Antiviral Research 145 (2017) 82–86

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Replication of the Zika virus in different iPSC-derived neuronal cells and implications to assess efficacy of antivirals

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ARTICLE INFO

Article history:
Received 27 March 2017
Received in revised form
29 June 2017
Accepted 13 July 2017
Available online 20 July 2017

Keywords:
Zika virus
iPSC
Antivirals
Neuronal cells

ABSTRACT

Infections with the Zika virus (ZIKV) are responsible for congenital abnormalities and neurological disorders. We here demonstrate that ZIKV productively infects three types of human iPSC (induced pluripotent stem cells)-derived cells from the neural lineage, i.e. cortical and motor neurons as well as astrocytes. ZIKV infection results in all three cell types in the production of infectious virus particles and induces cytopathic effects (CPE). In cortical and motor neurons, an Asian isolate (PRVABC59) produced roughly 10-fold more virus than the prototypic African strain (MR766 strain). Viral replication and CPE is efficiently inhibited by the nucleoside polymerase inhibitor 7-deaza-2′-C-methyladenosine (7DMA). However, ribavirin and favipiravir, two molecules that inhibit ZIKV replication in Vero cells, did not inhibit ZIKV replication in the neuronal cells. These results highlight the need to assess the potential antiviral activity of novel ZIKV inhibitors in stem cell derived neuronal cultures.

The Zika virus (ZIKV) is a mosquito-borne flavivirus that was first isolated in 1947 in Uganda (Dick et al., 1952). The last years this previously neglected virus has spread through the Pacific islands to South America, where it is currently causing a large epidemic (WHO, 2016). Infection during pregnancy has been shown to result in an increased chance of fetal abnormalities such as microcephaly, brain calcifications and ophthalmological problems (de Paula Freitas et al., 2016; Mlakar et al., 2016; Oliveira Melo et al., 2016). In adults, neurological complications such as Guillain-Barré syndrome, meningoencephalitis and acute myelitis have been reported (Cao-Lormeau et al., 2016; Carteaux et al., 2016; Mécharles et al., 2016). According to the WHO, the ZIKV epidemic associated with microcephaly and neurological complications continues to represent a major challenge for public health, therefore the Strategic Response Plan was set up to prevent and manage the complications of ZIKV (WHO, 2016). The exact mechanism of vertical transmission, teratogenic effects and neuro-invasion remains to be explored. To date ZIKV has been detected in the amniotic fluid (RNA) (Calvet et al., 2016; Sarno et al., 2016) and fetal brain tissue (RNA and viral particles) (Driggers et al., 2016; Mlakar et al., 2016) of fetuses that develop abnormalities upon ZIKV infection of the pregnant mothers. Several hypothesis of vertical transmission have been proposed (Adibi et al., 2016; Retallack et al., 2016; Tabata et al., 2016). Neural tropism is a characteristic of several flaviviruses: tick-borne encephalitis virus (TBEV) is among the common infective agents of meningoencephalitis in Europe, West Nile virus (WNV) and Japanese encephalitis virus (JEV) are known to cause encephalitis (Ludlow et al., 2016; Winkelmann et al., 2016), and neurological complications can also rarely occur in Dengue virus infection (Madi et al., 2014). In addition, TBEV has been found in brain tissue and spinal cord of infected patients (Gelpi et al., 2005), and WNV is associated with neuronal loss (Kelley et al., 2003). However, the birth defects caused by ZIKV have not been reported for other flavivirus infections.

To further explore biological processes underlying fetal infection and neurological complications, relevant cellular and animal models of ZIKV infection are being developed. Here we report on ZIKV infection, and inhibition thereof, in three types of induced pluripotent stem cell (iPSC)-derived neuronal cell types: cortical neurons, motor neurons and astrocytes. Cortical neurons form the human cerebral cortex responsible for higher nervous activity. The disturbance of proliferation and differentiation of these cells has been linked to various disorders including microcephaly (Manzini

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http://dx.doi.org/10.1016/j.antiviral.2017.07.010

and Walsh, 2011). Together with cortical neurons, astrocytes represent the predominant cell population in the human brain. Among other functions, astrocytes are involved in the neuro-inflammatory response during central nervous system (CNS) infection (Hamel et al., 2017; Molofsky and Deneen, 2015). Motor neurons control effector organs such as the muscles. In the context of ZIKV infection numerous cases of motor disability have been reported (Cao-Lormeau et al., 2016; Mécharles et al., 2016).

