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A novel antiviral inhibits Zika virus infection while increasing intracellular glutathione biosynthesis in distinct cell culture models.

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A B S T R A C T

We investigated the effects of a specific free-form amino acids formulation on Zika virus replication in two different cell culture model systems, one representative of humans and the other of Old World primates from whom Zika virus was first isolated. Here we present data demonstrating that the formulation of the specific free-form amino acid (FFAAP), comprising cystine, glycine, and a glutamate source, along with a minute concentration of selenium inhibited Zika virus replication by up to 90% with an ED_{90} (effective dose at which 90% of a dose of Zika virus was inhibited) of 2.5 mM in human cells and 4 mM Vero cells. The ED_{90} concentration of precursors was innocuous for uninfected cells, but resulted in reduced Zika virus replication by up to 90% at 2–5 mM concentrations in nonhuman primate cells and at 1–3 mM concentration in human placental cells.

Two important observations were forthcoming: 1) Zika virus production was decreased by up to 90% in Vero and JEG-3 cells treated with FFAAP (ED_{90} 4.0 mM, and 2.5 mM, respectively) throughout 48–72 h of post infection (hpi) compared to untreated infected cells and 2) Zika virus requires intracellular glutathione for replication in human placental cells, while showing enhanced replication in Vero cells with no glutathione. Relative increases in intracellular glutathione biosynthesis followed FFAAP treatment but blocking intracellular biosynthesis of glutathione in human cells resulted in virus inhibition in human placental cells. The blockade of biosynthesis actually increased Zika virus replication in Vero cells. These findings identify an efficacious inhibitor, FFAAP, of Zika virus replication in both human and nonhuman primate cells, while providing novel insight into the different roles of intracellular glutathione in Zika virus replication.

1. Introduction

Zika virus infection is an enormous global concern because of its destructive effect on human health and wellbeing in mosquito-infested regions (Zika virus, 2016; Al-Qahtani et al., 2016) (Fauci and Morens, 2016). During 2015–2016, at least three global outbreaks were recognized by WHO (Alera et al., 2015; Adams et al., 2016; Zika virus infection, 2016; Besnard et al., 2014; Borchardt, 2016; Brasil et al., 2016). The socio-economic impact of this arthropod-borne infection has been enormous, particularly due to its devastating effects on the fetus during pregnancy, and the demonstration that the virus persists in retinal cells, semen, and genitalia (Davidson et al., 2016) (Atkinson et al., 2016; Carod-Artal, 2016; Butler, 2016; Brent et al., 2016; Brooks et al., 2016). Innate defenses that may play a role(s) in Zika virus infections have not been extensively studied to date. One of the most primordial innate defense responses, validated in evolution dating back to Cyanobacteria, glutathione (GSH, GSSG) has been conserved across nearly every species, comprising part of the essential antioxidant defense system (Cameron and Pakrasi, 2010; Chardonnet et al., 2015). Glutathione negatively impacts certain viruses as reviewed by Fraternale and colleagues (Fraternale et al., 2006, 2008, 2009), suggesting in at least some cells, intracellular glutathione is an active cell defense. Here we employed cell culture models using two cell lines (placental and kidney) from different, but closely related species, human and Africa green monkey, respectively, to evaluate antiviral effects on Zika virus infection. The cell lines selected from humans, for whom health concerns are rampant, was JEG-3, to-date the most permissive human cell line for Zika virus replication and ultimately a target cell in pregnant women, while cells representative of nonhuman primates, from which Zika virus was first discovered, were Vero cells, the most permissive for Zika virus infection and an initial target cell following deposition of virus by mosquitos (Chan et al., 2016). Neither cell line produces Type I interferon, although JEG-3 cells do produce interferon ε. Fischer at al. reported this molecule is upregulated in endometrial cells during luteal phase in mares, and Nikodem et al. as well as Fung and colleagues each reported the presence of interferon ε was present in the reproductive tract pregnant women (Fischer et al., 2018; Nickodem et al., 2018a, 2018b). These reports, in addition to the fact that interferon ε is produced by the placental cytotrophoblasts, suggest that this Type I interferon plays an important role during pregnancy.

