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Castanospermine reduces Zika virus infection-associated seizure by inhibiting both the viral load and inflammation in mouse models

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1. Introduction

Since its discovery in 1947 in Uganda, the Zika virus (ZIKV) has spread throughout the world, causing significant outbreaks in Micronesia in 2007, French Polynesia in 2013, and Brazil in 2015. The spread of the virus to North America began in 2016 and was coincident with the World Health Organization declaring ZIKV a Public Health Emergency. Although the incidence of ZIKV infection has declined in recent years, the chances of another outbreak cannot be ruled out. Several vaccines and small molecules are in preclinical or clinical trials, but presently, there is no approved drug on the market for the treatment of ZIKV. Additionally, since antibody-mediated enhancement is reported reduced viral load and brain inflammation with the resulting appearance of delayed neuronal disorders, including seizures and paralysis in an Ifnar1\textsuperscript{−/−} mouse.

Zika virus (ZIKV) outbreaks have been reported worldwide, including a recent occurrence in Brazil where it spread rapidly, and an association with increased cases of microcephaly was observed in addition to neurological issues such as GBS that were reported during previous outbreaks. Following infection of neuronal tissues, ZIKV can cause inflammation, which may lead to neuronal abnormalities, including seizures and paralysis. Therefore, a drug containing both anti-viral and immunosuppressive properties would be of great importance in combating ZIKV related neurological abnormalities. Castanospermine (CST) is potentially a right candidate drug as it reduced viral load and brain inflammation with the resulting appearance of delayed neuronal disorders, including seizures and paralysis in an Ifnar1\textsuperscript{−/−} mouse.

ZIKV is a positive-sense, single-stranded RNA virus that belongs to the flaviviridae family, flavivirus genus, and transmitted by Aedes mosquitos. The genome is ~11,000 nt that encodes three structural proteins, including capsid (C), precursor membrane (prM), and envelope (E) as well as seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). Of these proteins, NS1, E and prM are glycosylated. The glycosylation of flaviviral NS1 appears to be essential for efficient secretion, virulence and viral replication (Annamalai et al., 2019; Crabtree et al., 2005; Pryor and Wright, 1994; Somnuke et al., 2011; Winkler et al., 1988). The ZIKV E protein is glycosylated at amino acid N154. In mouse models, recombinant N154Q ZIKV that lacks the E protein glycosylation results in lower viremia, decreased weight loss, and no mortality although it does not significantly affect neurovirulence (Fontes-Garfias et al., 2017). The prM of flaviviruses facilitates the folding and trafficking of the E protein at the time of virus particle biogenesis (Allison et al., 1995; Heinz et al., 1994). The cellular protease furin cleaves prM during particle egress, releasing an N-terminal fragment (pr) containing the single N-linked glycan of prM. This cleavage is required for infectivity in flaviviruses (Elshuber et al., 2003).

Only 20% of ZIKV infections are symptomatic, characterized by low-grade fever, pruritic maculopapular rash, myalgia, arthralgia, conjunctivitis, and headache. Although ZIKV symptoms are less severe than the closely related dengue virus (DENV), neurological abnormalities such as microcephaly in infants (Mikar et al., 2016; Moura da Silva et al., 2016), Gullian Barre syndrome (GBS) in adults (Brasil et al., 2016; Cao-Lormeau et al., 2016; Kassavetis et al., 2016; Rozé et al., 2017), and the possibility of sexual transmission (Petridou et al., 2019; Reyes et al., 2019) makes the ZIKV disease more complicated and of high concern. Among 35 ZIKV-infected infants with microcephaly in Brazil, other neurological abnormalities reported included hypertonia/spasticity (37%) and seizure (9%). Of the 27 available neuroimages from these infants, all presented with wide-spread brain calcification and evidence of cell migration abnormalities (Schuler-Faccini et al., 2016).

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https://doi.org/10.1016/j.antiviral.2020.104935
Received 27 April 2020; Received in revised form 2 September 2020; Accepted 11 September 2020
Available online 16 September 2020
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Encephalopathy and seizures were reported in a patient with ZIKV infection (Asadi-Pooya, 2016; Pastula et al., 2016; Rozé et al., 2016). Finally, motor abnormalities and epilepsy were reported in children with congenital ZIKV infection (Pessoa et al., 2018).

Seizure-related activities have also been reported in various animal models with ZIKV infection. Neonatal mice with blocked interferon signalling have been used in ZIKV viral infection studies. In addition to lethal infection, these mice also displayed neurological symptoms such as toe walking, tremors, loss of balance, paralysis and hunched posture (Aliota et al., 2016; Lazear et al., 2016; Manangeeswaran et al., 2016; Rossi et al., 2016). The seizure-related activity was seen in a ZIKV-infected immunocompetent neonatal mouse (Manangeeswaran et al., 2016). In adult AG129 mice, the seizures were independent of hind limb motor deficit levels (Zukor et al., 2018). The seizure-related activity has been reported in piglets with in-utero exposure to ZIKV (Darbellay et al., 2016). The potency of CST varies with cell type and the virus. CST exhibited an IC50 of 87 μM towards DENV2 in human hepatoma cells (Huh-7) and 1 μM in baby hamster kidney cells (Whitby et al., 2005). CST inhibits BVDV with IC50 110 μM and 567 μM in plaque inhibition assay and cytopathic effect assay, respectively in Madin-Darby bovine kidney (MDBK) cells (Whitby et al., 2004). The activity of CST against HIV has also been reported (Sunkara et al., 1990; Taylor et al., 1994; Walker et al., 1987). The weak activity in cellular assays is possibly due to its highly polar nature (Shi et al., 2011). Celgosivir (6-O-butanoyl castanospermine) is a pro-drug that is immediately converted to CST by esterase. Celgosivir is found to have better cell permeability, activity against glucosidase-I and anti-viral potency. In clinical studies among patients with secondary DENV infection, a small, non-statistical trend towards better outcome on platelet nadir and the difference between the maximum and minimum hematocrit was observed in celgosivir-treated patients (Sung et al., 2016). Herein we evaluated CST impact on another member of the flavivirus family, as we examined the anti-ZIKV activity of CST in vivo both in pre- and post-infection treatments in a murine model. In addition to antiviral activity, we also evaluated CST for its efficacy against ZIKV induced neurological abnormalities such as seizure, paralysis and motor function.