To investigate which cell types might be primary targets of ZIKV infection in the nervous system, we differentiated wild-type human iPSC into early cortical neurons, motor neurons and astrocytes and studied the susceptibility of these different cell types for ZIKV infection. To this end, the prototypic ZIKV strain MR766 from the African lineage and the recent clinical strain PRVABC59 from the Asian lineage (Puerto Rico, December 2015) were used each at a multiplicity of infection (MOI) of 10E-4 (as determined in Vero cells). We used a low inoculum since this may provide a more sensitive readout of susceptibility to infection than overloading cells with a high inoculum. Using a low inoculum also had the advantage that the special neuronal culture medium was not substantially diluted. Following infection, cell cultures were monitored microscopically and supernatant samples were collected at day 1, 3 and 6 post infection for cortical neurons and astrocytes and at day 1, 4 and 7 post infection for motor neurons until cytopathogenic effects (CPE) were observed. Viral RNA levels and the infectivity of viral progeny were quantified by means of qRT-PCR and end-point titration in Vero cells, respectively.

The three cell types proved susceptible to both ZIKV strains and produced infectious progeny virus (Fig. 1). The PRVABC59 strain produced significantly higher viral loads (both RNA and infectious virions) than the MR766 strain at the end of the experiment in cortical neurons (day 6 p.i.) and in motor neurons (day 7 p.i.). No significant difference in viral loads between the two ZIKV strains was observed in astrocytes. The highest titers of infectious virus were observed in motor neurons (up to 10E+7 $TCID_{50}$/ml for PRVABC59 and 10E+6 $TCID_{50}$/ml for MR766); titers in cortical neurons increased up to 10E+6 for PRVABC59 and 10E+5 $TCID_{50}$/ml for MR766, whereas the lowest viral production was observed in astrocytes. Similar results were obtained by measuring viral genome copies in culture supernatants by qRT-PCR (Fig. 1). The dynamics of ZIKV replication was comparable in cortical neurons and astrocytes with a rapid increase of viral RNA load during the first 3 days and complete destruction of the cell cultures by CPE at day 6 p.i. The infection in motor neurons progressed slower with first signs of CPE appearing at day 5–6 p.i. and complete destruction of the cultures by CPE at day 7–8 p.i. Our findings are in line with recent other studies where ZIKV was shown to infect human neural progenitor cells (hNPCs) and early cortical neurons (Tang et al., 2016; Dang et al., 2016; Xu et al., 2016). Infection of astrocytes was also demonstrated (Xu et al., 2016; Qian et al., 2016). When using a 10-fold lower MOI (10E-5) cortical neurons and motor neurons still proved susceptible to ZIKV infection, whereas this MOI proved too low to result in a productive infection in astrocytes (as monitored over a period of 6 days) (Fig. S1).

We next studied the potential antiviral activity of 3 molecules for which we demonstrated earlier that they inhibit ZIKV replication in Vero cells (Zmurko et al., 2016) namely T-705 (favipiravir), 7-deaza-2′-C-methyladenosine (7DMA) and ribavirin (Table 1). T-705 is a broad-spectrum inhibitor with antiviral activity against many RNA viruses including flaviviruses [the West-Nile virus and the yellow fever virus (Furuta et al., 2013)], and 7DMA was initially developed as a polymerase inhibitor of the hepatitis C virus (Olsen