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2. Materials and methods

2.1. Cells and virus

Each cell line was procured through ATCC®. Vero cells (ATCC® - CCL81) were grown in Eagles minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) as recommended by ATCC®. Cells used were passaged following trypsinization (Mediatech, Manasas, VA) no more than 20 times before use, with cells received from ATCC® between passage 127–130. Human placental cells JEG-3 (ATCC® HTB-36™) (Manassas, VA), were grown in Eagle's Minimum Essential Medium (ATCC, Catalog No. 30–2003) with 10% triple filtered fetal bovine serum premium (Atlanta biologicals, Flowery Branch, GA). Virus infections were initiated on monolayers of semi-confluent cells in log phase growth by removing medium, and adding minimum volume virus stock to achieve the specific multiplicity of infection used for adsorption during a 90-min incubation. Virus, which remained, was removed following adsorption for 1 h at 37 °C, and growth medium, i.e., MEM supplemented with 2% FBS in semi-solid medium was added for subsequent quantification of plaques described below.

2.2. Cell quantification and viability

Cell numbers were quantified using a hemocytometer to accurately count cells manually at 100X total magnification. An aliquot of each sample was placed into an equal volume of Trypan Blue and gently mixed; then 10 μL of cells were added to each side of the hemocytometer under the coverslip (Hoskins et al., 2012). Live, treated cells were counted to calculate the percent of viable treated cells versus the percent of live cells in the untreated samples. These methods were found to be superior with respect to consistency and sensitivity when compared to metabolic assays or automated microscopic analyses.

2.3. FFAAP treatment of cells

Uninfected Vero and JEG-3 cells were treated initially with 0, 2.5, 5.0, 13 mM concentrations of FFAAP for 72 h for safety studies, and for subsequent infections the highest concentration, up to 5 mM, demonstrated no detrimental effects on cell propagation and viability. Subsequent experiments to assess the effective doses at which 50% virus reduction was observed resulted in our selection of 4 mM and 2.5 mM for Vero cells and JEG-3 cells, respectively, in all subsequent experiments. Cells infected with Zika virus were treated with the above concentrations for 1.5 h adsorption for subsequent quantification of virus titers. For plaque enumeration assays, cells were treated with FFAAP at the time of adsorption and the treatment was continued for 3 days or the duration of the experiment within semi-solid medium. For these assays the final concentrations in the cell culture medium for the infected and uninfected cells were 0, 3.0, 6.0, and 12 mM FFAAP from solubilized stock solution prepared for FFAAP. Effective concentrations (ED50) for virus reduction were calculated as per GraphPad Prism for analysis of data with upward trends, in this case, viz., increasing reduction of virus concentrations/replication.

2.4. Plaque enumeration assays

In the first study exploring the effects of free-form amino acid precursors (FFAAP) on Zika virus, nearly confluent monolayers of treated and untreated cells were infected with approximately 150 plaque-forming units (pfu, or infectious particles) of Zika virus or mock-infected cell lysate, which adsorbed to the cells over 2 h at 37 °C in a humidified incubator with 5% CO_2: 95% air. Inoculum was removed, and cells overlaid with agarose in MEM supplemented with 2% FBS. Cells were then grown for 72 h in the presence of FFAAP, semi-solid medium removed, wells rinsed, then stained with crystal violet to count individual plaques to quantify replication of Zika virus with and without treatment. Numbers of plaques from treated wells were normalized against the numbers of plaques in untreated cells to establish percent reduction in infected cells.

2.5. Quantification of Zika virus titers

Approximately 95% sub-confluent Vero or JEG-3 cells were infected Zika in a 48-well plate. At 1.5 hpa (hours post-adsorption), virus inoculum was removed and media with or without FFAAP was added to the respective wells. At 1,2, 3 dpi, supernatants were collected and frozen at −80 °C until plaque assays were performed to quantify virus titers.

2.6. Glutathione precursors and glutathione assays

The FFAAP was provided by Proimmune Research Institute, and comprises a mixture of free-form glycine, an L-glutamate source, L-cystine, and L-seleno-methionine (Immune Formulation 200®, ProImmune Research Institute, Rhinebeck NY) as previously described (Crum, 2007, 2011, 2015; Sinha-Hikim et al., 2011). The FFAAP was solubilized in dilute NaOH to prepare a stock concentration, which was subsequently neutralized using HCl, with the final pH 7.5 in MEM in a 5% CO_{2-} -humidified incubator at 37 °C, and diluted to achieve the specific concentrations used in these studies. Intracellular glutathione levels (GSH) were measured to quantify the levels of newly biosynthesized intracellular glutathione with molar quantities calculated using standard curves prepared with reduced and oxidized glutathione standards in accordance with the assay instructions (Enzo Life Sciences, Farmingdale, NY). Standard error of the mean (SEM) was calculated using values from triplicate samples and coefficient of variance was calculated within each sample group for each specific treatment. Student t-test using Microsoft Excel was used to calculate p-values to assess whether significant differences exist. Multiple group analysis was also performed using multiple *t*-test and *t*-test for two groups at a time. The results were the same regardless of the assay used. Inhibition of the glutathione biosynthesis pathway was accomplished by using the well-documented inhibitor, BSO, or buthionine-(S,R)-sulfoximine (Sigma-Aldrich, St. Louis, MO). Effective blockade of intracellular glutathione biosynthesis was assessed by measurements of intracellular reduced and oxidized glutathione in the presence of inhibitor. Oxidized glutathione was negligible indicating cells were not in oxidative stress as a result of any of the treatments or infection.