2. Material and methods

2.1. Cell culture, virus strains, and reagents

Vero E6 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, HyClone) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich). SNB-19, human glioblastoma cells were maintained in RPMI-1640 medium (ATCC) with 10% FBS. Zika virus strains used were: FSS13025 (Cambodia, 2010), ZIKV PRVABC59 (Puerto Rico, 2015), and ZIKV MR766 (Uganda, 1947). ZIKV stocks were prepared by infecting Aedes albopictus (C6/36) cells at multiplicity of infection (MOI) of 0.1 and were incubated with the viral inoculum for 1 h at room temperature. Fresh medium was added to the culture flask and incubated at 28 °C till 8–10 days. The supernatant was collected and filtered through 0.45 μm, and frozen in aliquots at −80 °C. Virus stock was tittered in Vero E6 cells before infecting with mice and SNB-19 cells.
2.2. Extraction and purification of castanospermine

Castanospermine (1,6,7,8-tetrahydroxycotahydroindolizine) was isolated from the seeds of the Australian legume *C. australe* as per previously published protocol (Hohenschutz et al., 1981). Briefly, finely ground immature seed (3 kg) of *C. australe* was extracted with 75% ethanol (3 × 3 L). After filtration, the extract was mixed with strongly acidic cation exchange resin ( Dowex-50) in the H⁺ form and stirred overnight. After stirring, the resin was allowed to settle, and the liquid was poured off. The resin was bath washed extensively with deionized water until the washings were clear and the resin then poured into a column. The column was eluted with 2 M NH₄OH and the eluate fractions were concentrated by rotary evaporation until all ammonia was removed. The concentrated eluate was then loaded onto a column of Dowex-50, pyridinium ion form, washed extensively with deionized water and eluted first with 2 M pyridine followed by 2 M NH₄OH. Fractions containing CST were pooled, concentrated to near dryness, and crystallized in 95% ethanol. The white crystals were filtered, air dried, and used in these experiments. The purity and identity of the CST were determined by LC/MS and NMR analysis, respectively.

2.3. Viral titre by focus-forming assay (FFA)

Human glioblastoma-derived cells (SNB-19), were seeded in 96-well plates in RPMI-1640 medium containing 10% FBS, 100 units/ml penicillin and 50 μg/ml streptomycin. The cells were incubated at 37 °C in CO₂ incubator and grown to ~80% confluence. The media was replaced with fresh media containing either CST (0.3–300 μM, 3 fold dilutions) or phosphate buffer saline (PBS) as vehicle control. After 1 h of pre-treatment, cells were infected with ZIKV at a MOI of 1. The plates were incubated at 37 °C in a 5% CO₂ incubator for 24 h. Cell supernatant was titrated in duplicates onto a monolayer culture of Vero E6 cells in 96-well plates with log dilution and incubated at 37 °C for 2 h in CO₂ incubator. Virus inoculum was removed and replaced with RPMI medium with 1% methylcellulose overlay. Vero cells were incubated for an additional 48 h, removed the overlay before fixation with 4% formaldehyde, blocked and incubation with anti-flavivirus group antibody (G2) overnight at 4 °C. Cells were washed three times with PBS and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody for 1 h at room temperature. Plates were washed with PBS and incubated with DAB peroxidase substrate (SK4100, Vector Labs) for 10 min, and the reaction stopped by washing with water. Focus forming units (ffu) were counted by the naked eye and confirmed by counting by 40x microscopy. The IC₅₀ was calculated using Graphpad prism software version 8.

2.4. Cell toxicity assay

The tolerance level of CST to SNB19 was indirectly estimated by measuring the adenosine triphosphate (ATP) content of the SNB19 cells treated with various concentrations of the CST using CellTiter Glo reagent (G7571, Promega). Cells were seeded in opaque well plates in RPMI-1640 medium 10% FBS, 100 units/ml penicillin and 50 μg/ml streptomycin, incubated at 37 °C with 5% CO₂ until the cells reached ~80% confluence. Media was then replaced with fresh media containing CST (0.001–300 μM) or PBS as vehicle control. After incubating the cells for 24 h at 37 °C in CO₂ incubator, 100 μl of CellTiter Glo reagent was added. The luminescence was recorded using luminometer (9300–001, Turner Biosystems). The 50% cytotoxic concentration (CC₅₀) was calculated using Graphpad prism software version 8.

2.5. Western blot analysis

SNB19 cells were grown in 6-well plates in RPMI-1640 medium 10% FBS, 100 units/ml penicillin and 50 μg/ml streptomycin, to 80% confluence and treated with or without CST (100, 500 and 1000 μM) for 24 h. Cells were harvested by trypsinization, pelleted, lysed in 1X Laemmli buffer, and the lysates boiled. The lysates were run on SDS-PAGE, and separated protein bands were transferred to nitrocellulose membrane followed by blocking with 5% powdered skimmed milk in PBS with 0.1% Tween 20. Primary antibody anti–ZIKV NS1 (BF-1225-36, BioFront Technologies, Tallahassee, FL) was added and incubated for overnight at 4 °C. The reaction was visualized with HRP-linked anti-mouse secondary antibody followed by enhanced chemiluminescence (WBKLS0500 Millipore). For loading control, anti-GAPDH (sc-25778, Santa Cruz Biotechnology, Texas) was used as a primary antibody.

