Differential activity of nucleotide analogs against tick-borne encephalitis and yellow fever viruses in human cell lines

Binderup, Aleksander; Galli, Andrea; Fossat, Nicolas; Fernandez-Antunez, Carlota; Mikkelsen, Lotte S; Rivera-Rangel, Lizandro René; Scheel, Troels K H; Fahnøe, Ulrik; Bukh, Jens; Ramirez, Santseharay

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1. Introduction

Orthoflaviviruses are wide-spread vector-borne RNA viruses collectively infecting more than a hundred million people every year (Pierson and Diamond, 2020). Tick-borne encephalitis virus (TBEV) and yellow fever virus (YFV) are orthoflaviviruses that can cause diseases with significant mortality and morbidity. TBEV and YFV infect at least 12,000 and 200,000 people every year, respectively (Gardner and Ryman, 2010; “WHO,” 2023). Despite the existence of effective vaccines, the incidence of TBEV and YFV infections has risen during the last decades, and the viruses have spread to new previously non-endemic areas (“ECDC,” 2015; Gardner and Ryman, 2010; Lindquist and Vapalahti, 2008). There are currently no approved antiviral drugs for the treatment of orthoflaviviruses (Boldescu et al., 2017), thus identifying safe and effective drugs, ideally broad-spectrum anti-flavivirus compounds, is crucial. Drugs against the related hepatitis C virus (HCV), which lead to high cure rates for all HCV genotypes (Bukh, 2016), have been successfully developed.

Although numerous studies have found compounds that can inhibit orthoflaviviruses, including TBEV and YFV (Brzuska et al., 2020; Eyer et al., 2016; Guo et al., 2016; Zandi et al., 2019), only a few drugs have reached clinical trials (Komarasamy et al., 2022). Nucleos(t)ide analogs (nucs), such as remdesivir and 2′-C-methyladenosine, exhibit antiviral activity against several members of the *Flaviviridae* family (Carroll et al., 2003; Eyer et al., 2018; Ramirez et al., 2021). Nucs inhibit viral replication by targeting the viral RNA dependent RNA polymerase (RdRp), and can cause chain-termination of an elongating RNA strand or increase mutagenesis, which can lead to error catastrophe (De Clercq and Neyts, 2009; Graci and Cameron, 2008). The viral RdRp is located at the C-terminal of the non-structural protein 5 (NS5) of orthoflaviviruses and shares conserved domains with RdRps across RNA viruses (Choi and Rossmann, 2009), representing a good target for broad-spectrum antivirals. Numerous *in vitro* studies have investigated the anti-TBEV and anti-YFV activity of existing or novel nucs (Eyer et al., 2015, 2017a, 2017b, 2018; Gejji et al., 2020; Good et al., 2021; Mendes et al., 2019; Warren et al., 2014; Zandi et al., 2019), however, head-to-head determinations using comparable experimental conditions are lacking, making comparisons of antiviral activity between viruses and across studies difficult. Most nucs are developed as prodrugs and must first be converted intracellularly to an active 5′-triphosphate metabolite to enable their antiviral properties (Peterson and McKenna, 2009).
2. Materials and methods

2.1. Virus cell culture

All cell lines were kept at 37 °C in a humidified incubator with 5% CO₂ and grown with specific media, as described in detail in Supplementary Material. The TBEV stock (strain Neudoerfl) was generated from the pTNd/c-pBR322 plasmid (Mandl et al., 1997). Briefly, in vitro transcribed (IVT) RNA was produced with the T7 mMESSAGE mMACHINE Kit (Invitrogen) and purified RNA was electroporated in BHK-21 cells using a BioRad GenePulser Xcell (BioRad). Supernatant from the transfected cell culture was harvested after 6 days and used for two consecutive passages in Huh7.5 cells to generate a YFV viral stock, which was used for all experiments described here. Similarly, the YFV stock (strain 17D) was generated from the pACNR-FLYF-17D plasmid (Sanchez-Velazquez et al., 2020). IVT RNA was produced with the mMESSAGE mMACHINE SP6 Transcription kit (Promega, M6101), which was then electroporated in Huh7.5 cells. Supernatant from the transfected culture was harvested after 48 h and used in two consecutive passages in Huh7 cells to generate a YFV viral stock.

