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Cell-line dependent antiviral activity of sofosbuvir against Zika virus

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ABSTRACT

The recent epidemic of Zika virus (ZIKV) in the Americas and its association with fetal and neurological complications has shown the need to develop a treatment. Repurposing of drugs that are already FDA approved or in clinical development may shorten drug development timelines in case of emerging viral diseases like ZIKV. Initial studies have shown conflicting results when testing sofosbuvir developed for treatment of infections with another *Flaviviridae* virus, hepatitis C virus. We hypothesized that the conflicting results could be explained by differences in intracellular processing of the compound. We assessed the antiviral activity of sofosbuvir and mericitabine against ZIKV using Vero, A549, and Huh7 cells and measured the level of the active sofosbuvir metabolite by mass spectrometry. Mericitabine did not show activity, while sofosbuvir inhibited ZIKV with an IC_{50} of ~4 μM, but only in Huh7 cells. This correlated with differences in intracellular concentration of the active triphosphate metabolite of sofosbuvir, GS-461203 or 007-TP, which was 11–342 times higher in Huh7 cells compared to Vero and A549 cells. These results show that a careful selection of cell system for repurposing trials of prodrugs is needed for evaluation of antiviral activity. Furthermore, the intracellular levels of 007-TP in tissues and cell types that support ZIKV replication *in vivo* should be determined to further investigate the potential of sofosbuvir as anti-ZIKV compound.

Zika virus (ZIKV) is an arthropod-borne flavivirus and belongs to the family of *Flaviviridae*. It has gained global attention due to the recent emergence in the Americas, and the newly observed association with fetal and neurological complications (Lazear et al., 2016). Given this widespread emergence and the concern about neurological complications, ways to reduce the impact of infection are urgently needed. However, neither vaccines nor drugs are available, and their development requires a lengthy process before being available for use (Ekins et al., 2016). The repurposing of drugs, which are already FDA-approved or in clinical development, may shorten drug development timelines in case of emerging viral diseases like ZIKV (Mumtaz et al., 2016). For ZIKV, large libraries of FDA-approved drugs have been screened, including direct-acting antivirals of hepatitis C virus (HCV) which also belongs to the *Flaviviridae* family (Zmurko et al., 2016; Gane et al., 2013; Eyer et al., 2016; van der Eijk et al., 2016). Conflicting results were recently reported on the antiviral activity of the anti-HCV drug sofosbuvir when testing its effect on ZIKV replication: no anti-ZIKV activity was reported using Vero cells while others reported activity using Huh7, BHK-21, SH-Sy5y cells and neuronal stem cells (Eyer et al., 2016; Sacramento et al., 2016; Bullard-Feibelman et al., 2016). Though *in vitro* susceptibility testing of drugs against emerging viruses may seem straightforward, it should be carried out with certain considerations. Cell culture systems should be chosen with detailed knowledge about the pharmacodynamics and pharmacokinetic properties of the drugs along with information of active components and target cells. We hypothesized that these conflicting results could be explained by differences in the intracellular concentration of the active triphosphate form of sofosbuvir, GS-461203 or 007-TP, which were not provided in the publications.

We studied the antiviral activity of the anti-HCV compounds sofosbuvir and mericitabine against ZIKV, because it was previously shown that these two compounds inhibited replication of the closely related dengue virus using Huh7 cells (Bluemling et al.,

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2014). The inhibitory activities of sofosbuvir and mericitabine against two ZIKV strains belonging to the Asian lineage (H/PF/2013 [ZIKV$^{AS-FP13}$] and ZIKVNL00013 [ZIKV$^{AS-Sur16}$]) (van der Eijk et al., 2016) were tested using A549 cells (human pneumocyte type II carcinoma cells), Vero cells (African green monkey kidney epithelial cells) and Huh7 cells (human hepatocellular carcinoma cells). Virus isolates are available through the European virus archive with EVAg accession numbers 001V-EVA1545 and 011V-01621, respectively (https://www.european-virus-archive.com). Huh7 cells were included in view of the dengue virus studies (Bluemling et al., 2014), Vero cells because they are routinely used for ZIKV isolation, and A549 cells because these cells strongly expresses carboxylesterase 1 (CES1) which is needed for activation of sofosbuvir (Hosokawa, 2008; Murakami et al., 2010). We measured the intracellular concentration of 007-TP to further investigate the cell-line dependent antiviral activity of sofosbuvir against ZIKV.

