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A direct-acting antiviral drug abrogates viremia in Zika virus–infected rhesus macaques

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Abstract

Zika virus infection in humans has been associated with serious reproductive and neurological complications. At present, no protective antiviral drug treatment is available. Here, we describe the testing and evaluation of the antiviral drug, galidesivir, against Zika virus infection in rhesus macaques. We conducted four preclinical studies in rhesus macaques to assess the safety, antiviral efficacy, and dosing strategies for galidesivir (BCX4430) against Zika virus infection. We treated 70 rhesus macaques infected by various routes with the Puerto Rico or Thai Zika virus isolates. We evaluated galidesivir administered as early as 90 min and as late as 72 hours after subcutaneous Zika virus infection and as late as 5 days after intravaginal infection. We evaluated the efficacy of a range of galidesivir doses with endpoints including Zika virus RNA in plasma,

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Competing interests:

R.T., S.M., A.M., Y.S.B., and W.P.S. are employees of BioCryst Pharmaceuticals and own stock and stock options in BioCryst Pharmaceuticals Inc. Y.S.B. and W.P.S. are co-inventors on patent no. 9,492,452 entitled “Method and Compositions for Inhibition of Polymerase”, and patent no. 10,512,649 and 16/692/816 entitled “Methods and Compositions for Treatment of Zika Virus Infection.” All other authors declare no competing interests.

Data and materials availability:

All data associated with this study are in the paper or the Supplementary Materials. Requests for galidesivir, which can be made available by BioCryst Pharmaceuticals under a material transfer agreement, should be directed to R.T. at rtaylor@biocryst.com.

SUPPLEMENTARY MATERIALS

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saliva, urine, and cerebrospinal fluid. Galidesivir dosing in rhesus macaques was safe and offered postexposure protection against Zika virus infection. Galidesivir exhibited favorable pharmacokinetics with no observed teratogenic effects in rats or rabbits at any dose tested. The antiviral efficacy of galidesivir observed in the blood and central nervous system of infected animals warrants continued evaluation of this compound for the treatment of flaviviral infections.

INTRODUCTION

Zika virus (ZIKV), a member of the *Flaviviridae* family, was first isolated from a rhesus macaque in 1947 (1). The primary mode of ZIKV transmission is via an arthropod vector (2). Historic cases of human ZIKV infection describe only a portion of those infected presenting as clinically symptomatic (3–5). Contemporary ZIKV infections are associated with an increased spectrum of neurological complications (6, 7), including Guillain-Barré syndrome (8–11) and congenital Zika syndrome in the Americas (12–16).

In both humans and macaques, ZIKV can be detected in the blood for short periods after symptom onset (17–20). Infectious virus is also readily shed in the saliva (19) and urine (20, 21), in semen for protracted periods (22, 23), and in high amounts in the central nervous system, including cerebrospinal fluid (CSF) (11). At present, no licensed preventative drug or treatment is available for established ZIKV infection.

Galidesivir is a C-nucleoside analog of adenosine that exerts antiviral effects by impairing viral RNA-dependent RNA polymerase activity, thereby reducing ZIKV replication (24). Galidesivir was originally developed against filoviruses; however, it has been demonstrated to exert activity against numerous viruses including yellow fever virus, dengue virus, Japanese encephalitis virus, West Nile virus, and tick-borne encephalitis virus in either in vitro cell cultures or small animal models (24–28). Here, we have experimentally infected a total of 74 rhesus macaques using different ZIKV strains; 70 of these ZIKV-infected macaques were used to investigate the impact of various postexposure doses of galidesivir (BCX4430) on viral replication in the blood, CSF, and mucosal secretions of the adult macaques.

RESULTS

To evaluate the in vivo safety and efficacy of the broad-spectrum antiviral compound galidesivir against ZIKV infection, we conducted four sequential preclinical studies in 70 ZIKV-infected adult rhesus macaques. In study 1, 15 macaques were subcutaneously inoculated with 1×10^5 50% tissue culture infectious dose (TCID₅₀) of the ZIKV Puerto Rico isolate (fig. S1). Animals were then distributed into three groups ($n = 5$ per group), normalized by age, weight, and sex in each group. At 1.5 hours after subcutaneous infection, animals were administered either intramuscular doses of galidesivir or the formulation vehicle only as a control. Group 1 animals received an initial galidesivir loading dose of 100 mg/kg, twice per day (b.i.d.), and then galidesivir maintenance doses of 25 mg/kg (b.i.d.) for nine additional days. Group 2 animals received a loading dose of galidesivir at 100 mg/kg (b.i.d.) only. Group 3 control animals were administered equivalent volumes of formulation vehicle, also by the intramuscular route.