2.6. Mouse studies

All the studies were completed with adult (6–8 wk old) Ifnar1−/− mice which were of C57 background (Jackson lab). Upon arrival mice were quarantined for a week, fed ad libitum, provided 12 h dark/12 h light cycle. The mouse-adapted ZIKV FSS13025 strain was used for all the mouse studies. The virus and drug were dosed intraperitoneally (IP).

Stocks of the virus stored at –80 °C were diluted in serum-free EMEM media as required for each study. The CST was dissolved in physiological saline such that 100 μl will have required dose per mouse as indicated in individual studies. Five in vivo studies were conducted, and treatment groups in each study were compared by statistical analysis using unpaired Student’s *t*-test using Graphpad prism version 8.

2.6.1. Mouse-study-I: single dose of CST per day to check viral load

Ifnar1−/− mice (6–7 wk old, male and female) were dosed with CST (IP, 50 mg/kg or 100 mg/kg bw) or vehicle control (0.9% saline) once daily on day 0 to day 3 for a total 4 doses. Mice were infected with ZIKV (10⁶ ffu/in 100 μl EMEM media/mouse IP) on day 0. Mice were euthanized on day 3 dpi. Blood and liver tissue were collected, serum was separated from the blood and the NS1 antigen level in the serum was estimated by an enzyme linked immunosorbent assay (ELISA) kit (BioFront Technologies, Tallahassee, FL) as described in the manual. Liver tissue was stored in RNA-later (Qiagen) at 4 °C, around 30 mg of the tissue was homogenised in 600 μl of buffer RLT (79,216, Qiagen) with 10 μl/ml beta mercaptoethanol using Tissue Ruptor II (Qiagen). After centrifuging at 14,000 g, the supernatant was transferred to a new tube and added I volume of 70% ethanol. After mixing, 700 μl of the solution is loaded on to QIAamp Mini Spin Columns and RNA was extracted using the QIAamp viral RNA mini kit (52,904, Qiagen). RNA (2 μg) was converted to cDNA using SuperScript III First strand synthesis (Invitrogen), and expression levels of ZIKV-NS1 gene relative to mouse GAPDH gene was done using the specific primers (Supplementary Table1) by real time PCR.

2.6.2. Mouse-study-II: single dose of CST per day to study the survivability

Ifnar1−/− mice (6–7 wk-old, male and female) were randomly grouped into 4 groups with equal number of males and females in each group. Group 1: CST 200 mg/kg & media, Group 2: virus & saline, Group 3: virus & CST 100 mg/kg, Group 4: virus & CST 200 mg/kg. Mice were treated with CST (100 mg or 200 mg/kg bw) or saline once daily until seven doses starting from day 0 were given. Mice were infected with ZIKV (10⁶ ffu/mouse, IP) on day 0 in 100 μl EMEM media. Two to six mice were euthanized from each group on day 3 to estimate systemic and tissue viral load. The remaining mice were observed for death/morbidity until day 10. Endpoints were decided based on the published articles (Dowall et al., 2016; Morton, 2000; Offert and Godson, 2000) and our previous studies (Yang et al., 2018). Mice reaching endpoints (Table 1) were euthanized as per the approved protocol. Based on the previous study, the mice which reached endpoints before day 5 dpi were not considered for statistical analysis as death due to viral infection happens on or after day 5 dpi. The statistical analysis was done using Kaplan Meier equation and comparison of survival curves was done using log rank (Mantel-Cox) test.
Table 1

| Posture                  | Score |
|-------------------------|-------|
| Normal – 0; slightly hunched – 1; moderately hunched – 2; severely hunched – 3 |       |

Coat condition

| Score |
|-------|
| Normal/groomed – 0; rough – 1; ruffled/unkempt – 2 |

Activity

| Score |
|-------|
| Normal – 0; reduced exploratory activity – 1; slow moving, dull or depressed – 2; not moving – 3 |

Movement/Gait

| Score |
|-------|
| Normal – 0; slight incoordination/decreased righting response – 1; tip toe walking or altered gait involving 1 limb – 2; staggering or paralysis or tremors of 2 or more limbs – 3 |

End points.

a) Body weight loss is equal to or greater than 20% of baseline.
b) One limb paralyzed and body weight loss equal to or greater than 15% of baseline.
c) Both limbs paralyzed and body weight loss equal to or greater than 12% of baseline.
d) Assessment score of 3 for any of the following clinical observations: Activity/Alertness or Movement/Gait.
e) A total assessment score of 6 plus 10% body weight loss.
f) Found dead.

2.6.3. Mouse-study-III: split doses of CST to study survivability

In this study, the mice were dosed with 100 mg/kg CST twice a day 24 h before (preCST) or 24 h after (postCST) infection with lesser ZIKV load (10^5 ffu/mouse). Six to seven-week-old ifnar1^{-/-} (male and female) mice were grouped. G1: Saline and EMEM (n = 2), G2: preCST-100X2 + EMEM media (n = 1), G3: postCST-100X2 + EMEM media (n = 1), G4: ZIKV + Saline (n = 8), G5: ZIKV + preCST (n = 8), G6: ZIKV + postCST (n = 8). A survival blood sampling was done from the tail on day 3 for estimating the NS1 antigen by ELISA as a measure of viral load. The treatment was continued until day 6 dpi and scored as described in mouse-study-II above. Remaining mice were observed for morbidity/death until day 10. Mice reaching end points (Table 1) were euthanized and on day 10, all the remaining mice were euthanized. Seizure-related activity and hind limb paralysis were also noted. Statistical analysis was done with the Kaplan Meier equation and comparison of survival curves was done using log-rank (Matnel-Cox) test.