3. Results

Cell culture models using Vero cells and JEG-3, human placental cells, have each been validated by Chan and colleagues to support robust replication of Zika virus (Chan et al., 2016). Data shown in Fig. 1 demonstrated the titer of Zika virus in JEG-3 cells is ~5X less than virus in Vero cells with each infected using the same MOI. In order to initially assess the anti-viral capability of FFAAP, mid-log phase Vero cells were infected with a total of 150 PFU of Zika virus (strain PRVABC59). Following a two-hour adsorption of virus at 37 °C in a humidified environment of 5% CO_2: 95% air, semi-solid MEM growth medium with specified concentrations of FFAAP was added and infection in triplicate samples continued for 3 days. The efficacy of FFAAP on Zika virus infection was measured by counting plaques and ED50 was determined (Fig. 2). The FFAAP treatment at a concentration of 10 mM was toxic to cells, and therefore no plaques were detected. AT 3.0 mM FFAAP concentration, 42% reduction in plaque numbers was observed and at 6.0 mM concentration 85% plaque reduction was observed in this experiment.

FFAAP, which comprises free-form amino acids precursors for synthesis of intracellular glutathione, and with these, we initially hypothesized that biosynthesis of increased intracellular GSH inhibited virus replication in Vero cells. To test our hypothesis, first we

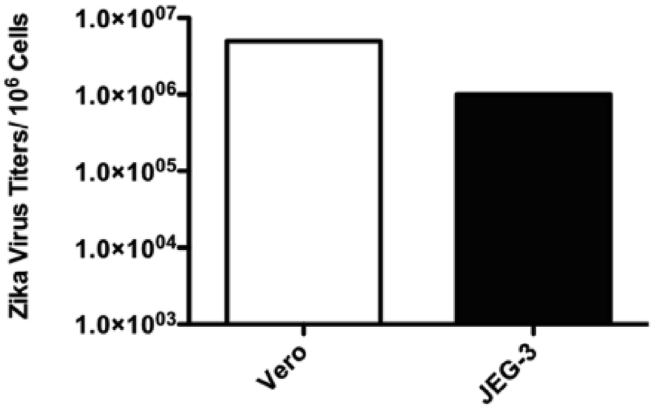

Fig. 1. Zika Virus Production in Human and Nonhuman Primate Cells. Cells from each species were grown to mid-log phase and infected with Zika virus (MOI 0.1), which was adsorbed to the monolayers of cells (triplicate wells) for two hours at 37 °C in a humidified environment of 5% CO2: 95% air. Subsequently, growth medium was added and infection continued for 3 days. Infected cells were harvested on day 3 to quantify virus replication. Plaque assays were performed subsequently to quantify the amount of virus produced in each cell line.

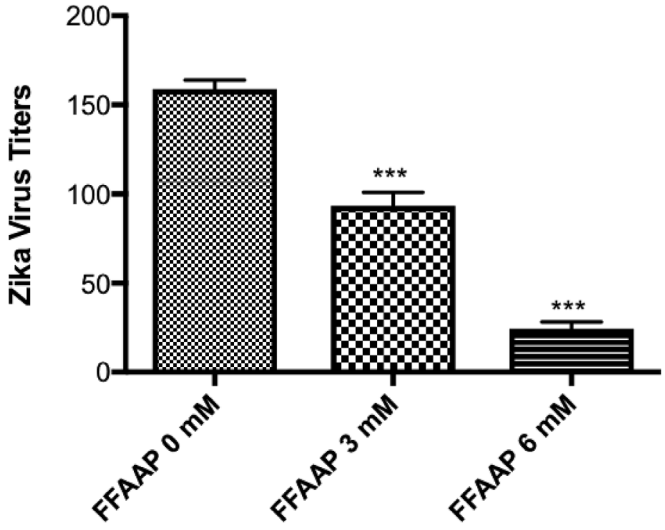

Fig. 2. Effect of FFAAP on Zika Virus Plaque Reduction. Vero cells were adsorbed for 2 h with approximately 150 infectious virions per well (triplicate) of Zika virus (MOI 0.02) and inoculum replaced with MEM containing 2% agarose and supplemented with 2% FBS and increasing concentrations of FFAAP. Cells were incubated for 3 days, semi-solid medium removed, and wells stained with crystal violet. Plaques were enumerated. Standard error of the means was calculated using Microsoft Excel.