After postexposure administration of galidesivir, animals were monitored longitudinally for detectable ZIKV RNA in the blood and bodily fluids, including saliva, urine, and CSF. In group 1, ZIKV RNA remained undetectable in plasma in all animals for the duration of the study (Fig. 1A). In group 2, three of the five animals remained negative for ZIKV RNA in plasma after a single (b.i.d.) dose of galidesivir. However, two animals in this group showed evidence of breakthrough viremia. In both cases, viremia was delayed in onset, reduced in peak magnitude, and was quickly suppressed to undetectable, as compared to control animals receiving only formulation vehicle (Fig. 1A). The animal 5964 had two detectable positive time points for viremia (days 3 and 4) after infection. The animal 5816 had a single positive time point for viremia at 10 days after infection. In contrast, animals in the control group displayed rapid onset and high-magnitude plasma viremia (Fig. 1A).

We also observed postexposure reductions in ZIKV RNA in other anatomical compartments, including CSF (Fig. 1B) and saliva or urine (fig. S2, A and B), relative to samples from sham-treated control animals. The comparison of plasma and CSF samples between treated and control animals showed significant differences in the proportion of macaques with viral breakthrough, calculated as the percentage of samples that tested positive over time ($P=0.008$; fig. S3). There were significant reductions in total plasma viremia calculated as area under the curve (AUC) after adjusting for multiple comparisons in treated macaques compared to vehicle controls (group 1, $P=0.002$; group 2, $P=0.025$) (Fig. 1C).

In the CSF samples, group 1 had three animals that remained completely negative for ZIKV RNA; in group 2, one macaque (5815) displayed persistently high CSF viral RNA over time, and the remaining four animals in this group had undetectable ZIKV RNA in CSF (Fig. 1B). After adjustment for multiple comparisons, no statistically significant difference was noted between treatment and control groups (group 1, $P=0.24$; group 2, $P=0.051$; Fig. 1C).

We next sought to determine whether the postexposure galidesivir therapy might affect the generation of antiviral immune responses against ZIKV. We assessed T cell activation from preinfection (baseline) through day 21 after primary ZIKV infection in all animals. After ZIKV infection, both CD4⁺ and CD8⁺ effector memory T cells exhibited early activation, as measured by expression of CD69, in both galidesivir-treated and control macaques to varying degrees in each group (Fig. 2A). Neither group 1 nor group 2 exhibited CD4⁺ or CD8⁺ T cell activation that was significantly different from control animals (Fig. 2A).

Natural killer (NK) cell activation rapidly increased in all groups after ZIKV infection (Fig. 2B). However, after adjustment for multiple comparisons, the peak activation response was significantly lower in group 1 compared to control animals ($P=0.016$), but there was no significant difference between group 2 and control animals ($P=0.22$). When we segregated the total number of NK cells into CD16⁺, CD56⁺, and CD16⁻CD56⁻ subsets, the CD16⁺ and CD16⁻CD56⁻ subpopulations exhibited the greatest increases in activation (fig. S4). However, the differences among groups were significant only for the CD16⁺ NK cell subset (group 1, $P=0.016$).

Next, we measured naïve and memory B cell activation using expression of the cellular phenotype marker CD38 (Fig. 2C). CD38 expression was observed to increase in all animals

after primary infection with ZIKV. Peak naïve B cell activation was significantly reduced in groups 1 and 2 compared to the control group (group 1, $P = 0.016$; group 2, $P = 0.016$). However, after adjustment for multiple comparisons, the mean peak activation for memory B cells was not significantly different between treated and control animals (group 1, $P = 0.11$; group 2, $P = 0.84$) (Fig. 2C).

Last, we examined the induction of neutralizing antibody responses in all groups against ZIKV using a plaque-reduction neutralization test (PRNT₉₀) assay. All postexposure plasma samples isolated from treated or control animals harbored virus-neutralizing ability after day 14 of ZIKV infection. Peak neutralizing activity was noted with dilutions of plasma from galidesivir-treated or control animals by day 21 after infection (fig. S5). However, the comparison of neutralizing antibody responses at the earliest time points showed subtle delays in the development of neutralizing antibody activity in the treatment groups (group 1, $P = 0.146$; group 2, $P = 0.146$) (table S1). The comparison of the values from the groups of animals was determined using a nonparametric Kruskal-Wallis test, and P values were adjusted for multiple comparisons.

To confirm that protective immunity had been established in galidesivir-treated animals, 15 macaques from groups 1, 2 and 3 were subsequently challenged with ZIKV at 70 days after primary ZIKV infection. The challenge viral dose was also delivered subcutaneously, but a 10-fold higher infection bolus of the heterologous Thai ZIKV isolate was used. After ZIKV challenge, we monitored blood plasma viremia for 28 days. Plasma viral RNA remained negative (<200 copies/ml) in most animals, with the exception of a single animal from each group displaying a small viremic blip after challenge virus infection (fig. S6). This has been observed by our group in prior rechallenge studies (29). Upon ZIKV challenge, both effector T cells and NK cells (fig. S7, A and B) exhibited detectable but low-magnitude activation, whereas B cells did not (fig. S7C). Neither the timing nor the magnitude of peak activation of NK cells and T cells was significantly different among the groups after ZIKV challenge.

