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Assessing the utility of antivirals for preventing maternal-fetal transmission of zika virus in pregnant mice

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ABSTRACT

Zika virus (ZIKV) infection during pregnancy has been associated with adverse outcomes and birth defects such as microcephaly in newborn children. Congenital malformations associated with ZIKV are believed to occur via direct infection of the fetus. Unfortunately, there are no licensed therapeutic or preventative tools to block maternal-fetal transmission of ZIKV. In this study, we developed a mouse model of ZIKV infection that specifically establishes vertical maternal-fetal transmission of ZIKV in 40–60% of fetuses when the dams acquire ZIKV infection during pregnancy. This mouse model somewhat mirrors the experience in humans at the peak of the epidemic in the Americas. Using this model, we demonstrate that a well-documented directly acting antiviral (DAA) compound that targets flaviviral RNA synthesis can completely prevent fetal infection when the treatment is started at the time of infection. Notably, we show that the treatment commenced at the time of peak viremia is still able to reduce the risk of fetal infection concomitant with significant reduction in placental viral load. Our results show for the first time the potential for clinical development of antiviral drugs for preventing vertical maternal-fetal transmission of ZIKV.

The recent Zika virus (ZIKV) outbreak in Brazil and subsequent global dissemination has focused on the risks associated with viral infection during pregnancy. Although ZIKV infection is primarily associated with mild self-limiting illness, there is now compelling evidence of its association with fetal brain and central nervous system abnormalities, such as microcephaly, eye lesions, hearing loss and craniofacial and musculoskeletal lesions, when mothers acquire the virus during pregnancy (Baud et al., 2017). The frequency of brain abnormalities at birth in infants born to mothers who had acquired ZIKV infection in pregnancy is estimated to be 2.3% based on meta-analysis of data collected during the epidemic in the Americas (Coelho and Crovella, 2017).

Microcephaly is the most severe birth defect associated with ZIKV and believed to occur via direct infection of the fetus mainly during the first or second trimester of pregnancy (Mlakar et al., 2016; Rasmussen et al., 2016). The mechanism(s) that cause microcephaly following infection is still unknown, however, recent studies show that infection of glial cells and neurons are associated with microcalcifications and this is suggested to dysregulate development of the fetal brain (Martines et al., 2016; Mlakar et al., 2016). Therefore, reducing the opportunity for maternal-fetal ZIKV transmission during pregnancy is an important step for preventing ZIKV-associated fetal or congenital abnormalities. At present, however, there are no approved antiviral drugs or vaccines to prevent vertical transmission of ZIKV.

An increasing number of mouse models of ZIKV infection in pregnancy have been reported with a variety of viral pathogenesis outcome depending on the genetic background of the animals (Caine et al., 2018). It has been challenging to establish a model for efficient vertical transmission of ZIKV in immunocompetent mice (Cugola et al., 2016; Gorman et al., 2018; Szaba et al., 2018), whereas the IFN-receptor deficient mice or immunocompetent mice treated with anti-IFN-alpha receptor antibody (Ab) have been shown to be readily permissive to fetal infection (Jagger et al., 2017; Miner et al., 2016; Richner et al., 2017). In these mouse models, the rate of fetal infection and/or mortality can be controlled by route of infection (Vermillion et al., 2017; Yockey et al., 2016), timing of infection at different gestational stage (Chen et al., 2017; Jagger et al., 2017; Vermillion et al., 2017), modulation of the amount of IFN-receptor blocking Ab (Miner et al., 2016), or using virus strains that have different levels of virulence in mice (Gorman et al., 2018).

During pregnancy, the placenta connects the maternal and fetal blood, and this critical interface is maintained and controlled by syncytiotrophoblasts in both humans (hemochorial) and mice (hemotrichorial) (Rossant and Cross, 2001). This common feature suggests

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that the mouse may be a useful model to study the mechanism of vertical virus transmission as a first step for evaluation of therapeutics for preventing fetal infection. Although pregnant mouse models have been actively used for the development of vaccines or therapeutic Abs (Richner et al., 2017; Sapparapu et al., 2016; Shan et al., 2017; Tharakaraman et al., 2018), only a few studies have explored the efficacy of antiviral drug during pregnancy in mice (Cao et al., 2017; Shiryaev et al., 2017). These studies showed that, although treatment of chloroquine (anti-malaria drug) in ZIKV-infected pregnant mice reduced the level of fetal viral load, the treatment still resulted in a high frequency of fetal infection in SJL mice (Shiryaev et al., 2017) or immunocompetent mice treated with anti-IFN-alpha receptor Ab (Cao et al., 2017). Thus, it is still unclear if efficient antiviral drugs can completely prevent vertical transmission of ZIKV. In this study, we characterized the fetus/infant status under the condition that induces high level of ZIKV infection but does not cause mortality in dams, and examined the utility of antiviral drug in blocking maternal-fetal transmission of ZIKV.