Cell viability assays (Cell Titer 96® Aq_{ueous} One Solution Reagent, Promega) with sofosbuvir and mericitabine showed 50% cell cytotoxicity concentrations (CC_{50}) of >100 μM for both drugs. Anti-ZIKV activity was tested by plaque reduction assay (PRA). In PRA, all three cell lines were challenged with ~0.001 MOI of ZIKV and incubated with 0.09 μM–50 μM (2-fold serial dilutions) of sofosbuvir and mericitabine for 3 days (37 °C, 5% CO_2). After 3 days of incubation, plaques were visualized with True Blue staining using mouse monoclonal antibody to ZIKV NS1 protein (Aalto Bio Reagents, USA) as primary antibody and HRP-labeled goat anti-mouse antibody as secondary antibody. Mericitabine did not show any inhibition of ZIKV (IC_{50} > 50 μM). Sofosbuvir inhibited ZIKV$^{AS-FP13}$ and ZIKV$^{AS-Sur16}$ replication in Huh7 cells with IC_{50} values around 4 μM, but not in Vero and A549 cells (Table 1). To validate the findings from the plaque reduction assays, we analyzed the dose-dependent inhibition of ZIKV replication by both drugs using cytopathic effect (CPE) reduction assays and virus yield reduction assays. Using a multiplicity of infection of 0.1, we again observed an IC_{50} of about 4 μM using the Huh7 cells, while no inhibition was observed with Vero cells. Supernatants from CPE reduction assay were titrated to quantify the new progeny virus titers, showing that ZIKV replication was inhibited ≥95% by 25 μM and 50 μM sofosbuvir using Huh7 cells, while no reduction in infectious titers was observed in Vero cells. To further understand the cell-line dependent inhibition of ZIKV by sofosbuvir, the active triphosphate form, 007-TP, was measured in all the three cell lines. Each cell line was treated with 5 μM and 50 μM of sofosbuvir and incubated for 48 h (37 °C, 5% CO_2). After 2 days of incubation, supernatants were removed and cell lysates were prepared for mass spectrometric analysis as described previously (Rower et al., 2015). Intracellular 007-TP concentrations were 11× and 158× lower in A549 and Vero, respectively, than in Huh7 cells when incubated with 5 μM sofosbuvir (see Table 2). The intracellular concentrations were 25× and 342× lower in A549 and Vero, respectively, compared to Huh7 cells when incubated with 50 μM sofosbuvir.

Sofosbuvir is a phosphoramidate nucleotide analogue and the metabolic pathway involves hydrolysis of the carboxyl ester moiety by cathepsin A (CatA) or carboxylesterase 1 (CES1) and phosphoramidate cleavage by histidine triad nucleotide-binding protein 1 (HINT1) followed by phosphorylation by uridine monophosphate-cytidine monophosphate kinase (UMP-CMP kinase) and nucleoside diphosphate kinase (NDPK) to its active metabolite 007-TP, a uridine-triphosphate analogue which inhibits the viral RNA dependent RNA polymerase (RdRp) (Serrano and Manns, 2012) (Hurwitz and Schinazi, 2013). Mericitabine, a nucleoside analogue, also inhibits the viral RdRp, but other enzymes are involved in the metabolism to the active triphosphate form (Ma et al., 2007). Bluemling et al. reported the inhibition of dengue virus, a virus closely related to ZIKV, by mericitabine and sofosbuvir using Huh7 cells, but this is still unpublished work (Bluemling et al., 2014). Mericitabine inhibits hepatitis C virus in Huh7 using a virus replicon system (Bassit et al., 2008). This implies that the active triphosphate forms of both drugs are formed in Huh7 cells, although these measurements were not included in these studies. We did not observe any inhibition of ZIKV by mericitabine using Huh7 cells, which suggests that the RdRp of ZIKV is not inhibited by the active triphosphate form of mericitabine. In contrast, sofosbuvir did inhibit the replication of ZIKV in Huh7 cells but not in Vero cells and A549 cells, which correlated with the intracellular concentration of 007-TP. One explanation for the difference in intracellular concentration could be that activation of sofosbuvir by cellular enzymes in A549 cells and Vero cells is less efficient than in Huh7 due to the absence of CES1 activity (Hosokawa, 2008). However, CES1 enzymes are thought to be present in the A549 cell line, which nevertheless metabolized sofosbuvir less well. However, the expression of other enzymes that are involved in the metabolic activation of sofosbuvir may be lower in A549 cells and Vero cells compared to Huh7 cells. Currently there is no data available on the comparative expression profiles of these enzymes in Huh7, A549 and Vero. Another possible explanation may be the overexpression of drug efflux pumps, such as the multi-drug resistance ABC-transporter, which might have cleared sofosbuvir and/or its metabolites from Vero and A549 cell lines (Guo et al., 2014; Sung et al., 2008). However, the role of drug efflux pumps still need further investigation as other nucleoside analogues like NITD008 exhibit antiviral activity against DENV and ZIKV in A549 and Vero cells (Deng et al., 2016; Yin et al., 2009).