determined the levels of intracellular glutathione in FFAAP-treated and untreated cells. We quantified intracellular glutathione levels in each cell line over 3 days. Growth and viability of the cells were also assessed (Supplementary Fig. 1). Vero cells were harvested 1, 2, and 3 days post treatment (dpt). No significant differences in viability were noted between FFAAP-treated cells using up to 5 mM concentrations when compared to untreated cells (Supplementary Fig. 1). Fig. 3 shows the total intracellular glutathione concentrations in triplicate cell culture wells grown in the presence or absence of FFAAP treatment. Little-to-no oxidized (GSSG) was detected in uninfected cells, therefore we represented only total intracellular glutathione for the remainder of the experiments. Analysis of the data confirmed that FFAAP-treated cells produced significantly greater levels of total intracellular glutathione than untreated cells as observed from quantification of total glutathione, in agreement with previously published data (Debieu et al.,

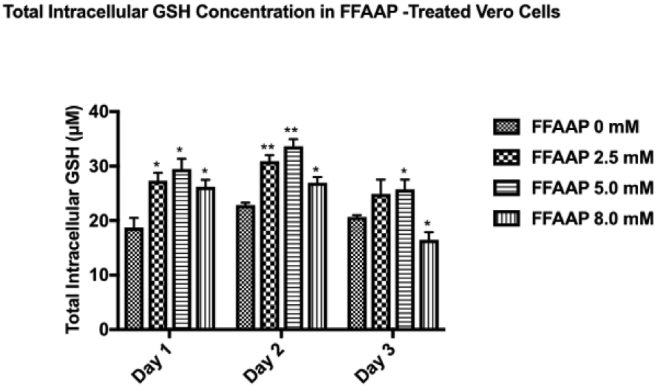

Fig. 3. Effect of increasing concentrations of FFAAP on levels of total intracellular glutathione in healthy Vero cells on days 1, 2, and 3. All experiments shown were done in triplicate and the data shown are representative data from multiple independent experiments performed. Standard error bars were calculated using Microsoft Excel and significance assessments were performed using GraphPad® Prism. $p < 0.05$ and > 0.01 = *, $p < 0.01$ and > 0.001 = **, and $p < 0.001$ = ***.

1987; Aalto and Raivio, 1993; Buckman et al., 1993; Cheng et al., 2013) (Volk et al., 2014; Abdo et al., 2010). On day two, untreated cells showed concentrations of 15–20 μM of reduced glutathione, whereas FFAAP-treated cells produced up to 30% more GSH on 1 and 2 dpt. With increasing concentrations of FFAAP (5–8 mM), biosynthesis of total intracellular GSH increased slightly by 2 dpt (days post treatment) and by 3 dpt levels and began to approach values comparable to those observed in untreated cells, suggesting that cells were depleted of FFAAP by 3 dpt. An alternative possibility is that between 2 and 3 dpt, glutathione was conjugated to cellular or viral molecules for removal, and therefore was not detectable by the assay utilized. Glutathione biosynthesis is one of the principal defenses for protecting the cell against excess oxidative events. It also conjugates cellular constituents for biotransformation, and subsequent removal of toxic cell constituents (Parker and Ankel, 1992). In the conjugated form, glutathione is no longer detectable in the assays used here, which may also explain the reduction in total intracellular glutathione at 3 dpt as shown in Fig. 3. Whether or not re-addition would stimulate another round of biosynthesis is under study. The current data demonstrate that FFAAP increases intracellular biosynthesis of total intracellular glutathione relative to untreated cells in an FFAAP dose-dependent manner, plateauing at 5 mM concentrations. Higher levels had no effect on increasing intracellular biosynthesis. Thus, subsequent experiments were performed in the presence of the ED_{50} FFAAP concentration determined for each cell line.