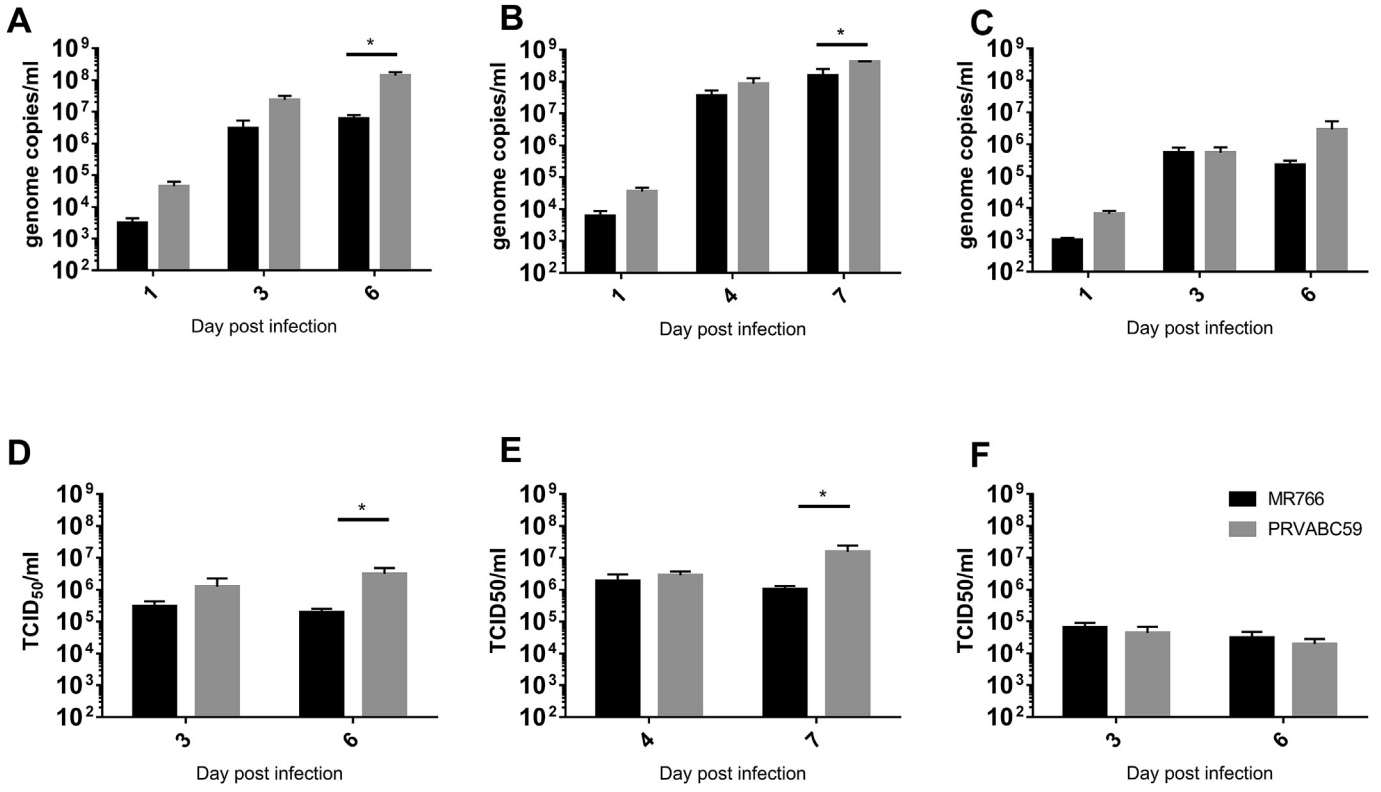

Fig. 1. hiPSC-derived cells of neural lineage are susceptible to ZIKV infection. Infectious virus and viral RNA levels of ZIKV MR766 (black) and PRVABC59 (grey) strains following infection of hiPSC-derived (A, D) cortical neurons, (B,E) motor neurons and (C, F) astrocytes as quantified by qRT-PCR or by end-point titration respectively. Data are mean values of three independent experiments and are presented as mean (±) SEM. *p < 0,05.

Table 1
Antiviral activity of 7DMA, T-705 and Ribavirin in neuronal cultures.

	Cortical neurons			Motor neurons			Astrocytes		
	EC50		CC50	EC50		CC50	EC50		CC50
	MR766	PRVABC59		MR766	PRVABC59		MR766	PRVABC59	
7DMA	2,2 ± 0,6	1,6 ± 0,3	52 ± 1	1,2 ± 0,1	1,7 ± 0,01	52 ± 1	1,7 ± 0,3	1,4 ± 0,1	>100
T-705	NA	NA	>100	NA	NA	>100	NA	NA	>100
Ribavirin	NA	NA	>100	NA	NA	>100	NA	NA	>100

*EC50 and CC50 values in μg/ml. Antiviral activity was determined by measuring reduction of viral RNA load in culture supernatants. Data represent median values ± standard deviations (SD) of three independent experiments. NA – not active.