In study 2, we evaluated dose reductions of galidesivir. Twenty early adult male macaques were divided into five groups ($n = 4$ per group), normalized by age and weight in each group, and were then infected with the same dose of ZIKV (Puerto Rico isolate) via the same route, as described for study 1. At 1.5 hours after infection, galidesivir treatment was administered at various doses ranging from 100 to 25 mg/kg (delivered b.i.d.). Group 1 animals received 100 mg/kg, group 2 received 75 mg/kg, group 3 received 50 mg/kg, and the group 4 received 25 mg/kg, followed by maintenance doses of 25, 19, 13, or 6 mg/kg (b.i.d.), respectively, for 9 days after the initial loading dose (fig. S1). Control animals received equivalent volumes of formulation vehicle only. The reduced doses of galidesivir affected the timing and magnitude of detectable plasma viremia. Animals in groups 1 and 2 were negative for ZIKV RNA in plasma, and only a single viremic blip was detected in one macaque from the group 2 (Fig. 3A and fig. S8). Animals in groups 3 and 4 displayed viral breakthrough subsequent to galidesivir dosing, which was more prominent in group 4. Two of four treated animals in group 3 showed detectable plasma viremia, albeit of reduced magnitude and duration compared to controls. Animals in group 4, treated with the lowest dose regimen, had readily detectable viremia that was not significantly diminished compared to infected, untreated control macaques (Fig. 3 and fig. S8).

To evaluate the effect of galidesivir treatment at different doses on plasma ZIKV RNA copies, we used a nonparametric Kruskal-Wallis test. Our analysis indicated that all three doses had significant effects on the reduction in maximal viral burden assessed at peak viremia (group 1, $P = 0.002$; group 2, $P = 0.002$; group 3, $P = 0.002$) except for the lowest doses of 25 mg/kg and 6 mg/kg b.i.d. (group 4, $P = 0.183$) compared to control animals (Fig. 3B). A nonlinear least square regression analysis of log total dose versus virus titer showed that galidesivir controlled ZIKV replication in vivo in the groups of treated macaques with a median effective concentration of 304.1 mg/kg (groups 1 to 4, $P = 0.002$; maximum plasma virus titer, $R^2 = 0.849$) (Fig. 3C).

From the galidesivir dose-reduction experiment (study 2), we also assessed the development of ZIKV-specific neutralizing antibody responses after challenge in each study group. We noted that animals in groups 1 through 3, which displayed the greatest reductions in blood viremia, showed subtle delays in PRNT90 titers on day 14 after infection according to the total galidesivir dose administered (group 1, $P = 0.018$; group 2, $P = 0.079$; group 3, $P = 0.47$) (fig. S9 and table S1). The kinetics and magnitude of PRNT90 titers in group 4 ($P > 0.999$) macaques receiving the lowest galidesivir dose appeared indistinguishable from that of infected, untreated controls (fig. S9 and table S1).

In study 3, we sought to assess galidesivir's effect on viremia after administering treatment at delayed times after ZIKV infection. We used 20 early adult macaques divided into four groups ($n = 5$ per group), normalized by age and weight in each group. All animals were infected subcutaneously with a dose of 1×10^5 TCID₅₀ of the ZIKV Puerto Rico isolate. The administration of galidesivir was initiated at 24, 48, or 72 hours after ZIKV infection of groups 1, 2, and 3, respectively. The dosing regimen comprised an initial loading dose of 100 mg/kg (b.i.d.), followed by maintenance dosing of 25 mg/kg b.i.d. for nine additional days (fig. S1). Control animals received equivalent volumes of formulation vehicle only. Plasma remained negative for viral RNA (<200 copies/ml) in animals treated with galidesivir at 24 hours after infection. A single viral blip (435 ZIKV RNA copies/ml) was detected before galidesivir treatment in one macaque in group 1 (Fig. 4A and fig. S10).

We observed a reduction in ZIKV shedding into the CSF in galidesivir-treated animals. ZIKV RNA in the CSF was detected in 100% of control animals on day 14 after infection; however, it was not detected in any animals in the group 1 and detected only in 20 to 40% of animals in groups 2 and 3 on days 7 and 10 after infection (Fig. 4B and fig. S10). Reductions in total ZIKV burden, calculated as AUC, in both plasma (group 1, $P = 0.0003$; group 2, $P = 0.035$) and CSF (group 1, $P = 0.002$; group 2, $P = 0.009$; group 3, $P = 0.044$) were significant for treated animals compared to controls (Fig. 4C). Plasma viral RNA was substantially decreased after the administration of galidesivir. We observed a log median reduction in plasma ZIKV RNA of greater than 2.8 and 3.7 for groups 2 and 3, respectively, within 24 hours after treatment initiation. All treated animals, irrespective of group, showed undetectable plasma ZIKV RNA within 48 hours of treatment initiation, whereas median plasma ZIKV RNA at the same time points from control macaques exceeded 6.0 logs of virus (Fig. 4D).