In a previous study, we showed that H/PF/2013 (French Polynesian), referred to as ZIKV-FP, is lethal at 10^3-10^5 pfu in adult (7–12 weeks old) A129 mice (IFN-α/β receptor-deficient Sv/129), while Paraiba (Brazilian), referred to as ZIKV-B, is not lethal even at a dose of 10^6 pfu (Watanabe et al., 2019). As shown in Fig. 1, intravenous (iv) infection with 1,000 pfu or even a dose as little as 50 pfu of ZIKV-FP caused 100% mortality around day 10 post-infection (pi) (Fig. 1A) accompanied with acute weight reduction (Fig. 1B) and high levels of serum viral RNA (viremia) (Fig. 1C). By comparison, although iv inoculation with ZIKV-B at 1,000 pfu induced high level of viremia, this strain only caused a small decrease in weight (up to 10%) and did not cause mortality (Fig. 1A–C). We previously evaluated the potential of neutralizing Ab to prevent vertical infection and fetal mortality using ZIKV-FP in A129 mice, and showed that Ab treatment successfully prevented (or reduced) fetal infection and mortality (Tharakaraman et al., 2018). However, this model had limited utility for examination of the long-term consequences of ZIKV-infection such as fetus/infant growth and malformation since ZIKV-FP infection caused 100% fetal infection and mortality.

In this study, we therefore used ZIKV-B to characterize the fetus/infant status under non-lethal infection in pregnancy. Pregnant A129 mice were iv inoculated with 1000 pfu of ZIKV-B on day 14 post-mating (10–14 days after gestation: E10-14). The fetuses were harvested on days 4 and 8 pi, and also the physical status of pups was monitored until 21 days after birth (Fig. 2A). The fetuses were observed to grow in ZIKV-infected dams without any remarkable abnormalities (Fig. 2B) and 95.8% (23/24) of fetuses were found to be alive on day 8 pi (data not shown). The infected dams could give birth on day 7–12 pi, however, most of the pups were dead or died within 2 days post-birth (Fig. 2B and Table 1). Only 7 pups born to 1 dam survived more than 2 days post-birth (7/60: 10.4%), however, 4 out of the 7 pups died within 8–13 days post-birth with signs of neurological disorders in the form of excessive hyperactive movement for 1–2 days prior to death. This suggests that these pups may have been directly infected with ZIKV and appears to be consistent with the detection of high level of placental viral load on day 4 pi that further increased on day 8 pi (Fig. 2C). Surprisingly, however, fetal viral load was almost undetectable on day 4 pi, whereas virus was detected in some of the brains (4/17: 23.5%) and other body tissues (7/24: 29.2%) on day 8 pi (Fig. 2D), indicating that vertical transmission of virus occurs later than 4 days pi under this condition. The low frequency of fetal infection (< 30%) even on day 8 pi suggests that high mortality of the pups within 2 days post-birth was not caused by direct virus infection, and that other factors, such as reduced care due to the dam's unusual condition caused by infection, may affect the survival of the pups. Furthermore, the reduction in the number of pups (3.9 per dam) compared with uninfected mice (6.3 per dam) (Table 1) may be due to the dam's predisposition to prey on dead/weakened pups.