et al., 2004). Ribavirin was previously shown to have moderate antiviral activity against flaviviruses (Crance et al., 2003; Leyssen et al., 2006). Following infection of the differentiated cells, fixed concentrations of the compounds were added to the medium (10 μg/ml 7DMA, 40 μg/ml favipiravir or 20 μg/ml ribavirin). No cytotoxic effects in any cell type were observed. Interestingly, only 7DMA resulted in an antiviral effect as shown by an inhibitory effect on ZIKV-induced CPE in all three cell types and a reduction of infectious viral yield and viral RNA levels by 5 $\log_{10}$ and 4 $\log_{10}$, respectively (at a concentration of 10 μg/ml) (Fig. 2). 7DMA treated cultures did not show signs of CPE at the end of experiment. The strongest inhibition by 7DMA was observed in astrocytes, in which the titers of both strains were reduced to undetectable levels in treated cells. The more pronounced antiviral effect in astrocytes may possibly be the result of the overall lower production of ZIKV in this type of cells. The inhibitory effect of 7DMA was more pronounced against the MR766 strain than against the PRVABC59 strain in all three cell types. Surprisingly, whereas T-705 and ribavirin inhibited ZIKV replication in Vero cells, these antiviral molecules did not inhibit the replication of both MR766 and PRVABC59 ZIKV in the neuronal cells (Fig. 2). It may be assumed that differences in the uptake or cellular metabolism of the compounds (ribavirin needs to be phosphorylated and T-705 is converted to a nucleotide) between Vero cells on the one hand and neurons and astrocytes on the other hand explain this difference.

Microcephaly can be caused by many factors, among them the disruption of differentiation, and the proliferation and apoptosis balance in neurons (Manzini and Walsh, 2011). Increased cell death caused by ZIKV infection might therefore directly contribute to the development of microcephaly. The infection of astrocytes, although