2.6.4. Mouse-study-IV: ZIKV induced inflammation of the brain and treatment with CST

The study design follows mouse-study-III but was terminated at day 4 dpi. Survival blood sampling was conducted daily from day 2 to day 4 dpi from the tail vein to estimate viral load using the ZIKV-NS1 ELISA kit. Following euthanization on day 4 dpi, brain tissues were collected in Trizol, snap-frozen and stored at -70°C. RNA was isolated, converted to cDNA and the relative expression of genes of ZIKV-NS1 and inflammation associated genes (IL1β, TNFa, IL6, IL10, GFAP, iNOS1) were determined using real time PCR. Mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a house-keeping gene. The estimation of ZIKV RNA copy number from blood collected on day 4 dpi is made by Inverted grid suspension test. Inverted grid suspension test was done as based on the published protocol (Deacon, 2013) in mouse-study-V on day (−1), day 3, day 5 and day 6. A rectangular screen made of stainless steel (18 × 12 inch) with 1 cm² mesh was used. The side of the screen was covered with sealing tape to prevent mice climbing to the top of the mesh. For testing, the mouse was placed in the centre of the wire mesh screen; a timer was started, and the screen was rotated to an inverted position over 2 s with the mouse’s head declining first. The screen was held steady 40 cm above a padded surface. Time was noted when the mouse fell off or was removed when the criterion time of 120 s was reached (Rabl et al., 2016). Each mouse was trained two times before the actual reading was taken. Video recording was done using a handycam (Sony) mounted on a stand.

2.6.5.2. Paralysis test. The test was done during mouse-study-V on day (−1), day 3, day 5, day 6 and day 7. Each mouse was held on its tail and lifted to a height of 30 cm, and movements of the limbs were noted, and video recorded. Further, the mouse was allowed to walk on a mounted flat surface and observed for paralysis or any other walking disabilities. Video recording was done using a handycam (Sony) mounted on a stand.

2.6.5.3. Provoked seizure test. The test was done during mouse-study-V on day (−1), day 3, day 5, day 6 and day 7. The provoked (acute symptomatic) seizure test was conducted based on the observation in previous studies that there is a seizure-related activity in mice during the opening of the cage lid or handling of mice, especially in the morning. Hence, we decided to observe the mice for seizure and related activity in the morning from 9 to 11 a.m. The mouse cages were brought to the hood between 9 and 10 a.m., and the camera was focussed on the cage for video recording. To induce uniform handling stress, mice were picked up by the tail and lifted to a height of 10 inches and dropped to the cage bedding three times. Seizure and related activity, if any, was noted and scored (Racine, 1972) along with the addition of other symptoms such as toe walking, hunched position, erratic running and jumping upon touching as indicated in Table 2. (Lütjohann et al., 2009). Mice with a Racine score of at least one (Score 5 and above in Table 2) were considered as exhibiting a seizure. Some of the mice exhibited the seizure and related activity while opening the cage lid while others showed this activity after dropping to the cage bed.

2.6.5.4. Measure of spontaneous seizure and related activity. During mouse-study-V, the mice in their cages were observed randomly for any seizure, and related activity between 8 a.m. and 7 p.m. On day 6 of pdi, 1 h video recording of the mice in their cages were done using a handycam mounted on a stand.

2.6.5.5. Rotarod test. During the mouse-study-V, rotarod testing was done using rotarod test equipment (Panlab, 76–0770, rod dia 1 inch, fall height 20 cm) as per published protocol (Rabl et al., 2016). The mice were habituated to the testing system before stating the study until they were able to stay on the rotating rod for at least 1 min at a constant speed of 4 rpm. Mice were trained 2–3 times before conducting the actual test. Each mouse was lifted from the cage by their tail and placed on the rod, and the rod was rotated with an initial speed of 4 rpm until the mouse stabilized itself, and then the speed was accelerating up to 40 rpm. The mice falling before 5 s were not considered in the analysis. The trial

Table 2

| Sl. No | Neurological Abnormalities | Score |
|-------|---------------------------|-------|
| 1     | Toe walking               | 1     |
| 2     | Hunched dorsal position   | 2     |
| 3     | Erratic running           | 3     |
| 4     | Jumping upon touching     | 4     |
| 5     | Mouth and facial movement (RS-1) | 5 |
| 6     | Head nodding (RS-2)       | 6     |
| 7     | Forelimb clonus (RS-3)    | 7     |
| 8     | Rearing with forelimb clonus (RS-4) | 8 |
| 9     | Rearing and falling with forelimb clonus (RS-5) | 9 |
began when acceleration was started and ended when the animal fall-off the rod. If the animal clung to the rod and completed the full passive rotation, the timer was stopped for the animal, the passive rotation was noted, and the animal was returned to its home cage. The rod and floor were wiped with alcohol between each test. On test-day, 2–3 trials and three actual tests were conducted for each mouse with a gap of 5 min between each test. The average time taken to fall from the rod on day (−1) is considered as 100% for each mouse. The percentage change duration to fall on the subsequent testing day is calculated and compared using the unpaired Student’s t-test using Graphpad prism version 8.