Our data shows that selection of cell lines to screen prodrugs for

Table 1
Summary of *in vitro* assays and IC_{50} values.

Cell systems	VERO				Huh7				A549			
ZIKV strains	ZIKV$^{AS-FP13}$		ZIKV$^{AS-Sur16}$		ZIKV$^{AS-FP13}$		ZIKV$^{AS-Sur16}$		ZIKV$^{AS-FP13}$		ZIKV$^{AS-Sur16}$	
Antivirals	SB	MB	SB	MB	SB	MB	SB	MB	SB	MB	SB	MB
CC_{50} (μM)	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
PRA (IC_{50} μM)	>50	>50	>50	>50	3.9	>50	4.0	>50	>50	>50	>50	>50
CPE (IC_{50} μM)	>50	>50	>50	>50	4.0	>50	4.1	>50	–	–	–	–

In the cell viability assay, cell lines were incubated for three days with sofosbuvir and mericitabine. In PRA, all three cell lines were challenged with ~0.001 MOI of ZIKV$^{AS-FP13}$and ZIKV$^{AS-Sur16}$ and incubated with different concentrations of sofosbuvir and mericitabine for 3 days using 1.6% carboxyl methyl cellulose (CMC) overlay. After 3 days of incubation the overlay was aspirated and cells were fixed with formalin for immuno-histochemical staining to visualize the plaques. In CPE reduction assay cells, cells were infected with 0.1 moi of ZIKV$^{AS-FP13}$and ZIKV$^{AS-Sur16}$ and after three days cells were scored for CPE using a scale from 0 to 4 (0 meaning no CPE and 4 meaning 75%–100% CPE). For the virus yield reduction assay (see text), supernatants from the CPE reduction assay were titrated and incubated for 5 days to quantify new progeny virus titers using CPE as read-out. For all experiments medium with 10% FBS was used. PRA = Plaque Reduction assay. SB = Sofosbuvir. MB = Mericitabine.

Table 2
Measurement of intracellular active metabolite 007-TP by LC-MS/MS.

Sofosbuvir Conc. (μM)	007-TP (PMOL/10^6 Cells)		
	Huh-7 cells	A549 cells	Vero cells
5	416 (SD = 13.5)	36.20 (SD = 0.95)	2.63 (SD = 0.05)
50	5174 (SD = 158)	204 (SD = 7.90)	15.11 (SD = 0.28)

For all experiments medium with 10% FBS was used.

activity against ZIKV can strongly affect the final outcome, and may give false negative results in compound screening studies (Eyer et al., 2016). The average plasma C_{max} in humans using a single dose of 1200 mg sofosbuvir is nearly equivalent to the IC_{50} of 4 μM that we found in the Huh7 liver cell line (Kirby et al., 2015). Thus, using this dosage, sufficiently high plasma concentrations of sofosbuvir may be reached to inhibit ZIKV in humans. It should however be noted that sofosbuvir has been developed for treatment of a hepatotropic virus, and is designed to facilitate the intracellular penetration in liver tissue, whereas there is lack of data on the uptake and intracellular activation of sofosbuvir in other tissues. For this, understanding the cell tropism of (early) ZIKV infection is important in order to select cell lines relevant for drug repurposing screening. Bullard et al. reported an anti-ZIKV EC_{50} of 1–5 μM for sofosbuvir using Huh7 cells and an EC_{50} of 32 μM using neuronal stem cells (Bullard-Feibelman et al., 2016). The higher EC_{50} of sofosbuvir in neuronal stem cells may reflect the lower CES1 activity in brain tissue compared to liver tissue (Satoh et al., 2002). Thus, to further investigate the potential of sofosbuvir as anti-ZIKV compound, intracellular concentrations of the active metabolite 007-TP in cell types and tissues known to support ZIKV replication *in vivo* should be taken into account. Since measuring 007-TP levels in various tissues is technically challenging, measuring expression levels of enzymes involved in the metabolic activation of sofosbuvir in these cell types and tissues may be a good alternative. Furthermore, given the high uptake of sofosbuvir in liver tissue, further pursuing the activity of sofosbuvir against other flaviviruses, in particular those with liver tropism like yellow fever virus, is warranted.

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