In study 4, we evaluated the impact of galidesivir treatment on the transmission of ZIKV by a mucosal route. We first modeled the kinetics of intravaginal infection in four naïve female macaques, evaluating two viral dilutions (1×10^5 or 1×10^6 TCID₅₀) of our ZIKV Puerto Rico isolate. After intravaginal infection, we observed a substantial delay in the detection of plasma viremia, with the first emergence of detectable ZIKV RNA occurring about 3 to 7 days after that observed for subcutaneous infection (fig. S11).

To evaluate the efficacy of galidesivir treatment against intravaginal ZIKV challenge, we infected 15 adult female macaques intravaginally with 1×10^5 TCID₅₀ of our ZIKV Puerto Rico isolate and then distributed animals into three groups ($n = 5$ per group), normalized by age and weight in each group. We then initiated the postexposure galidesivir treatment at 4 or 5 days after intravaginal ZIKV infection in group 1 or group 2 animals, respectively. Group 3 control animals received formulation vehicle only. Animals in both treatment groups received an optimal galidesivir dosing regimen, as determined from prior studies, comprising a 100 mg/kg (b.i.d.) loading dose followed by a maintenance regimen of 25 mg/kg b.i.d. for nine additional days (fig. S1). After treatment, plasma viral RNA remained below the limit of detection (<200 copies/ml) in all macaques from both treatment groups. In control macaques, four to six logs of ZIKV RNA in plasma were detected in all animals (Fig. 5A and fig. S12). We also monitored the emergence of ZIKV RNA in the central nervous system through weekly monitoring of CSF samples isolated from macaques in all three groups. All control animals were positive for ZIKV RNA in the CSF, typically by 1 week after infection (Fig. 5B and fig. S12). Macaques from either treatment group showed reductions in total ZIKV burden in both plasma (group 1, $P = 0.003$; group 2, $P = 0.011$) and CSF (group 1, $P = 0.011$; group 2, $P = 0.061$; Fig. 5C).

In addition to efficacy studies, we also conducted reproductive toxicology studies in both pregnant rats and pregnant rabbits dosed with galidesivir to evaluate effects on embryo-fetal development. Galidesivir was administered daily via intravenous infusion; pregnant rats received daily doses from pup gestation day 6 (GD6) to GD17; pregnant rabbits received daily doses from GD7 to GD19. In both pregnant rats and rabbits, there was related embryonic lethality, fetotoxicity, or teratogenicity at dosages $75 \text{ mg kg}^{-1} \text{ day}^{-1}$ administered over 12 days in rats and at dosages $25 \text{ mg kg}^{-1} \text{ day}^{-1}$ administered over 13 days in rabbits. The observed maternal and fetal drug exposures were similar to each other in each species, indicating that galidesivir was effectively transferred across the placenta (table S2).

To better understand drug effects on viral dynamics in a combination of loading and maintenance doses, we used a nonlinear mixed effects modeling approach to analyze the plasma viral loads of the macaques infected subcutaneously (studies 1 to 3). The model and model parameters are described in detail in Materials and Methods. In particular, we were interested in determining the drug's effectiveness in blocking viral production from infected cells, ϵ , where $\epsilon = 1$ implies a 100% effective drug and $\epsilon = 0$ implies no drug effect. Because drug effectiveness can vary among animals, we estimated the population distribution of the drug's effectiveness (ϵ) for the different study protocols. Our analysis demonstrated that both increasing the drug's plasma exposure by including a maintenance dose and increasing the total dosage provided a decrease in virus production (fig. S13A).

The estimated population mean values of e for macaques treated with each drug dosing regimen are shown in table S3, and the individual estimates are depicted in fig. S13A. We calculated the $\log_{10}$ drop in viral load that would be expected in a steady state system due to drug-induced reduction of the viral production rate (fig. S13B), as has been done to estimate the effectiveness of antiviral drug therapy for hepatitis C virus (30). Our modeling analysis confirmed that the highest dose was most effective, inhibiting 99.9% of viral production, yielding an expected three-log viral load reduction. We also estimated that the dosing regimen with a high loading dose (100 mg/kg b.i.d.) and no maintenance dose was as efficient at reducing the viral production rate as a halved loading dose (50 mg/kg b.i.d.) plus 9 days of maintenance doses (13 mg/kg b.i.d.).

From the estimated population distribution of parameters, individual parameters and viral loads for each macaque were predicted (31), as shown in fig. S14 (A to C). The model was able to recapitulate the major features of the observed viral loads, including breakthrough viremia in some animals. This analysis demonstrated that both undetectable and breakthrough viremia under the same dosing regimen could be explained by the distribution of parameters among the population rather than any inherent differences between animals. The predicted viral loads in some macaques suggested that with reduced doses of galidesivir, or with treatment started later, limited viral replication (rather than immediate clearance) could still be occurring below the limit of assay detection.