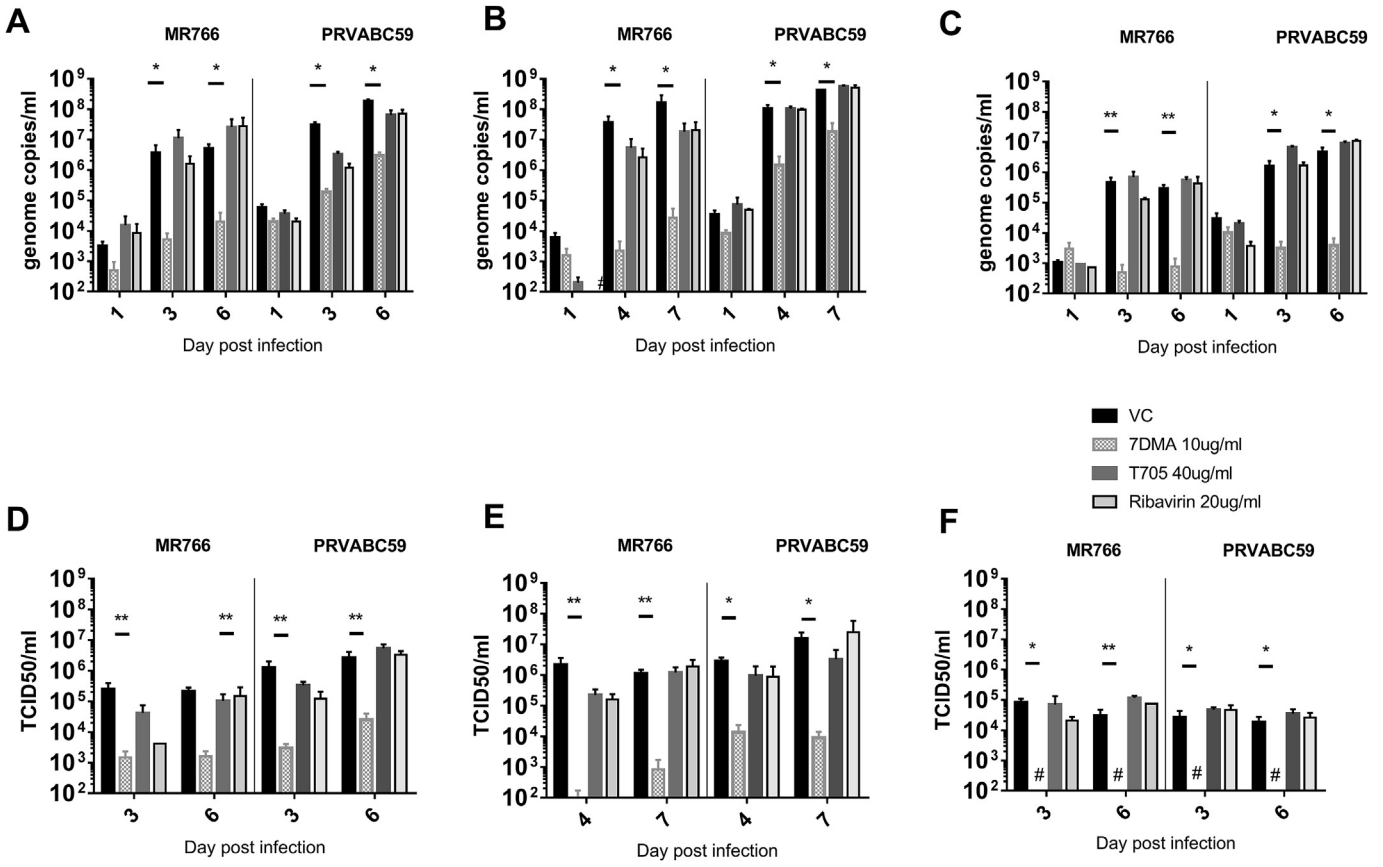

Fig. 2. ZIKV replication in hiPSC-derived neural cells is inhibited by the nucleoside polymerase inhibitor 7DMA, but not by favipiravir or ribavirin. Inhibitory effect of 7DMA (10 μg/ml, dotted bar), favipiravir (40 μg/ml, dark grey bar) and ribavirin (20 μg/ml, light grey bar) on the production of ZIKV RNA of strain MR766 or PRVABC59 (black bars) in hiPSC-derived (A, D) cortical neurons, (B, E) motor neurons and (C, F) astrocytes as quantified by qRT-PCR or by end-point titration respectively. Data are mean values for three (7DMA) or two (favipiravir, ribavirin) independent experiments and are presented as mean (±) SEM. *p < 0,05 **p < 0,005 # not detected.

less robust than in cortical neurons, may contribute to the development of ZIKV associated CNS abnormalities, since these cells represent the predominant population in the brain (Sofroniew and Vinters, 2010).

To date 21 countries have reported an increased incidence of Guillain-Barré syndrome (GBS) in coincidence with an ongoing ZIKV outbreak (WHO, 2017). GBS is usually caused by an autoimmune attack on the nerves damaging myelin or axons and is often triggered by infection (Hardy et al., 2011). It is not yet known whether Guillain-Barré syndrome associated with ZIKV infection is caused by an autoimmune reaction or by direct action of the virus on the nerves. We here demonstrate as the first that ZIKV is able to infect motor neurons. This suggests the potential for a direct effect of the virus on nerves, which may possibly contribute to/trigger the induction of GBS in ZIKV infected patients.

It is not clear yet why cortical and motor neurons are more susceptible to ZIKV than astrocytes. This could be due to cell-type specific expression of ZIKV receptors (Nowakowski et al., 2016) or different innate immune programs (Cho et al., 2013). Further studies are thus needed to reveal the exact mechanism determining ZIKV neurotropism.

It is subject of further investigation whether particular differences between strains of the Asian and African lineages contribute to differences in ZIKV neurotropism (Wang et al., 2016). Infection rates in human forebrain organoids infected with the MR766 strain (African lineage) and FSS13025 (Asian lineage) proved comparable (Qian et al., 2016). Another study showed also comparable infection rates of these strains in human brain microvascular endothelial cells (HBMECs) (Bayer et al., 2016). Infection of primary human astrocytes with the H/PF/2013 strain (Asian lineage) and the HD 78788 strain (African lineage) resulted in comparable levels of production of infectious virus (Hamel et al., 2017). Hamel and colleagues observed higher titers of infectious virus in primary human astrocytes then we report in iPSC-derived astrocytes. This might be attributed to the differences in experimental design (cell system, virus isolates, MOIs). The isolates used in our study may induce stronger antiviral response in astrocytes; it is also possible that low MOI and slower infection kinetics allow enough time for innate immunity activation, resulting in lower production of infectious virus. The low passaged African strain ArB41644 showed higher infectivity in human neural stem cells (NSC) and astrocytes compared to Asian strain H/PF/2013 (Simonin et al., 2016). We here observed that the Asian isolate PRVABC59 produced roughly 10-fold more virus than the prototypic African strain (MR766 strain) in cortical and motor neurons. A larger panel of several low passaged clinical isolates will need to be studied in parallel in the future.

In conclusion, we demonstrate (i) for the first time that motor neurons support ZIKV replication and these cells are as a consequence of ZIKV replication destroyed by the virus and (ii) that human iPSC derived neuronal cells offer a physiologically relevant system to assess the potential antiviral effect of small molecule inhibitors of viral replication that are being developed to clear ZIKV infections in the nervous system.

Acknowledgements

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie grant agreement No 642434, ZIKAlliance grant agreement No 734548 and ZikaPLAN grant agreement No 734584.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.antiviral.2017.07.010.

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