Since higher risk of fetal abnormalities is associated with the first or second trimester in humans (Honein et al., 2017; Pomar et al., 2017), we next infected ZIKV-B on day 8 post-mating (E4-8). This enables the monitoring of the fetal status for a longer period of time after infection (6–12 days pi) (Fig. 2E). As observed for the E10-14 infected dams, the fetuses also could grow in the ZIKV-infected dams (Fig. 2F), however, an increase in the number of dead fetuses was observed on days 9 pi (4/38: 10.5%) and 12 pi (7/30: 23.3%) compared with the fetuses harvested from uninfected control dams (1/24: 4.2%) (Fig. 2G). In this infection condition, many of the pups successfully survived longer than 2 days (57/73: 78.1%) which is comparable (or even higher) to uninfected control mice (89/129: 69.0%) (Table 1). We note that a high rate of dead pups born to uninfected A129 dams (31%) in this study is unusual compared with AG129 mice (12% mortality) reported in a previous study (Julander et al., 2018). This may be due to inherent behavioral differences between the two strains of mice. Since the uninfected A129 dams were treated exactly the same way as the treated dams, it is likely that the stress caused by experimental handling may have resulted in the reduction in the care of pups. About 10% of the pups born to the ZIKV-infected dams showed abnormal behavior (excessive hyperactive movement for 1–2 days) followed by death (6/57: 10.5%) around day 10 post-birth (Table 1), however, there was no obvious evidence of apparent malformations such as microcephaly in any fetuses/infants. High level of placental viral load was detected even on day 12 pi (Fig. 2H). Notably, virus was detected in 59.1% (26/44) and 65.8% (25/38) of fetuses on days 6 and 9 pi, respectively (Fig. 2I), indicating that fetal ZIKV infection is more likely to occur during the earlier stage of pregnancy, as has been suggested in humans as well as other mouse models (Chen et al., 2017; Jagger et al., 2017; Vermillion et al., 2017). In addition, high viral load was detected in dead fetuses on day 9 pi (Fig. 2I), suggesting that these fetuses may have died from direct ZIKV infection. ZIKV was still detectable in the head (11/23: 47.8%) and body tissues (11/23: 47.8%) of the live fetuses on day 12 pi. Surprisingly however, virus was almost undetectable in the head (0/30) and body tissues (1/30) of live pups on the days of birth (13–18 days pi) (Fig. 2I). This may be explained by the high rate of prenatal death due

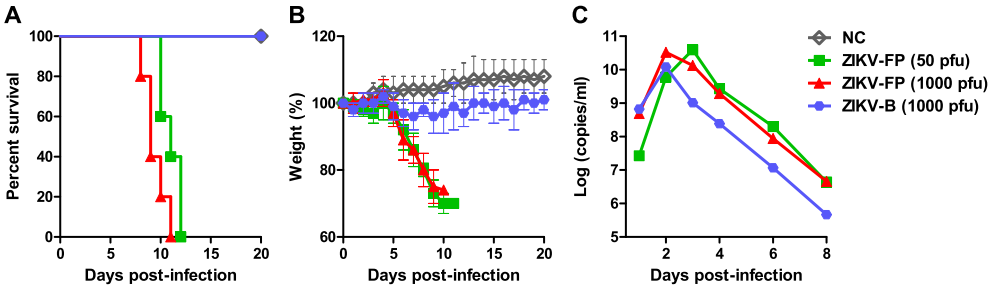

Fig. 1. ZIKV infection in non-pregnant adult female A129 mice. Eight to 10 weeks old female A129 mice were inoculated intravenously (iv) with ZIKV H/PF/2013 (ZIKV-FP) (50 or 1,000 pfu) or Brazilian (ZIKV-B) (1,000 pfu). Mouse survival rate (A) and weight change (B) were monitored until day 20 post-infection (pi). NC indicates uninfected control group. Blood samples were collected on days 1, 2, 3, 4, 6 and 8 pi and mixed serum from each group were subjected to real-time RT-PCR to obtain the average viral genome copy numbers using standard methods for virological analysis as described in our previous report (Watanabe et al., 2019). The number of mice per group is 5. All animal experiments in this study were approved by the Institutional Animal Care and Use Committee at Singapore Health Services (protocol 2016/SHS/1197) and conformed to the National Institutes of Health (NIH) guidelines and public law.