2.2. Determination of viral stock titers

Infectivity titers were determined using tissue culture infectious dose 50% (TCID₅₀) assays, as described in detail in Supplementary Material. In brief, virus stocks were 10-fold serially diluted in appropriate cell culture medium and was added in quadruplicates to monolayers of cells and incubated 48 h. Cells were then fixed and stained with anti-Flavivirus group antigen antibody (R&D Systems), HRP-linked anti-body NA991V (Cytiva), and visualized with the Bright-DAB substrate kit (ImmunoLogic). An automated ImmunoSpot series 5 UV analyzer (CTL Europe GmbH) was used to produce detailed image files of each well (Gottevin et al., 2010), which were then used to estimate the number of wells harboring antigen positive cells (infected cells) at each dilution.

2.3. Cytotoxicity and treatment assays

Antivirals were purchased from Acme Bioscience or Sigma. Cytotoxic concentration 50% (CC₅₀) was determined using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega), as described in detail in Supplementary Material. Concentration-response assays were performed with a high-throughput assay used previously for HCV (Jensen et al., 2019) with modifications (Supplementary Material). In brief, cells were seeded in 96-well plates one day prior to being infected at a multiplicity of infection (MOI) resulting in fully infected monolayers for each cell line. Drug dilutions were immediately added afterwards in triplicate wells and cells were incubated for 72 h. After viral specific immunostaining performed as described above, images of each well were obtained with the ImmunoSpot series 5 UV analyzer and a customized ImageJ (version 1.52p) macro script was used to determine the relative number of infected cells in each well. EC₅₀ and CC₅₀ values were obtained with non-linear regression analysis using GraphPad Prism (version 9.3.1).

2.4. Sequencing of virus stocks

The sequence of viral stocks was confirmed with next generation sequencing (NGS) of the complete virus open reading frame (ORF). Methodologies for sample preparation and NGS analysis were adapted from protocols previously described for HCV (Fahnoe and Bukh, 2019; Jensen et al., 2019), as detailed in Supplementary Material. In short, viral RNA was extracted from cell culture supernatants and used as template for reverse transcription (RT) followed by a polymerase chain reaction (PCR)-amplification spanning the complete viral ORF with virus specific primers (Supplementary Material). PCR products were purified and prepared for NGS using the NebNext Ultra II FS DNA Library Prep Kit (NEB). NGS was performed with MiSeq (Illumina) using the v2 kit (500-cycles).

3. Results

3.1. Initial screening

An initial screening of the antiviral activity of fourteen nucs, remdesivir, GS-6620, sofosbuvir, uprifosbuvir, ribavirin, galidesivir, mericitabine, valopicitabine, molnupiravir, favipiravir, tenofovir alafenamide, tenofovir disoproxil, entecavir and lamivudine (Supplementary Table 1), was conducted in the human hepatoma derived Huh7.5 cells. Drugs were found active if they led to at least 50% inhibition of viral propagation at concentrations equal to or below 100 μM. Eight drugs were found active against TBEV and/or YFV: remdesivir, GS-6620, sofosbuvir, uprifosbuvir, valopicitabine, galidesivir, ribavirin and molnupiravir (Fig. 1, Table 1, and Supplementary Fig. 1). Huh7.5 cells were chosen since most nucs, including prodrugs, are well metabolized by the liver (Hecker and Erion, 2008), and these cells support TBEV and YFV replication and propagation. Moreover, the liver is relevant for the pathogenesis of YFV (Gardner and Ryman, 2010) but also of TBEV by supporting high level of virus replication early after infection (Rüzek et al., 2010).

3.2. Confirmation of activity in different human cell lines

During the initial viremic phase, TBEV may breach the blood-brain barrier and enter the central nervous system to infect neuronal cells (Rüzek et al., 2010). Cases of neurologic disease have also been reported for yellow fever, particularly after vaccination with some live attenuated virus strains (Cohen et al., 2022; Ribeiro et al., 2021). Thus, to further investigate, quantify and compare the antiviral activity of the eight drugs in liver and also in neuronal cells, detailed analyses of cytotoxicity and potency (determinations of cytotoxic concentration 50% [CC₅₀] and of effective concentration 50% [EC₅₀], respectively) were performed in Huh7.5, SH-SY5Y, and in Daoy cells (Table 1, Fig. 1 and Supplementary Fig. 1). SH-SY5Y cells originated from a patient with neuroblastoma (Biedler et al., 1978) and have been used to study orthoflaviviruses (Breitkopf et al., 2021; Kalia et al., 2013). Daoy cells originated from a medulloblastoma patient (Jacobsen et al., 1985) and have been used in TBEV studies (Rüzek et al., 2009). In addition, for YFV we also

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**Abbreviations:**
- CO-HEP: Copenhagen Hepatitis C Program
- TBEV: Tick-borne encephalitis virus
- YFV: Yellow fever virus
- Nucs: Nucleos(t)ide analogs
- HCV: Hepatitis C virus
- RdRp: RNA dependent RNA polymerase
- EC₅₀: 50% effective concentration
- CC₅₀: 50% cytotoxic concentration
- PS: Porcine Stable kidney cells
- 2′-C-MeC: 2′-C-methylytidine
- Cmax: Plasma maximum concentration
- SI: Selectivity index

Accordingly, their in vitro activity is cell-dependent (Byer et al., 2016).