Next, we quantified intracellular total glutathione during Zika virus infection of Vero cells. Intracellular glutathione levels diminished rapidly over 3 dpi in the absence of FFAAP (Fig. 4A). On the other hand, FFAAP-treated infected cells showed marked increases in intracellular glutathione over 3 dpi when compared to untreated cells. At 5 mM concentrations of FFAAP, levels of intracellular glutathione exceeded three standard deviations of the mean of untreated cells, with Zika virus yields reduced significantly in the presence of each concentration of FFAAP, most notably between 5 and 8 mM by 3 dpi (Fig. 4B). Over 90% reduction in virus levels was observed in the presence of 2.5–5 mM FFAAP in Vero cells.

Finally, to evaluate whether intracellular glutathione was responsible for the reduction in Zika virus titers in Vero cells, we quantified virus reduction while inhibiting biosynthesis of glutathione by using buthionine sulfoxime (BSO) (200 μM). We predicted that the inhibition would restore the Zika virus replication to nearly the levels observed in untreated cells. We also rationalized that full restoration would require at least 36 h post infection given that the reported half-

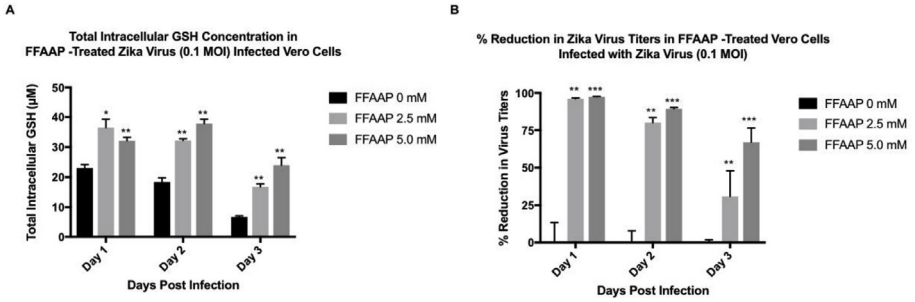

Fig. 4. Effect of FFAAP on total intracellular glutathione and Zika virus replication in Vero cells. Vero cells, grown to approximately 90–95% confluence (mid-log phase), were treated with and grown in the presence of increasing concentrations of FFAAP for 3 days. At each time point, medium was discarded and cells were harvested to measure intracellular concentrations of total glutathione. Panels A and B show data from these studies. All experiments shown were done in triplicate and the data shown are representative data from multiple independent experiments performed. Standard error bars were calculated using Microsoft Excel and significance assessments were performed using GraphPad Prism. $p < 0.05$ and > 0.01 = *, $p < 0.01$ and > 0.001 = **, and $p < 0.001$ = ***.