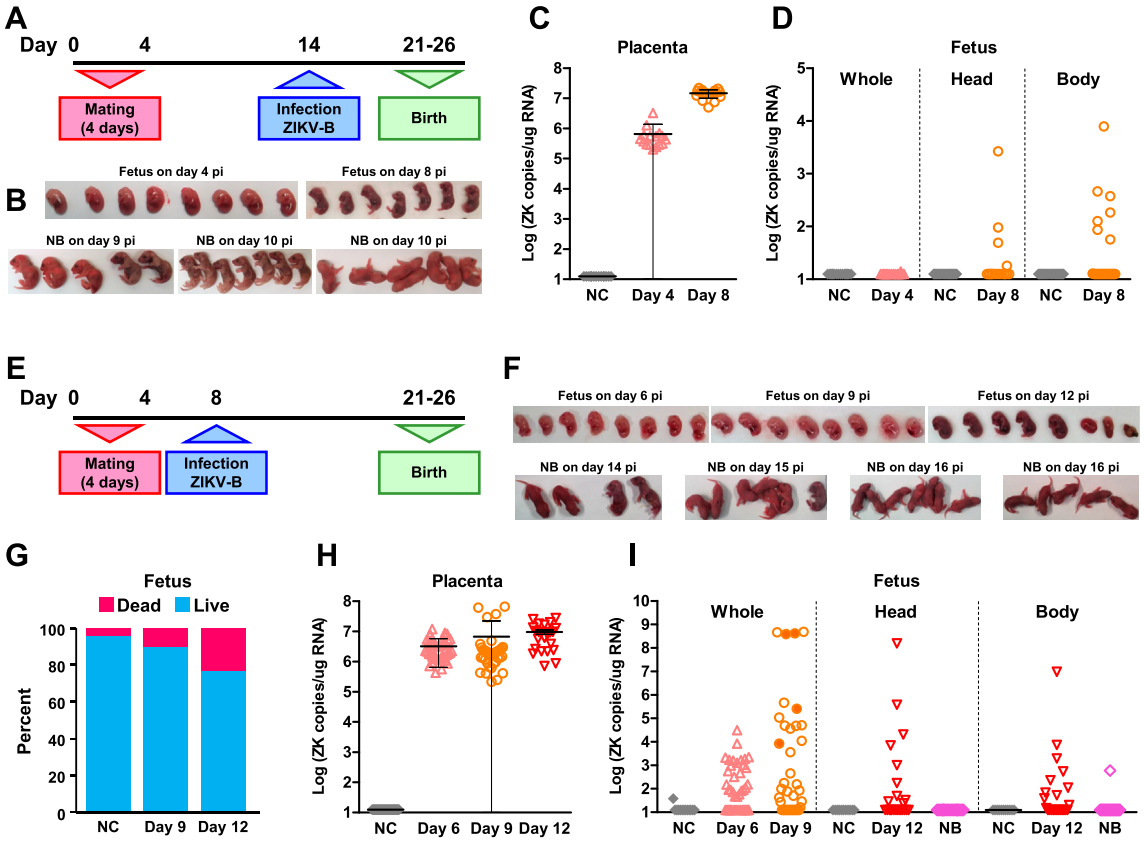

Fig. 2. Status of fetuses/infants after non-lethal ZIKV infection in pregnant A129 mice. (A–D) Eight to 10 weeks old female A129 mice were housed with adult male A129 mice for 4 days, and female mice were inoculated iv with 1,000 pfu of ZIKV-B on day 14 post-mating (corresponding to E10-14) (A). Fetuses and placentas were harvested on days 4 and 8 pi and the representative pictures of the fetuses harvested on day 4 (1 out of 3 dams) and day 8 (1 out of 5 dams) pi or newborns (NB) (3 out of 17 dams) are shown in panel B. Viral load in placentas (C) and fetuses (D) was measured by real-time RT-PCR after homogenization as described in our previous report (Watanabe et al., 2019). The limit of PCR detection is 12.5 copies/μg RNA. The fetuses harvested on day 8 pi were separated into head (without skin) and other internal body organs for viral RNA measurement (D). Negative control samples (NC) were harvested from uninfected pregnant mice on the corresponding days. The results of NC placentas (C) harvested on days 4 and 6 pi are shown as one group. (E–I) ZIKV-B was inoculated into pregnant mice on day 8 post-mating (E4-8) (E) as described above. Fetuses and placentas were harvested on days 6, 9 and 12 pi, and the representative pictures of the fetuses harvested on day 6 (1 out of 6 dams), day 9 (1 out of 6 dams) and day 12 (1 out of 6 dams) pi or newborns (NB) (4 out of 23 dams) are displayed in panel F. Percentages of live/dead fetuses on days 9 and 12 pi are shown in panel G, and fetuses of uninfected control dams (NC) were harvested on the day corresponding to day 12 pi. Viral load in placentas (H) and fetuses (I) was measured by real-time RT-PCR, and viral load in dead fetuses on day 9 pi is displayed as filled circles. The fetuses harvested on day 12 pi and newborns (NB) were separated into head and other internal body organs for viral RNA measurement. Negative control (NC) for placenta harvested from uninfected control mice on the corresponding days (days 6, 9 and 12 pi) is shown as one group (H). Similarly, NCs for whole fetuses, fetal head and body organs harvested on different days are displayed as one group (I). The number of mice used for each experiment is described in the text.