2.6.6. ZIKV-NS1 antigen level as a measure of viral load

ZIKV-NS1 antigen levels in the serum were estimated using ZIKV-NS1 sandwich ELISA kit (BioFront Technologies) as an indirect estimation of viral load. Briefly, 5 μl of the serum was diluted in 1:300 or 1:1500 in dilution buffer, added to antibody-coated plates, washed and HRP-labelled antibody added. Absorbance was read at 450 nm after the addition of substrate tetramethylbenzidine and stop solution. NS1 was quantified using the standard curve and multiplying by the dilution factor. Statistical analysis was done by unpaired Student’s t-test using Graphpad prism version 8.

2.6.7. Viral copy number in serum

Serum stored at −70 °C was thawed, and 80 μl of the serum was used to isolate RNA using QIAamp viral RNA mini kit (52,904 Qiagen). Briefly, the isolated RNA was converted into cDNA using Superscript III First-Strand synthesis (18080051 Invitrogen) and quantitative real time PCR was performed using SYBR Green PCR master mix (4,344,463 Invitrogen) and ZIKV specific primers (Supplementary Table 1, ZIKV-NS1-F & ZIKV-NS1-R). For the standard curve, the ZIKV viral RNA was synthesized in vitro and used in real-time PCR. The cycle threshold (Ct) values of the test samples were converted into viral copy numbers using the standard curve. Statistical analysis was done by unpaired Student’s t-test using Graphpad prism version 8.

2.6.8. Ethics statement

The in vivo efficacy study in Ifnar1−/− mice was approved by the Institutional Animal Care and Use Committee of Florida State University and was in accordance with Public Health Service Policy and the Guide for the Care and Use of Laboratory Animals (8th edition).

3. Results

3.1. Purification of CST

The CST was purified from seeds of C. australe with an overall yield of 0.5%. As per the LCMS analysis, the purity was >98% and the NMR spectrum is shown in Supplementary Fig 1.

3.2. Viral load reduction and survivability in mice

In the initial study (mouse-study-I) in ifnar1−/− mice there was 46% (p = 0.003) ZIKV–NS1 antigen level in serum and 68% (p = 0.011) reduction of ZIKV-NS1 gene in liver tissue on day 3 dpi at CST 100 mg/kg dose given IP once daily (Fig. 1 D and Supplementary Fig 2A). We followed up this with a survivability study where mice were challenged with a ZIKV load of 10^6 ffu/mouse given IP at CST doses of 100 and 200 mg/kg once daily for a total of 7 doses (mouse-study-III). CST was given 100 mg/kg bw twice daily up to day 6 dpi. A survival blood sampling from tail was done on day 3 dpi. 1A. Viral load in serum was estimated by ELISA using ZIKV-NS1 kit. Statistical analysis was done by unpaired Student’s t-test. p < 0.05 is considered as significant. 1B and 1C: Survival curves of pretreatment (1B) and post treatment (1C) were plotted. Statistical analysis was done with the Kaplan Meier equation and comparison of survival curves was done using Log rank (Mantel-Cox) test using Graphpad prism version 8. dpi-days post infection.
may mask the efficacy of the test compounds, and hence ZIKV at a reduced dose (10^3 ffu/mouse) was given IP in mouse-study-III. Also, to optimize the dose regime, instead of a single dose of 200 mg/kg, CST was given in split doses of 100 mg/kg/dose twice daily. We also wanted to determine the efficacy of CST when given post-infection. Hence, separate treatment groups were made wherein CST treatment started at 24 h before or 24 h after infection in mouse-study-III. Along with the 36% reduction in systemic viral load in serum taken from pre (p = 0.015) and post (p = 0.016) treatment groups on day 3 dpi (Fig. 1A), there was a marginal improvement in survival in both 24 h pre (p = 0.021) and 24 h post (p = 0.043) infection treatments (Fig. 1B and C). Pre-treatment of CST did not have any significant additional effect as compared to post-infection treatment. In mouse-study-IV, the ZIKV-NS1 antigen in the serum was estimated from day 2 to day 4 dpi, and the CST could inhibit the serum NS1 antigen levels from day 2 dpi (Fig. 2A). There was also a reduction of ZIKV-RNA copy number in the serum on day 4 dpi in both pre and post-infection treatments (Fig. 2B). Additionally, CST inhibited ZIKV-NS1 gene levels in tissues such as liver, spleen, and brain (Fig. 2C, D, 3C), indicating a decrease in the viral load.

3.3. Effect of CST on seizure, brain inflammation and motor coordination in ZIKV infected mice

When we analyzed the endpoints of survival studies, in mouse-study-II, four out of eight mice (50%) had hind limb paralysis, and 3 out of 8 (37%) had seizure. However, neither paralysis nor seizure was observed in CST 100 mg/kg, or 200 mg/kg treated infected mice (Table 3). Similarly, in mouse-study-III, the seizure and hind limb paralysis were observed in 40% and 50% of the ZIKV infected-untreated mice, respectively, as compared to none in CST treated-infected mice (Table 3). To reconfirm the above observations, a separate detailed study (mouse-study-V) was conducted to see the effect of CST on neuronal abnormalities such as seizure and paralysis. In mouse-study-V, mice were watched for unprovoked seizure from day 1–10 dpi by random observation and 1 h video recording on day 6 occurred when there was the first appearance of seizure while handling the mice. There was no unprovoked seizure observed in any of the mice. On day 6, some of the mice exhibited a seizure while opening the cage lid in ZIKV-infected untreated mice. To have a uniform provocation of seizure, a method was developed as described under “materials and methods” based on the previous observation. There was seizure in 50% of the untreated-infected mice (n = 8) on day 6 dpi, whereas there was no seizure observed in CST treated mice on day 6 dpi (Fig. 3A). However, initial stages of neuronal abnormalities such as toe walking, and hunched positions were seen in a few of the mice (Table 4) CST treated mice. On day 7 dpi, out of the total infected mice, only 4 mice were surviving in CST treated groups. As the body weight decreased to 80% of starting weight, we euthanized these four mice as per the protocol. The unaffected mice were healthy and euthanized on day 10 dpi.