DISCUSSION

ZIKV and other flaviviral infections have affected susceptible populations globally, but virus spread has been particularly rapid in the Americas, with important public health consequences (3, 4, 6, 7, 12). There are major concerns regarding ZIKV-induced neurological complications in adults; however, there is no licensed preventative or antiviral treatment available. To advance the preclinical development of galidesivir as a candidate antiviral drug for ZIKV indications in nonpregnant adults, we have examined its activity in vivo using a tractable nonhuman primate model (29). We demonstrate in this study that galidesivir has antiviral activity against ZIKV, with a favorable safety profile. We show that treatment under various experimental scenarios prevented or rapidly reduced viral burden in the blood and anatomical compartments such as the central nervous system. Notable was the demonstration of reduced ZIKV RNA in macaque tissues known to harbor infectious virus, particularly the central nervous system.

There are several limitations to the present study. First, the treatment regimen involved up to 20 intramuscular doses. Future dosing regimens might optimally include a single intramuscular loading dose followed by oral delivery of maintenance doses. A second limitation is the timing of postexposure prophylaxis, particularly given that clinical diagnosis of ZIKV infection within the first 72 hours is difficult. This limitation combined with the small ($n = 5$) sample sizes of the various macaque groups limits the clinical relevance of these studies.

We demonstrated that galidesivir treatment, when applied after ZIKV infection, did not obstruct the generation of adaptive immune responses against the virus. Under a single-dose

regimen of galidesivir, we did observe viral breakthrough, albeit with subsequent rapid control of viremia, implying that host innate immunity may aid in viral suppression. Despite reductions in ZIKV RNA after treatment, viral antigen appeared to be sufficient to engender durable immunological protection from subsequent viral challenge by the same delivery route. Combined, these findings imply that drug-mediated viral suppression, calculated as greater than 3 logs, was not absolute and that the host immune response could be an important contributing factor in optimal viral suppression.

Prior studies suggest that anatomical sites such as the central nervous system or male genital tract are particularly susceptible to ZIKV-induced pathology and may harbor persistent reservoirs of ZIKV (32) long after the resolution of symptomatic infection (33, 34). We also conducted studies of sexual acquisition of ZIKV through an intravaginal route and found that the kinetics of infection spread were delayed compared to subcutaneous routes of infection. Although the biological rationale for the delayed appearance of plasma viremia after intravaginal infection has not been precisely defined, it may offer an expanded treatment window for individuals after a possible sexual exposure to ZIKV and for those presenting with severe neurological complications of ZIKV infection.

Ongoing pharmacological and long-term outcome studies will help to optimize route and frequency of dosing to avoid intramuscular injection as the sole route of delivery. Here, we did not conduct experiments using pregnant rhesus macaques; future studies using this model will help to assess and optimize the possible application of galidesivir as a prophylaxis or treatment for ZIKV infection during fetal development. Our findings also highlight the need for continued assessment of candidate preventative drugs, such as galidesivir, for treating ZIKV infection and other viral diseases.

MATERIALS AND METHODS

Study design

Seventy-four Indian-origin rhesus macaques (*Macaca mulatta*) were used for these studies, and 70 macaques (male and female) were treated with various regimens of galidesivir or formulation vehicle as a control. Animals were infected by subcutaneous or intravaginal routes using contemporary ZIKV isolates from Puerto Rico (KU501215) or Thailand (K993678). Before infection, animals were assigned on the basis of sex, age, and body weight across groups to assess antiviral drug efficacy (study 1, $n = 15$), dose-ranging antiviral drug efficacy (study 2, $n = 20$), and time-delayed dosing after subcutaneous infection (study 3, $n = 20$) or intravaginal infection (study 4, $n = 15$). Galidesivir treatment regimens for each study are shown in fig. S1. Formulated galidesivir was administered twice daily at various concentrations for both loading and maintenance doses, which were always delivered by the intramuscular route. After infection, ZIKV RNA in blood, saliva, urine, and CSF was monitored by quantitative reverse transcription polymerase chain reaction (qRT-PCR) for at least 1 month. Macaques were bled on days 1, 2, 3, 4, 5, 7, 10, and weekly until day 28 after infection for the evaluation of ZIKV RNA concentrations in plasma, saliva, and urine, as described previously (29). CSF samples were acquired by lumbar puncture at weekly intervals. Immune responses in naïve and ZIKV-infected animals were monitored longitudinally, as described previously (29). ZIKV-specific neutralizing antibody responses

were monitored longitudinally using a PRNT90 assay (29). Technical staff were blinded during data acquisition and unblinded for analysis. The four studies were each approved by the appropriate Institutional Animal Care and Use Committee of the Wisconsin National Primate Research Center. No animals were excluded from analysis in any of the four studies.

Antiviral synthesis and formulation

Galidesivir was synthesized by BioCryst Pharmaceuticals. Galidesivir was formulated in a vehicle of sterile 0.9% saline for intramuscular administration for all in vivo applications.