to infection, and/or clearance of virus by maternal humoral immunity. Indeed, the number of pups born to infected mice (4.7 per dam) was found to be lower than uninfected mice (6.3 per dam) although there were no differences in the weight of newborns or their survival rate (Table 1). Taken together, non-lethal ZIKV infection in pregnant A129 mice at earlier embryonic stage (E4-8) resulted in a higher rate of fetal infection and successful long-term survival of the pups, however, there was no evidence of apparent malformations even though about 10% of the pups exhibited neurological disorders after birth. Since not all human fetuses are suggested to be infected with ZIKV when the mothers experience infection during pregnancy, this new model may mimic more closely the human situation than lethal mouse models (Sapparapu et al., 2016; Tharakaraman et al., 2018) and be suitable for the assessment of therapeutics for preventing vertical transmitted infection.

Next, we decided to investigate the ability of antiviral drugs in preventing vertical transmission of ZIKV in the mouse model by using a well-established flaviviral inhibitor NITD008, an adenosine analog, that has been shown to inhibit the replication by directly inhibiting the RNA-dependent RNA polymerase activity through chain-termination (Yin et al., 2009), and also shown to reduce viremia in mice infected with ZIKV (Deng et al., 2016). Based on the results of fetal infection rate observed for the E10-14 (14 days post-mating; Fig. 2D) and E4-8 (8 days post-mating; Fig. 2I) infected dams, we expected around 50% fetal infection when the dams are infected on day 10 post-mating. Therefore,

Table 1
Status of neonates born to ZIKV-infected and uninfected A129 mice.

	Uninfected control	Infection on day 14 post-mating	Infection on day 8 post-mating
Number of neonates (per dam)	6.3 (± 1.6) (n = 27)	3.9 (± 2.3) (n = 17)	4.7 (± 1.5) (n = 23)
Survival of neonates (> 2 days)	69.0% (89/129)	10.4% (7/60)	78.1% (57/73)
Mortality of neonates (2–21 days)	0% (0/89)	57.1% (4/7)	10.5% (6/57)
Weight of neonates (including dead)	1357 (± 160) mg	1219 (± 178) mg	1346 (± 150) mg