Further, to test the effect of ZIKV infection on motor neurons, the mice were subjected to the rotarod test. There was significant improvement in motor coordination in treated mice on day 3 (p = 0.036, p = 0.006 pre- and post-infection treatment, respectively), day 5 (p = 0.047, p = 0.0005 pre- and post-infection treatment) and on day 6 (p = 0.041, p = 0.012 pre- and post-infection treatment) dpi as measured by the rotarod test (Fig. 3B). In order to compare the muscle strength, we conducted an inverted grid suspension test. We did not see any difference in the time of fall between infected and untreated mice up to 120 s (Data not shown). There was no hind limb paralysis in any of the mice in mouse-study-V, and hence we could not analyze the effect of CST on paralysis in this study. However, data from the previous studies (mouse-study-II and mouse-study-III) indicate that there was no paralysis with
Fig. 3. Effect of CST on seizure and brain inflammation. Ifnar1−/− mice were treated with CST 100 mg/kg twice daily starting at 24 h before and 24 after the infection with ZIKV on day 0 as explained in mouse-study V. Mice were observed for seizure or seizure related activities. 3A: Mice with seizure 3B: Motor coordination is assessed by rotarod test. The time taken to fall from the rod on day (-1) is taken as 100% for each mouse. Percentage change in the time is calculated for subsequent testing day. 3C–3I: To check the effect of CST on inflammation of brain, the ifnar1−/− mice were treated and infected as explained in mouse-study-IV. RNA was extracted from the brain tissue, converted to cDNA, the relative expression levels of ZIKV-NS1 gene (3C), Astrocyte marker GFAP (3D), inflammation associated cytokines IL-1β (3E), TNFα (3F), IL-6 (3G), IL-10 (3H), inducible nitric oxide synthase iNOS1 (3I). Statistical analysis is done by unpaired Student’s \( t \)-test. \( p < 0.05 \) is considered as significant.
Castronospermine abolishes or delays ZIKV induced seizure or related activities. In mouse study V, mice were observed before and after opening the cage lid for behaviour, body position, activities and scored accordingly. Mice were lifted by its tail and dropped to cage bed from a height of 30 cm three times. Seizure related activities were noted.

Table 4

| Seizure stages                        | Number of mice showing seizure related activities |
|--------------------------------------|--------------------------------------------------|
|                                      | Mock infection (n = 4) ZIKV (n = 8) ZIKV + postCas (n = 8) ZIKV + preCas (n = 7) |
| Toe walking                           | 0 7 0 1                                           |
| Hunched dorsal                        | 0 3 1 0                                           |
| Erratic running                       | 0 1 0 0                                           |
| Jumping upon touching                 | 0 2 0 0                                           |
| Mouth and facial movement (RS-1)      | 0 4 0 0                                           |
| Head nodding (RS-2)                   | 0 3 0 0                                           |
| Fore limb clonus (RS-3)               | 0 3 0 0                                           |
| Rearing with forelimb clonus (RS-4)   | 0 1 0 0                                           |
| Rearing and falling with forelimb clonus (RS-5) | 0 1 0 0                                        |

| Seizure stage | Number of mice with seizures and paralysis | Paralysis of at least one limb |
|---------------|--------------------------------------------|-------------------------------|
| Mouse Study   | Treatment groups                           |                               |
|               | Saline + ZIKV mg/kg | ZIKV + C100 mg/kg | ZIKV + C200 mg/kg |
| seizures*     | 4/8 (50%) | 0/8 (0%) | 0/8 (0%) |
| Seizure**     | 3/8 (37%) | 0/8 (0%) | 0/8 (0%) |
| Hind limb paralysis** | 3/8 (40%) | – | 0/8 (0%) |
| Mouse Study II| Treatment groups                           |                               |
| seizures*     | 4/8 (50%) | 0/8 (0%) | 0/8 (0%) |
| Hind limb paralysis** | 4/8 (50%) | – | 0/8 (0%) |

RS- Racine scale.

4. Discussion

Zika viral disease has become a concern because of its effect on neuronal tissues both in new-born children and adults. Attempts have been made to repurpose or develop anti-virals that can reduce the viral load. ZIKV infection is associated with neuronal abnormalities such as microcephaly, GBS, and seizures in clinical as well as experimental animal models. Encephalitis (inflammation of the brain), meningitis (Inflammation of membrane covering the brain), and myelitis (inflammation of spinal cord) are known to cause seizures. ZIKV infection causes encephalitis (Hayashida et al., 2019). We thought that CST, with its anti-viral and anti-inflammatory activity, can be useful in reducing the seizure-related neuronal disorders apart from viral load reduction and, thus, the severity of the infection. Dosing (IP) rats with CST was able to decrease the α-glucosidase activity in the brain (Suat et al., 1985), indicating that the CST is permeable to the brain-blood barrier. α-Glucosidase is a host enzyme that removes glucose units from N-linked glycans and thereby participates in the maturation and folding of flaviviral glycoproteins (Courageot et al., 2000). CST, an α-glucosidase inhibitor, is known for its anti-viral activity (Sayce et al., 2016; Taylor et al., 1994; Warfield et al., 2017; Whitby et al., 2004). The CST pro-drug celgosivir has better pharmacokinetics compared to CST, and clinical trials indicate that the CST-prodrug celgosivir is not having any known adverse effects (Sung et al., 2016). We explored the anti-viral and anti-inflammatory properties of CST to combat ZIKV infection in vivo in ifnar1−− mouse model and in vitro in SNB19 cells.