Viruses

Sequence-defined ZIKV isolates of Thai and Puerto Rican (PR) origin (GeneBank accession nos. KF993678 and KU501215) expanded on Vero cell lines were used in the studies. Each ZIKV stock was thawed on ice, diluted in phosphate-buffered saline to 1×10^6 plaque-forming units (PFU)/ml (Thai) and 1×10^5 TCID₅₀/ml (PR), and maintained on ice until inoculation.

Sample collection and processing

Viral RNA was monitored in the blood, urine, saliva, or CSF for 4 weeks after infection. Blood, saliva, and urine were collected daily for the first 7 to 10 days and then weekly. CSF samples were collected weekly. Blood was collected into EDTA anticoagulant and then subjected to Ficoll processing to obtain peripheral blood mono-nuclear cells (PBMCs) and plasma. Saliva was collected by cotton roll Salivette (Sarstedt). Clean urine was obtained by cystocentesis. CSF was obtained by lumbar puncture. Samples were immediately frozen at -80°C after processing.

ZIKV detection in blood, CSF, and mucosal fluids

Viral RNA in peripheral blood, urine, saliva, and CSFs during infection was monitored by qRT-PCR using primer/probe sets specific for ZIKV, as described (29).

Plaque-reduction neutralization test

Macaque plasma samples were collected and stored at -80°C . Macaque plasma and control ZIKV-neutralizing sera (of known titer from our previous study) were heat inactivated and serially diluted. Undiluted and diluted samples were added at an equal volume of ZIKV (2000 PFU/ml; Thai, National Center for Biotechnology Information accession no. KF993678). The virus-antibody mixture was incubated at 37°C for 90 min before 100 μl (about 100 PFU ZIKV) was added to each well of a confluent six-well tissue culture dish seeded with Vero cells. After a 1-hour virus adsorption at 37°C , cells were overlaid with 1% noble agar in supplemented minimal essential medium (MEM). At 4 days after infection, an additional overlay with 0.02% (w/v) neutral red in MEM with 1% agar was added, and plaques were counted and recorded the following day. The assay was performed in duplicate; control wells incubated with the virus-plasma from naïve macaques were also incorporated to calculate the mean percent plaque reduction for each respective plasma dilution. The neutralizing antibody titer was expressed as the lowest dilution of plasma that yielded at least a 90% plaque reduction.

Immunophenotyping

PBMC and plasma were collected from each treatment and control group after Ficoll density gradient centrifugation. PBMCs (0.5×10^6) were stained with three different antibody cocktails to assess T cells, B cells, and NK cell subpopulations, as described in Osuna *et al.* (29). Single cells were selected by forward scatter area (FSC-A) and forward scatter height (FSC-H), and lymphocytes were identified by FSC-A and side scatter area (SSC-A). T cells were identified as CD3⁺CD20⁻ lymphocytes and then as either CD4⁺ or CD8⁺, which were each then categorized as naïve (CD28⁺CD95⁻), central memory (CD28⁺CD95⁺), or effector/effector memory (CD28⁻CD95⁺). B cells were identified as CD20⁺CD3⁻ lymphocytes and then categorized as either naïve (CD27⁻) or memory (CD27⁺). NK cells were identified as CD3⁻CD8⁺NKG2A⁺ and then as either CD16⁺, CD56⁺, or CD16⁻CD56⁻. Percent CD69 was used as an activation marker for T and NK cell subsets. The geometric mean of fluorescence (GMF) intensity of CD38 staining was used as an activation marker of B cells. GMF was monitored longitudinally by normalizing the GMF on each time point to that of Sphero Daily QC Particles (BD Biosciences) also run on each time point.

Evaluation of galidesivir reproductive toxicity

To detect the potential adverse effects of galidesivir on pregnant animals and on the development of the embryo and fetus, female rats (Wistar) and rabbits (New Zealand White) were administered galidesivir from implantation to closure of the hard palate. These studies followed the ICH Harmonised Tripartite Guideline for the detections of toxicity to reproduction for human pharmaceuticals [S5(R3)] and were compliant with a Good Laboratory Practice regulations. Galidesivir was administered daily via a 30-min intravenous infusion; rats received 0 (saline), 25, 75, and 150 mg kg⁻¹ day⁻¹ from GD6 to GD17; rabbits received 0 or 25 mg kg⁻¹ day⁻¹ from GD7 to GD19. Because of maternal toxicity at higher doses, two groups of pregnant rabbits were administered galidesivir 45 mg kg⁻¹ day⁻¹ in a split dosing schedule to cover the entire critical organogenesis period; one group received galidesivir from GD7 to GD13 and the other group from GD13 to GD19. To measure plasma concentrations of galidesivir, blood was sampled on the first and last day of dosing from dams and on the last day of dosing from pups.