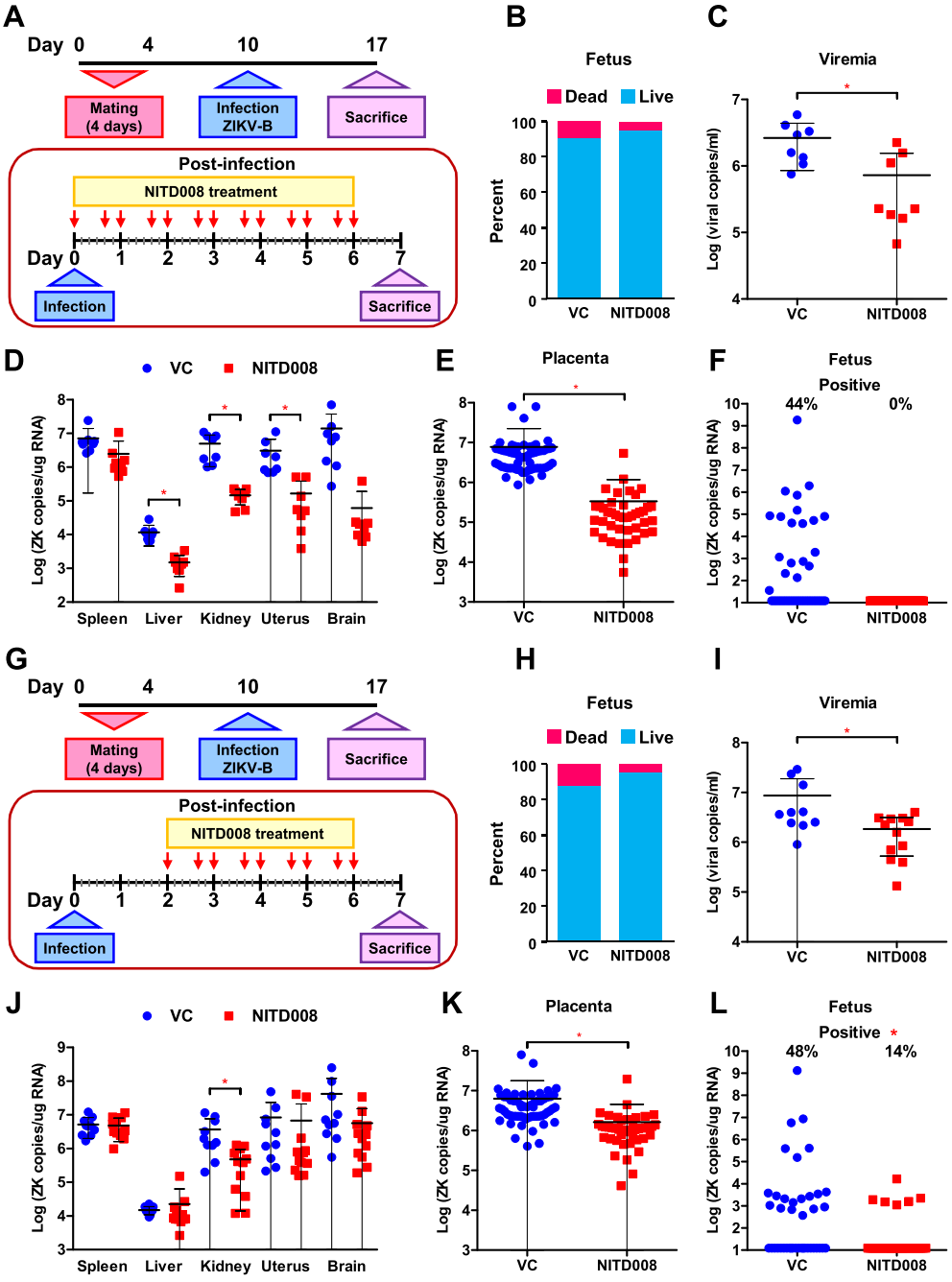

Fig. 3. Effect of antiviral treatment on preventing fetal-maternal transmission of ZIKV. (A–F) Eight to 10 weeks old female A129 mice were housed with adult male A129 mice for 4 days, and female mice were inoculated iv with 1,000 pfu of ZIKV-B on day 10 post-mating (E6-10). Mice were treated orally with NITD008 (0.5mg/mouse) at the time of infection followed by twice daily administration (16/8 h cycle) until day 6pi. Mice were then sacrificed on day 7 pi to harvest fetuses and tissues for the measurement of viral RNA copy number (A). NITD008 in 100% DMSO (100 mg/ml) was diluted 40-fold with RPMI1640 with 10% FBS just prior to each dosing and 200 µl of the solution was administered into each mouse (0.5mg/mouse). Vehicle control mice (VC) were received 200 µl of 2.5% DMSO in RPMI1640 with 10% FBS. Percentage of live/dead fetuses on day 7 pi is shown in panel B. Viremia (C) and viral load in various tissues (D), placentas (E) and fetuses (F) on day 7 pi were measured by real-time RT-PCR. The number of dams per group is 8. (G–L) ZIKV-B was inoculated into pregnant mice as described above, and mice were treated orally with NITD008 (0.5mg/mouse) at 48 h after infection followed by twice daily administration (16/8 h cycle) until day 6pi.Then, mice were sacrificed on day 7 pi to harvest fetuses and tissues (G). Percentage of live/dead fetuses on day 7 pi is shown in panel H. Viremia (I) and viral load in various tissues (J), placentas (K) and fetuses (L) on day 7 pi were measured by real-time RT-PCR. The number of dams is 10 for vehicle control and 12 for NITD-treated group. Significant differences between data groups were determined by 2-tailed Student *t*-test analysis for viral load comparison and Fisher's exact test for the frequency of fetal infection. **P* value less than 0.05 was considered significant.