Ifnar1−− mouse model does not have active IFN α/β signalling, which is one of the major anti-viral pathways. Due to this reason, the Ifnar1−− is highly susceptible to ZIKV and even adult mice when infected IP can give consistent mortality rates (Marzi et al., 2018). We have used Asian lineage of ZIKV (FSS13025), which is the lineage that has spread to the Americas and has raised serious concerns about human ZIKV-induced brain dysfunctions (Pol et al., 2017).

4.1. Reduction of viral load and improvement in survivability

We have estimated ZIKV-NS1 antigen levels in the blood as an indirect measure of viral load due to the limited availability of blood samples in a survival blood sampling from the tail vein. NS1 antigen level correlated significantly with viremia in dengue patients (Duong et al., 2011). In another study where Ifnar1−− mice were injected with ZIKV intraperitoneally, the viremia peaks were on 2–4 days post-infection (Aliota et al., 2016) which is in correlation with our results on ZIKV-NS1 antigenemia (Fig. 2A) and in our previously published data (Yang et al., 2018). In mouse-study IV there was significant positive correlation (Pearson r = 0.66, p = 0.0004) between ZIKV-NS1 antigen levels and ZIKV-RNA copy number in blood (Supplementary Fig 4A).

In our initial studies, the treatment with CST was started the same time as that of ZIKV challenge, and there was a reduction in viral load both at the systemic level and tissue level as measured by secreted ZIKV-NS1 antigen and ZIKV-NS1 gene expression respectively. The reduction of viral load was observed even after CST treatment started 24 h post-infection. Pre-treatment of CST did not have a significant additional advantage over the post-treatment. This may be because the CST acts predominantly post-translationally affecting the proper glycosylation as compared to the entry-level of the virus. However, glycosylated moieties are important for host viral interaction too. Host cell surface glycans correlated strongly with the glycomic features of ZIKV E protein indicating ZIKV uses glycosylation of its E protein to interact with host cell receptors to facilitate entry (Routhu et al., 2019). High-throughput
Fig. 4. CST inhibits Zika virus in vitro in glial cells. 4A. Cytotoxicity of CST. SNB19 cells were treated with CST (0.0003–300 mM) for 24 h and ATP levels were estimated as relative luminescence units (RLU) using CellTiter Glo kit from Promega. 4B-D: Efficacy of CST in inhibiting various strains of ZIKV. SNB19 cells were seeded in 96 well plates and infected with ZIKV strains (MOI = 1) for 24 h. ZIKV load in culture supernatants were tittered on Vero E6 cells by focus forming assay. Percentage of focus forming units (ffu) compared to DMSO control is calculated. CC50 and IC50 were calculated using Graphpad prism version 8. IC50 values are average of two repeats ± standard deviation. 4E: ZIKV-NS1 protein levels in SNB19 cells as tested by Western blot with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as loading control.

fitness profiling of ZIKV E protein reveals different roles for glycosylation during infection of mammalian and mosquito cells (Gong et al., 2018). Interference in the processes of glycosylation can generate non-infectious viral particles without decreasing the viral RNA. We have shown that treatment with CST has systemic viral RNA levels in vivo. When CST was given a single dose per day, at 200 mg/kg, there was an only marginal improvement in survivability. A split dose of CST (Watanabe et al., 2012) and an optimized dose regime can improve CST efficacy (Sung et al., 2016). Next, we tried to optimize the dose regime by dosing the mice 100 mg/kg twice daily instead of a single dose of 200 mg/kg per day. From two different studies, it indicates that split-dose did not have significant improvement in survivability as compared to a single dose. The level of survivability may also depend on the mouse model used in the study. In immunocompetent A/J mice infected with another member of flavivirus, dengue virus (DEN-2) injected intracranially; there was 90% survivability with 50 mg/kg CST (Whitby et al., 2005).

We also demonstrated the anti-ZIKV activity of CST in vitro in SNB19 cells using Western blot and focus forming assay. The CST could inhibit all the three strains of ZIKV in focus forming assay, although the potency is less in vitro. CST was tolerable to SNB19 and was toxic to cells only at high concentrations. The CC50 of castanospermine and celgosivir in MDCK cells was >1000 μM (Whitby et al., 2004).

4.2. Reduction in brain inflammation and related neurological abnormalities

Following ZIKV infection, neurological abnormalities were reported in mouse models (Figueiredo et al., 2019) as well as in clinical conditions (Capasso et al., 2019; Moura da Silva et al., 2016). In our initial studies, we observed seizure in ZIKV infected mice while handling it, but not in CST treated ones. Moreover, none of the CST treated mice had hind limb paralysis giving us an indication that the CST may play a role in the reduction of neuronal abnormalities. As inflammation of the brain known to cause seizures, we studied, ZIKV induced inflammation of the brain and effect of CST on seizure-related activity and other neurological disorders such as paralysis and motor co-ordination.

Apart from systemic inflammation, ZIKV cause inflammation of brain (Vieira et al., 2018) human retinal pigment epithelium (Simonin et al., 2019), ovary (Caine et al., 2019). Inflammation and seizure-like activities may not be specific to ZIKV, as it is also reported in immunocompromised mice infected with DENV too (Getts et al., 2007; Tsai et al., 2016). The inflammation might be a common factor that cause seizures. Apart from immunocompetent neonatal mice, ZIKV induced seizures were also reported in immunocompromised adult mice (Zukor et al., 2018). In our study, the immunocompromised adult mice (6–7 wk old) show seizure in 40–50% of infected untreated mice while handling them, and hence we considered it as provoked seizures. Spontaneous seizures were reported when 3-day old Swiss mice were infected with ZIKV 10⁶ ffu/mouse (Souza et al., 2018). They also observed increased susceptibility to chemically induced seizures in adult mice. However, in our studies with adult mice, we did not observe the spontaneous seizures during random observation and during 1 h video recording on day 6 dpi.