ZIKV dynamic modeling load, including galidesivir treatment

We describe the plasma viral dynamics in rhesus macaques subcutaneously infected with ZIKV and treated with a polymerase inhibitor with a system of ordinary differential equations:

$$\begin{aligned}\frac{dT}{dt} &= -\beta VT \\ \frac{dI_1}{dt} &= \beta VT - kI_1 \\ \frac{dI_2}{dt} &= kI_1 - \delta I_2 \\ \frac{dV}{dt} &= (1 - \epsilon)pI_2 - cV\end{aligned}$$

In this model, target cells T are infected by circulating virus V at a rate βVT . Initially infected cells do not produce virus, i.e., are in an eclipse phase, and are denoted I_1 . These

cells transition to a state of productive infection, I_2 , at rate k . Productively infected cells die at rate δ . In the absence of antiviral treatment, virus is produced from these cells at per capita rate p and is cleared from the circulation at rate c per virion. The effect of a polymerase inhibitor is to reduce the virus production rate p by a factor $(1-\epsilon)$, where ϵ (between 0 and 1) represents the drug effectiveness. When $\epsilon = 1$, the drug is 100% effective.

We adopt a nonlinear mixed-effects model fitting approach to these data, obtaining estimates of the distribution of each of the parameters across the population. We use the expression $R_0 = \beta T(0)p/\delta c$ to reparameterize the model, obtaining estimates for R_0 , δ , ϵ , p , and $V(0)$, where R_0 is the basic reproductive number. We assume that R_0 , δ , and p are log-normally distributed in the population, that $\log_{10} V(0)$ is normally distributed, and that ϵ is logit-normally distributed. We test a range of fixed values for c and k , selecting those that provide the best fit to the data. We fix $T(0) = 10^4$ cells/ml and $I_1(0) = I_2(0) = 0$ cells/ml.

We assume that before drug treatment is started in these macaques, the value of ϵ is 0 and that after initiation of therapy, ϵ takes a constant value for the remainder of the time course of the data. We allow the estimated population distribution of ϵ to be centered around a different value for each drug regimen, where a drug regimen is defined as a combination of loading and maintenance doses.

The optimal model fit to the data was found with $c = 10/d$ and $k = 4/d$, corresponding to a mean eclipse phase length of 6 hours. We did not find any evidence of different distributions of parameters R_0 , δ , p , or $V(0)$ between studies 1, 2, and 3 when all monkeys are considered. The population median basic reproductive number of 15.9 (fig. S14A) implies that one infected cell introduced into a population of susceptible cells would cause almost 16 secondary infected cells. This is a higher estimate than has been previously seen for ZIKV and may, in part, be due to the low estimated initial plasma viral load, with a median of about 0.1 RNA copies/ml of plasma. Why this estimate is so low is not clear, but is required to account for the undetectable viral load 1 day after infection. Then, with a low initial viral load, to reach the peak (about 10^6 copies/ml at days 3 or 4 in the untreated macaques) the up-slope of the viral load, primarily determined by the value of R_0 , needs to be large.

Statistical analyses

Analyses of virological and immunological data were conducted using a two-tailed Mann-Whitney test. P values reported were adjusted for multiple comparisons using the Holm's method. All statistics between grouped variables or transformed $\log_{10}$ values were calculated using GraphPad Prism. All P values were two-tailed and considered significant when <0.05 .

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A Plasma **B CSF** **C**

Fig. 1. Therapeutic administration of galidesivir to macaques abrogates ZIKV infection. Fifteen rhesus macaques ($n = 5$ per group) were infected subcutaneously with 1×10^5 TCID₅₀ of a ZIKV Puerto Rico isolate and then were treated with galidesivir or formulation vehicle 1.5 hours after infection by intramuscular injection. Group 1 animals received 100 mg/kg (b.i.d.) on day 0 (1.5 hours after infection) and then received galidesivir maintenance treatments for nine additional days at 25 mg/kg (b.i.d.). Group 2 animals received 100 mg/kg (b.i.d.) on day 0 (1.5 hours after infection) only. Control animals in group 3 received formulation vehicle only. ZIKV replication was monitored in the blood and CSF between days 1 and 28 after infection. (A) Log ZIKV RNA copies per milliliter of plasma are indicated. (B) Weekly longitudinal ZIKV RNA measurements in CSF from individual animals are shown as log ZIKV RNA copies per milliliter. The limit of detection (200 RNA

copies/ml) for the assay is indicated by the horizontal black dotted line. (C) The combined plasma or CSF ZIKV burden calculated as the area under the curve (AUC) was compared between each treatment group and the control group. The results are expressed as the minimum and maximum for all data points, with the line representing the median. Comparison between groups was determined using a one-way analysis of variance (ANOVA) on ranks (Kruskal-Wallis test), and *P* values were adjusted for multiple comparisons.

Fig. 2. Therapeutic administration of galidesivir does not inhibit antiviral immunity in ZIKV-infected macaques.