pregnant mice were iv infected with ZIKV-B (1000 pfu) on day 10 post-mating (E6-10), and NITD008 (0.5mg/mouse) or vehicle control was administered orally twice daily from the time of infection until day 6 pi. On day 7 pi the mice were sacrificed and various tissues and fetuses were harvested on day 7 pi to measure the viral load (Fig. 3A). Since the weight of pregnant mice is changeable, the drug concentration (0.5mg/mouse) was correspondingly adjusted to approximate 16–30 mg/kg. This concentration range was shown to be effective in reducing viremia and mortality in dengue virus (DENV)-infected mice when the treatment commenced on day 0 pi (Yin et al., 2009). The fetuses harvested from NITD008-treated dams showed higher survival rate (95%: 39/41) compared with vehicle control fetuses (VC: 91%: 40/44) (Fig. 3B), indicating that NITD008 is not harmful to fetal viability in mice under this regimen. The treatment was found to reduce viremia (3.6-fold: $p = 0.017$) (Fig. 3C) and tissue viral load with varying degree [spleen (2.9-fold: $p = 0.112$), liver (7.8-fold: $p = 0.001$), kidney (34.1-fold: $p = 0.004$), uterus (18.6-fold: $p = 0.042$) and brain (230.9-fold: $p = 0.117$)] (Fig. 3D) on day 7 pi. Significant reduction in placental viral load (34.5-fold: $p = 0.002$) was noted in the treated dams (Fig. 3E), and remarkably, while the vehicle control mice showed infection in 44.2% (19/44) of the fetuses, the NITD008 treatment completely prevented the fetal infection (0/41) (Fig. 3F). The average weight of the live fetuses was 239 ± 91 mg ($n = 40$) for vehicle control group and 210 ± 80 mg ($n = 39$) for NITD008-treated group ($p = 0.137$), suggesting that the absence of fetal infection in treated group is not due to the difference of gestation period. These results suggest that successful suppression of placental viral load by the antiviral treatment resulted in prevention of the maternal-fetal transmission of virus.

In order to address the potential use of antivirals in a clinical setting, we next tested NITD008 treatment starting on day 2 pi (Fig. 3G) when viremia already reaches the peak level (Fig. 1C). The delayed treatment

was also capable of reducing viremia (4.7-fold: p = 0.031) (Fig. 3I), whereas it failed to induce significant reduction in viral load in many tissues except kidney [spleen (1.1-fold: p = 0.780), liver (0.7-fold: p = 0.559), kidney (7.7-fold: p = 0.011), uterus (1.3-fold: p = 0.790) and brain (7.4-fold: p = 0.131)] (Fig. 3J). Importantly, however, the treatment still induced the significant reduction in placental viral load (3.4-fold: p = 0.005) (Fig. 3K), and successfully reduced the risk of fetal infection [positive fetus: 47.6% (20/42) in vehicle control dams vs 13.6% (6/44) in treated dams: p < 0.001] (Fig. 3L). Once again, the average weight of live fetuses was 216 ± 95 mg (n = 38) for vehicle control group and 201 ± 59 mg (n = 41) for NIT008-treated group (p = 0.411), suggesting that the difference of the rate of fetal infection is not due to the gestation period-dependent susceptibility. Collectively, these results suggest the potential clinical utility of safe and efficacious DAA drugs for preventing maternal-fetal ZIKV transmission.

In this study, we established a mouse model of ZIKV infection that stably induces 40–60% of fetal infection and demonstrated the utility of an antiviral drug for preventing maternal-fetal transmission of ZIKV. In the case of dengue, when dengue fever patients present at a clinic, viremia is usually at a peak level (Low et al., 2014; Nguyen et al., 2013; Tricou et al., 2010) and this makes it difficult to assess the antiviral efficacy since the rate of viremia clearance varies depending on the immune status of the patients and virus serotype (or genotype) (Duyen et al., 2011; Tricou et al., 2011). In fact, the challenges of using of viremia reduction as a primary endpoint to assess the drug efficacy when the treatment was commenced at peak viremia have been investigated for DENV (Chacko et al., 2017; Watanabe et al., 2016). On the other hand, in the current ZIKV study, “prevention of fetal infection” can be used as a key criteria to evaluate the potential use of drug candidates in pre-clinical animal studies as well as clinical settings.

To conclude, our results with NITD008 treatment in pregnant mice showed that, even though the levels of viral load reduction differs among the various tissues tested, significant reduction in placental viral load could be achieved even when the treatment was commenced on day 2 pi, and this directly corresponded to lower frequency of fetal infection. Thus, our results encourage the approach to advance antiviral drugs for clinical use to reduce the risk of maternal-fetal transmission of ZIKV.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.04.013.

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