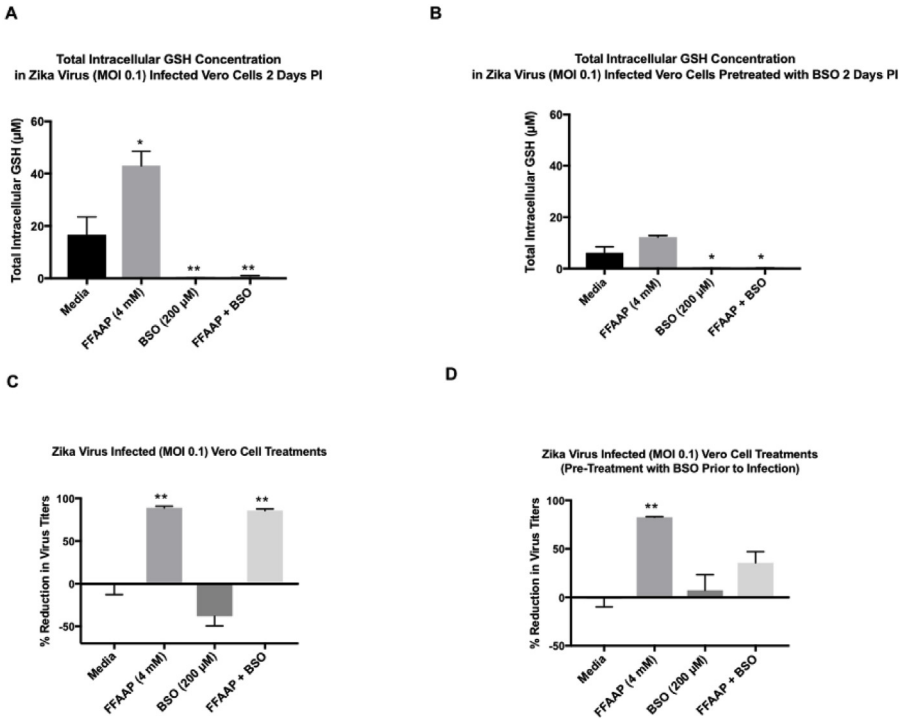

Fig. 5. Effects of Inhibition of Glutathione Biosynthesis on Zika Virus Replication in Vero cells. **A**. Zika virus infected Vero cells were either untreated or treated with BSO (200 μM), FFAAP (4 mM), or BSO + FFAAP, and the levels of intracellular glutathione measured. **B**. Vero cells pre-treated with BSO (200 μM) were infected with Zika virus and treated with the above treatments to quantify inhibition of total intracellular glutathione synthesis. **C**. Percent Zika virus titer reduction in Vero cells treated with BSO, FFAAP, or BSO + FFAAP. **D**. Percent reduction in Zika virus titers in Vero cells pretreated with BSO and then treated with BSO, FFAAP, or BSO + FFAAP post infection. All experiments shown were done in triplicate and the data shown are representative data from multiple independent experiments performed. Standard error bars were calculated using Microsoft Excel and significance assessments were performed using GraphPad® Prism. $p < 0.05$ and > 0.01 = *, $p < 0.01$ and > 0.001 = **, and $p < 0.001$ = ***.

life of intracellular glutathione was approximately the same. To test this, we used two different experimental conditions. In the first setup, cells were seeded in growth medium and once cells were ~90% confluent, these were infected with Zika virus (MOI 0.1). At 1.5 h post adsorption cells were treated with viral growth medium, medium containing FFAAP at 4 mM, medium containing BSO at 200 μM, or medium containing both FFAAP and BSO at 4 mM and 200 μM concentrations, respectively. In the second set-up, cells were seeded in the presence of BSO (200 μM) until they were ~90% confluent. Cells were then washed and Zika virus inoculum or uninfected cell lysate of equal protein concentration was added. Two hours post-adsorption, each inocula was removed and cells were treated identically to those as described in the first setup. The results of replicate experiments are shown in Fig. 5. Supplementary Fig. 2 shows data validating that BSO treatment reduced intracellular glutathione levels in Vero cells to undetectable levels in uninfected cells, even if the cells were pretreated with BSO. Data demonstrated also that the intracellular levels of glutathione dramatically decreased and were undetectable by 48 h post BSO treatment in Zika virus-infected Vero cells (Fig. 5A and B). We also analyzed Zika virus replication in infected (MOI 0.1) cells that were untreated or pre-treated with BSO prior to infection and subsequent treatments with FFAAP, BSO, or FFAAP and BSO (Fig. 5C and D). Data in Panel C demonstrate that Zika virus replication was unaffected when BSO was present in Vero cells, suggesting that the virus did not require glutathione for replication. We, however, observed that administration of FFAAP in the presence of BSO partially restored inhibition of Zika virus production in Vero cells indicating FFAAP reduced virus titers independently of total intracellular glutathione levels. We observed the same results in cells that were pretreated with BSO for 48 h prior to infection. Thus, even in the absence of measurable intracellular glutathione, FFAAP alone reduced Zika virus replication Vero cells. Together these data suggested for the first time the direct antiviral effects of FFAAP.

To determine whether the effects of FFAAP and intracellular GSH during Zika virus infection in a susceptible and relevant human cells line each had a role in virus inhibition, we performed similar experiments using JEG-3 cells, FFAAP was found to be toxic at 4 mM concentration in JEG-3 cells, therefore all experiments were performed with FAAPP treatments at 2.5 mM concentrations in which cells remain healthy as shown in Supplementary Data Fig. 1. BSO (200 μM) treatment blocked intracellular glutathione biosynthesis in Zika virus infected and uninfected JEG-3 cells similar to observations in Vero cells (Fig. 6A and B). Remarkably, in JEG-3 cells we discovered that blocking intracellular biosynthesis of glutathione resulted in inhibition of Zika virus replication, suggesting for the first time that at least these human cells depended on intracellular glutathione biosynthesis for Zika virus replication, supporting observations reported from another study indicating that other flaviviruses utilize reduced glutathione (GSH) to

