Cell-line dependent antiviral activity of sofosbuvir against Zika virus

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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 IC50 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.

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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 (Lalezar 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.,
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 and incubated with 0.09 M sofosbuvir and incubated for 48 h and 100% cell CPE with sofosbuvir and mericitabine showed 50% cell cytopathic effect (CPE) reduction assays and virus yield reduction assays dependent inhibition of ZIKV replication by both drugs using 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 (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) (Hurst 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).}

**Table 1**

| Cell systems | VERO | ZIKV\(^{AS-\text{FP13}}\) | ZIKV\(^{AS-\text{Sur16}}\) | HuH7 | ZIKV\(^{AS-\text{FP13}}\) | ZIKV\(^{AS-\text{Sur16}}\) | A549 | ZIKV\(^{AS-\text{FP13}}\) | ZIKV\(^{AS-\text{Sur16}}\) |
|--------------|------|-----------------|-----------------|------|-----------------|-----------------|------|-----------------|-----------------|
| ZIKV strains |      | ZIKV\(^{AS-\text{FP13}}\) | ZIKV\(^{AS-\text{Sur16}}\) |      | ZIKV\(^{AS-\text{FP13}}\) | ZIKV\(^{AS-\text{Sur16}}\) |      | ZIKV\(^{AS-\text{FP13}}\) | ZIKV\(^{AS-\text{Sur16}}\) |
| Antivirals   |      | SB               | MB               |      | SB               | MB               |      | SB               | MB               |
| C\(_{50}\) (μM) |      | >100             | >100             |      | >100             | >100             |      | >100             | >100             |
| PRA          |      | >100             | >100             |      | >100             | >100             |      | >100             | >100             |
| (C\(_{50}\) μM) |      | >50              | >50              |      | >50              | >50              |      | >50              | >50              |
| CPE          |      | >50              | >50              |      | >50              | >50              |      | >50              | >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\(^{AS-\text{FP13}}\) and ZIKV\(^{AS-\text{Sur16}}\) 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 were infected with 0.1 moi of ZIKV\(^{AS-\text{FP13}}\) and ZIKV\(^{AS-\text{Sur16}}\) and after three days cells were scored for CPE using a scale from 0 to 4 (0 meaning no CPE and 4 meaning 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.
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 $C_{\text{max}}$ 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 dosing, 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 activity against 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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Table 2

| 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.