The activation of PBMC subsets in ZIKV-infected macaques was monitored by flow cytometry in treated and control animals in study 1 ($n = 5$ per group). **(A)** T cell activation was measured as a change from day 0 in percent expression of CD69 on effector memory (EM; CD28⁻CD95⁺) T cells expressing CD4⁺ or CD8⁺. **(B)** NK cell activation was measured as a change from day 0 in percent expression of CD69. **(C)** Geometric mean fluorescence (GMF) of CD38 expression (×10⁴) was measured on naïve (CD27⁻) and memory (CD27⁺) B cells. All values are expressed as the difference in percent expression from day 0, which is indicated by the horizontal dotted line.

Fig. 3. Antiviral efficacy of galidesivir is dose dependent.

(A) Plasma ZIKV RNA was monitored in macaques challenged subcutaneously with 1×10^5 TCID₅₀ of a ZIKV Puerto Rico isolate after intramuscular administration of decreasing loading doses of galidesivir ($n = 4$ per group) on days 0, 1, 2, 3, 4, 5, 7, 10, 14, 21, and 28 after infection. Group 1, 100 mg/kg; group 2, 5 mg/kg; group 3, 50 mg/kg; group 4, 25 mg/kg; vehicle was delivered b.i.d. on day 0 (1.5 hours after infection). Maintenance treatment with galidesivir was continued for 9 days: group 1, 25 mg/kg; group 2, 19 mg/kg; group 3, 13 mg/kg; group 4, 6 mg/kg; vehicle control (b.i.d.) group. Log₁₀ virus RNA copies/ml of plasma from individual animals are shown. The limit of detection (200 RNA copies/ml) is indicated by the horizontal black dotted line. (B) Peak plasma viral burden expressed as the maximum viral titer was compared between groups 1 through 4. The box and whiskers represent the minimum and maximum for all data points. The median value of four animals is shown as a line in the box plot. Comparisons of grouped variables were analyzed by an ANOVA on ranks, and P values were adjusted for multiple comparisons. (C) Nonlinear (least square) regression analysis to evaluate the dose effect of galidesivir on the control of plasma viremia in ZIKV-infected macaques is shown.

Fig. 4. Antiviral efficacy of galidesivir in ZIKV-infected macaques.

ZIKV-infected rhesus macaques ($n = 5$ per group) were treated with galidesivir (100 mg/kg, b.i.d., intramuscular administration) at either 24 (group 1), 48 (group 2), or 72 hours (group 3) after infection. Control animals were treated with vehicle at 24 hours after infection. Galidesivir maintenance treatment was continued for 9 days with groups 1, 2, and 3, each receiving 25 mg/kg (b.i.d.); vehicle control was administered b.i.d. to control animals. ZIKV replication was monitored by qRT-PCR at days 0, 1, 2, 3, 4, 5, 7, 10, 14, 21, and 28 after infection in plasma and was monitored in CSF weekly. Log₁₀ virus RNA copies per milliliter of plasma (A) and CSF (B) for individual animals are shown. (C) The combined plasma or CSF ZIKV burden calculated as AUC is shown as the minimum and maximum for all data points. The median value of five animals is indicated as a black dotted line.

Comparisons between groups were determined using a one-way ANOVA on ranks (Kruskal-Wallis test), and *P* values were adjusted for multiple comparisons. (D) ZIKV RNA in both plasma and CSF in each treated group was compared to controls at days 1, 2, 3, 4, 5, and 7 after infection. The box and whisker plots represent the maximum and minimum for all data points. Median values are depicted by a line in the box plot. The statistical significance was determined using multiple *t* tests, and *P* values were adjusted for multiple comparisons. The time of galidesivir initiation and the limit of detection (200 RNA copies/ml) are denoted by the vertical and horizontal black dotted lines, respectively.

A Plasma **B CSF** **C**

Fig. 5. The therapeutic window for galidesivir administration is extended during intravaginal ZIKV challenge.

Virus replication was monitored after intravaginal challenge of 15 macaques with 1×10^5 TCID of a ZIKV Puerto Rico isolate in two groups of galidesivir-treated macaques or a vehicle control group ($n = 5$ per group). Group 1, galidesivir administration at 96 hours after infection; group 2, galidesivir administration at 120 hours after infection; control group, vehicle administration at 96 hours after infection. Treatment with galidesivir was continued for 9 days and groups 1 and 2 both received 25 mg/kg (b.i.d.); the control group received vehicle b.i.d. ZIKV replication was monitored by qRT-PCR between days 1 and 28 after infection in plasma (A) and weekly after infection in CSF (B). Longitudinal ZIKV RNA measurements from individual animals are shown. Time of galidesivir initiation in each

group and the limit of detection (200 RNA copies/ml) are indicated by the vertical and horizontal black dotted lines, respectively. (C) The combined plasma or CSF ZIKV burden calculated as AUC is shown as the minimum and maximum for all data points. Median values are indicated. Comparisons between groups were determined using a one-way ANOVA on ranks (Kruskal-Wallis test), and *P* values were adjusted for multiple comparisons.