the findings of this study. Autophagy can function both as a defense mechanism, degrading viral components, and as a supportive platform for viral replication. The specific interaction between SFV and the autophagic machinery in HeLa cells may create a balance that 3-MA is unable to disrupt. Generally, both RNA and DNA viruses can activate autophagy, though the exact pathways remain incompletely understood (Nakashima et al., 2006).

Three potential mechanisms for virus-induced autophagy have been proposed:

1. Double-stranded RNA may stimulate autophagosome formation by inhibiting PKR activity and downregulating mTOR.
2. Viral infection may trigger autophagy through endoplasmic reticulum (ER) stress.
3. The inhibition of mTOR can suppress cellular translation and subsequently induce autophagy (Wullschleger et al., 2006).

For instance, Lee et al. (2008) reported that several viral proteins can suppress host translation and transcription by inhibiting mTOR activity, which in turn promotes autophagosome formation. During DENV-2 infection, mTOR and its downstream effector p70S6K were inhibited. The effect of this inhibition was modulated by 3-MA, confirming that mTOR is involved in DENV-2-induced autophagy (Lee et al., 2008).

Some viruses, however, have evolved mechanisms to suppress autophagy entirely. For example, herpes simplex virus type 1 (HSV-1) encodes the neurovirulence protein ICP34.5, which inhibits the autophagy protein Beclin-1. Mutant HSV-1 lacking the Beclin-1 binding domain fails to suppress autophagy in neurons and exhibits reduced neurovirulence in mice. This effect is reversed in $pkr^{-/-}$ mice, showing that ICP34.5-mediated suppression of autophagy via Beclin-1 is essential for HSV-1 neurovirulence, with PKR acting upstream in the host antiviral pathway (Krejbich-Trotot et al., 2011; Orvedahl and Levine, 2008).

This experiment was part of a four-student study that examined pharmacological autophagy (chloroquine, wortmannin, rapamycin, and 3-methyladenine). Notably, all compounds failed to result in substantial alterations in SFV replication, indicating a consistent trend across various pharmacological methods.

A notable limitation of this study is the relatively small sample size and limited replication due to time constraints and project scope, which may reduce statistical power. Although the observed trends were consistent, future research should incorporate more biological replicates and direct autophagy markers (e.g., LC3-II, p62) to strengthen the conclusions and clarify underlying mechanisms.

CONCLUSION

In summary, this study enhances the understanding of the effect of 3-MA's role in SFV infection and demonstrates that 3-MA does not significantly influence SFV replication in HeLa cells. The results showed no statistically significant differences in plaque counts between the control and 3-MA-treated groups, supporting the null hypothesis and rejecting the alternative hypothesis that 3-MA inhibits SFV replication. Further investigation into autophagy modulators and their virus-specific effects could advance the development of novel therapeutic strategies targeting autophagic pathways in viral infections.

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