CST is also known for its immunosuppressive properties (Bartlett et al., 1994; den Dulk et al., 2004). CST inhibited passively induced allergic encephalomyelitis in a dose-dependent manner when administered continuously for 7 days, beginning at the time of lymphocyte transfer (Willenborg et al., 1989). In our study, ZIKV infected mice have shown an increase in inflammatory marker genes, mainly IL-1β and TNFα on day 4 dpi. Treating with CST has reduced the level of these cytokine genes in the brain. In dengue patients, the TNFα was reduced following treatment with celgosivir (Sung et al., 2016). There was an increase in expression GFAP gene, an astrocyte marker indicating infiltration of astrocytes or changes in expression pattern. Astrocytes induce neuroinflammation through pro-inflammatory cytokines mediating synaptic and cognitive changes (Stefanik et al., 2018). Although there is an increase in gene expression of IL-6 with ZIKV infection, no reduction was observed with CST treatment. No changes were observed in the gene expression of anti-inflammatory cytokine IL-10 with ZIKV infection. Carbohydrate moieties play an important role in some critical steps of
the neuro-immunologic inflammatory process of allergic encephalomyelitis, and CST inhibited passively induced allergic encephalomyelitis in a dose-dependent manner (Willenborg et al., 1989). Blocking of TNFα using neutralizing monoclonal antibodies prevented seizures in young Swiss mice and normalizes susceptibility to chemically induced seizures in adult mice submitted to neonatal ZIKV infection (Souza et al., 2018). Acute myelitis due to ZIKV infection was reported in a 15-year old girl, and treatment with immunosuppressant drug methylprednisolone for 7 days improved her neurological condition (Mechamers et al., 2016).

Along with inflammatory markers, there was an increase in iNOS1 mRNA expression in ZIKV infected mice, and CST was able restore the normal level in pre-infection treatment mice, although it was not statistically significant in post-infection treatment of mice. Early in the course of systemic inflammation, there is a profound induction of iNOS mRNA in vascular, glial and neuronal structures of the rat brain, accompanied by the production of nitric oxide metabolites in the brain parenchyma and cerebrospinal fluid (Wong et al., 1996).

The reduction in brain inflammation can be either due to an overall reduction in viral load or a combined effect of anti-viral and immunosuppressive activity of CST. Although the group size is small (n = 2), CST did not suppress the basal level of inflammation in control mice, which were not infected. Iminosugar N-buty1-1-deoxynojirimycin and N-(9-methoxyxynonyl-1-DNJ) counteracted IFN-γR, which is down-regulated by DENV infection in human monocyte-derived macrophages (Miller et al., 2019). Hence, CST might play anti-viral activity at various stages of ZIKV infection.

Inhibition of glucose trimming with CST promoted degradation of the alpha subunit of the nicotinic acetylcholine receptor (nAChR) (Keller et al., 1998). Although not tested, another possible mechanism by which the CST reduces seizure may be by inhibiting the nAChR, which is an excitatory ligand-gated channel.

From mouse-study-II and mouse-study-III, it is clear that the CST treatment could delay hind limb paralysis. The hind limb paralysis observed in ZIKV infected mice, may be the result of infection and inflammation in motor neurons. In our study, we found the motor dysfunction in ZIKV infected mice, as demonstrated by the rotarod test. The CST treated mice could remain on the rod for a more extended period as compared to untreated ones. The grip strength test is a widely used non-invasive method designed to evaluate mouse limb strength that has been used to investigate the effects of neuromuscular disorders and the effect of drugs. Inverted grid suspension test measures the ability of the mouse to hang on a mesh with its forepaws for a pre-set length of time or until grip fails. We assessed grip strength for 2 min, and all the mice could complete 2 min irrespective of ZIKV infection or CST treatment.

In conclusion, we have reported anti-ZIKV activity of CST in vivo. Moreover, CST abolished inflammation-mediated neuronal abnormalities such as seizure and paralysis till day 7 dpi. Since, we must euthanize the mice on day 7 dpi because of the bodyweight loss; we do not have data for subsequent days and hence further studies may require to understand whether CST can abolish or delay the seizure. Although there are reports of a few clinical trials about the anti-viral efficacy of CST-prodrug celgosivir, no report of clinical trials on effectiveness of CST or celgosivir on neuronal abnormalities such as seizure and paralysis. The combined anti-viral and anti-inflammatory properties of castanospermine are possibly effective in treating neuronal disorders in ZIKV infection. Hence, it will be worth testing the pro-drug celgosivir for controlling seizure-related issues in ZIKV infection, either alone or in combination with other potent anti-virals. The combinational therapy of anti-viral and anti-inflammatory drugs is gaining importance as a new strategy in the treatment of many viral diseases, including COVID-19.

Authors’ contributions

A.M.T., H.T., E.H.H., G.K.O Design the study and wrote the manuscript, A.M.T. and Y.C. conducted the study, E.H.H. extraction and purification of castanospermine.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Acknowledgements

The authors thank Dr. Kathleen Harper for her guidance in in-vivo studies. Suggestions from Dr. Sanjay Kumar for seizure studies are greatly appreciated. Help from Jason Nipper in the animal room maintenance is gratefully acknowledged. Support from Dr. Brian K. Weshburn and Kristina Poduch in performing PCR of samples is appreciated. The study is supported by Zika seed funding to H.T. from Florida State University Office of Research.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2020.104935.

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