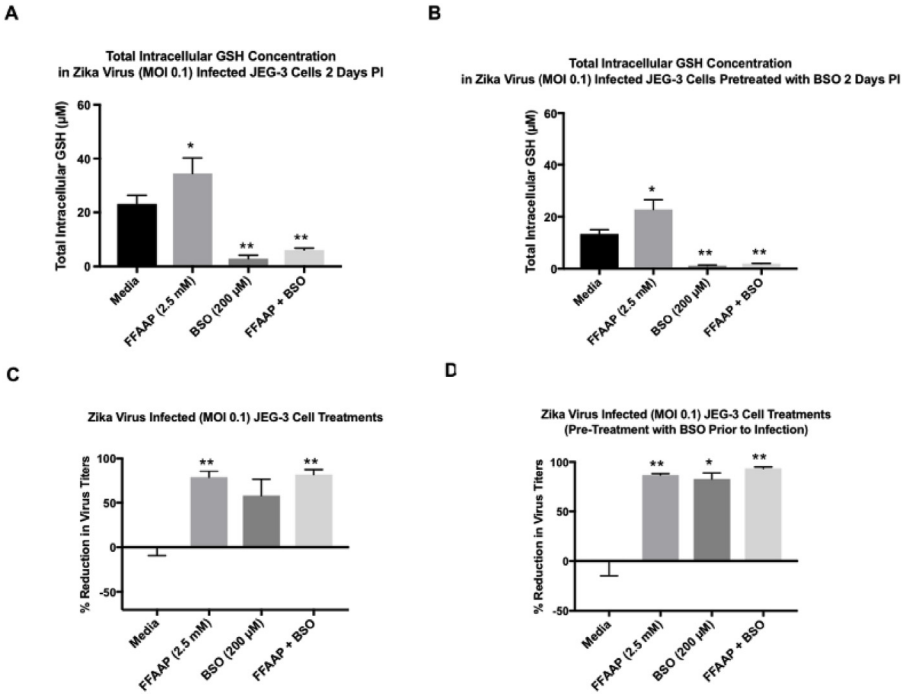

Fig. 6. Effects of Inhibition of Glutathione Biosynthesis on Zika Virus Replication in JEG-3 cells. **A**. Zika virus infected JEG-3 cells were either untreated or treated with BSO (200 µM), FFAAP (2.5 mM), or BSO + FFAAP to verify the levels of intracellular glutathione. **B**. JEG-3 cells pre-treated with BSO (200 µM) were infected with Zika virus and treated under conditions described above to quantify inhibition of total intracellular glutathione synthesis. **C**. Percent Zika virus titer reduction in JEG-3 cells treated with BSO, FFAAP, or BSO + FFAAP. **D**. Percent reduction in Zika virus titers in JEG-3 cells pretreated with BSO and then treated with BSO, FFAAP, or BSO + FFAAP post infection. All experiments shown were done in triplicate and the data shown are representative data from multiple independent experiments performed. Standard error bars were calculated using Microsoft Excel and significance assessments were performed using GraphPad Prism. $p < 0.05$ and > 0.01 = *, $p < 0.01$ and > 0.001 = **, and < 0.001 = ***.

inhibit stress-granule formation (Basu et al., 2017). The addition of extracellular glutathione in the absence of FFAAP had no effects (data not shown) on virus yields, which was expected since uptake of exogenous glutathione across the membrane has been shown to be poor. Regardless, in the presence of FFAAP as well as FFAAP plus BSO, JEG-3 cells showed 90% inhibition of Zika virus replication (Fig. 6C and D).

Collectively, these data demonstrate for the first time that FFAAP-treated cells demonstrated increased intracellular biosynthesis of glutathione as evidenced by the levels of intracellular of glutathione shown in each of the experiments presented. Furthermore, up to 90% reduction of Zika virus yields was observed with FFAAP (2.5–5 mM) treatment of both JEG-3 cells and Vero cells. The reduction of Zika virus titers in FFAAP-treated Vero and JEG-3 cells is independent of total intracellular GSH concentrations. These data also mark the first time investigators have shown that as Zika virus infection progresses there is a steady reduction in the biosynthesis of new intracellular glutathione, which can be circumvented in the presence of FFAAP in Vero and JEG-3 cells. Most interesting, however, is that Zika virus appears to require intracellular glutathione to replicate in human JEG-3 cells, as do other flaviviruses, but nonetheless, FFAAP effectively inhibits replication.

4. Discussion

These studies demonstrate that by increasing biosynthesis of intracellular levels of glutathione slightly beyond that normally observed levels remarkably impacts infectious Zika virus production differently in the two types of mammalian cells evaluated in this study. The effect of intracellular glutathione on virus infections has been previously described using HIV over two decades earlier (De Rosa et al., 2000; Raju et al., 1994; Roederer et al., 1992), and more recently in flaviviruses, Sendai virus, and herpesviruses (Basu et al., 2017; Fraternale et al., 2014; Palamara et al., 1995, 2004; Mumtaz et al., 2017; Garaci et al., 1992). With the sounding global alarms of concern about emerging pathogens, developing insight into how Zika virus can be naturally controlled is imperative and our results show that Zika virus replication in human placental cells is dependent upon intracellular glutathione, supporting the findings of Mumtaz et al. (2017) We show here, however, that regardless of how a cell utilizes intracellular glutathione, e.g., stress granule reduction and restricted translational processes, FFAAP inhibits virus replication independently. Although it seems counter-intuitive to provide an antiviral that enhances glutathione biosynthesis in cells for viruses that require glutathione, our data demonstrates that FFAAP inhibits virus production regardless of the concentrations of intracellular glutathione in each cell line used. Further, with previously published reports, one can speculate that other cellular factors that are modulated by glutathione can easily provide counter-measures against viral exploitation of cell defenses. Cell culture models are invaluable for identifying such defenses that can control emerging and re-emerging viruses, e.g., Zika virus replication, while simultaneously enhancing our understanding of the pathogenesis of this re-emerging infection. Such models also provide a rapid, efficient way to identify the efficacy of potential therapeutics for controlling infection, while revealing any apparent differences between Zika virus infected humans and animal models. Glutathione, the smallest sulfur-containing molecule biosynthesized in the cell, is one such highly conserved innate defense, a pleiotropic molecule with the capacity to reduce reactive oxygen species and subsequent stress granule formation generated in cells under stress, as well as to chelate divalent cations that may be essential to viruses. (Mutoh et al., 1990; Younes and Strubelt, 1990) (Vilas et al., 1976; Lopez-Torres et al., 1991). The glutathione system is recognized as one of the earliest, if not the earliest primordial defense system selected and maintained throughout evolution in most living systems dating back from the time of cyanobacteria (Carod-Artal, 2016; Chardonnet et al., 2015; Babu et al., 2011; Masip et al., 2006). As such, it is not surprising that it plays a dynamic defense role for some cells during virus infections. The design of the experiments presented here facilitated assessment of the differential role(s) intracellular glutathione plays in different cell lines infected with Zika virus. To our knowledge, this is the first study to demonstrate that Zika virus rapidly depletes intracellular biosynthesis of glutathione, perhaps as a countermeasure to thwart its defense strategies, and this depletion may partly explain why Zika virus grows to relatively lower levels in human cells. [21] It is also the first report, to our knowledge, that demonstrates the free-form amino acids formulation provided by FFAAP reduces Zika virus replication up to 90% at doses that are completely safe for cells.

Robbins and colleagues showed over three decades ago the role of glutathione in the antiviral state induced in cells sensitive to Type I interferons (Robbins et al., 1981). Since it is well understood that Type

1 interferons reduce Zika virus replication, future studies are warranted to identify mechanisms by which this process may occur. The absence of Type 1 interferons in each of the cell lines used for these studies provided us with the opportunity to directly assess antiviral actions that are independent of this critical cellular defense, which adds another complex layer of interactions between Zika virus and its host cells.

Finally, reduction of virus production in JEG-3 cells and Vero cells by ~90% in the presence of 2.5–5 mM concentrations of FFAAP throughout 48–72 hpi demonstrates the efficacy of FFAAP as a significant antiviral and underscores the value of extending this research using *in vivo* models. The fact that these precursors result in direct reduction of infectious Zika virus production suggests that FFAAP may prove useful for minimizing Zika virus replication in humans, particularly in those individuals for whom the consequences of the infection can be life-altering.

Acknowledgements

*Authors, Vasireddi and Hilliard contributed to this work as follows, Drs. Vasireddi and Hilliard conceived the experiments and MV executed these in the laboratory. Vasireddi and Hilliard analyzed data and established conclusions for additional experiments and Dr. Katz provided critical reviews, important contributions as the work progressed, and was invaluable during manuscript preparation. Dr. Hilliard drafted the manuscript, while Dr. Vasireddi took charge of statistical analyses and graphics. Drs. Crum and May provided critical discussions and insight into the mechanisms of the free-form amino acid precursors, which was quality assessed and provided. Dr. Crum originally conceived of the formulation of free-form amino acids, and is the president of the ProImmune Company, LLC. The work performed here was suggested by Julia Hilliard and subsequently a collaboration was discussed and formed with the Drs. Crum and May, as well as Drs. Vasireddi and Katz. This work was supported by special project resources from the Viral Immunology Center and special interest funds from the endowment of the Georgia Research Alliance to whom the authors are greatly appreciative. The Viral Immunology Center also gratefully acknowledges the support of the Georgia Research Alliance for the facilities in which these studies were performed.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.antiviral.2018.09.004.

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