The aim of this study was to screen the antiviral activity of a panel of existing nucs in cell culture, which could be repurposed for the treatment of TBEV and YFV, using high-throughput virus propagation assays and relevant human cell lines.

2. Materials and methods

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performed the experiments in African green monkey kidney cells (Vero E6) to directly compare the results with previously published work (Supplementary Table 2 and Supplementary Fig. 1). In concentration-response assays (Fig. 1 and Supplementary Fig. 1), we observed that the activity of most drugs varied between human cell lines and viruses, but all eight drugs inhibited both viruses in at least one human cell line. Most drugs were more potent against TBEV than YFV in the human cell lines tested. For YFV, overall drug activity was significantly reduced in Vero E6 cells, with only two drugs significantly more potent in all human cell lines, similarly inhibiting TBEV and YFV, with sub-micromolar EC_{50} values of 0.2 and 0.3 μM in Huh7.5 cells and 0.5 and 0.7 μM in Daoy cells, respectively, but was more than 15-fold

**Figure legend.** Graphs show a representative example of concentration-response curves used to determine the EC_{50} value for the different compounds for TBEV and for YFV in Huh7.5 cells. The y-axis shows the amount of intracellular virus antigen in treated wells normalized to non-treated controls (%) at each drug concentration (μM, Log10) indicated in the x-axis. Compounds are depicted in different colors. Dots represent the mean of triplicate infections and error bars represent the standard error of the mean (SEM). Curves were constrained to the y-axis with a top of 100% and bottom of 0%.

**Table 1**

<table>
<thead>
<tr>
<th></th>
<th>TBEV</th>
<th>YFV</th>
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<tbody>
<tr>
<td><strong>CC_{50} (μM)</strong></td>
<td><strong>EC_{50} (μM)</strong></td>
<td><strong>SI (CC_{50}/EC_{50})</strong></td>
</tr>
<tr>
<td>Remdesivir</td>
<td>Huh7.5</td>
<td>12.6</td>
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<td></td>
<td>Daoy</td>
<td>2.7</td>
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<td></td>
<td>SH-SYSY</td>
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<td>Huh7.5</td>
<td>148.5</td>
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<td></td>
<td>Daoy</td>
<td>75.0</td>
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<td></td>
<td>SH-SYSY</td>
<td>97.0</td>
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<tr>
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<td>Huh7.5</td>
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<td></td>
<td>SH-SYSY</td>
<td>&gt;125.0</td>
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<td>Uprifosbuvir</td>
<td>Huh7.5</td>
<td>&gt;500.0</td>
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<td>SH-SYSY</td>
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<td>Valopicitabine</td>
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<tr>
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<td>Daoy</td>
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<tr>
<td></td>
<td>SH-SYSY</td>
<td>&gt;125.0</td>
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**Table note.** Summary of drug 50% cytotoxic concentration (CC_{50}) and of 50% (antiviral) effective (EC_{50}) concentration values obtained in all cell lines for both viruses. Selectivity indexes (SI) were calculated as indicated (CC_{50}/EC_{50}). CC_{50} is the mean of quadruplicate treatments in a single assay. For EC_{50}, the mean with 95% confidence interval of triplicate infections in a single assay are shown. In cytotoxicity assays, all drugs were tested at concentrations ranging from 1 to 500 μM, except for remdesivir for which concentrations ranged from 0.08 to 40 μM. Drug concentrations associated with cytotoxicity from DMSO alone (drug solvent) were excluded. In virus treatment assays, all drugs were tested at maximum concentrations of 100 μM, except for remdesivir, for which maximum concentrations were 2 μM in Huh7.5 and Daoy cells and 40 μM in SH-SYSY cells, and uprifosbuvir with a maximum concentration of 10 μM in Huh7.5 cells. For drugs where the CC_{50} or EC_{50} value could not be reached, the highest drug concentration tested is instead shown (e.g., >500.0 μM). For SI a greater-than symbol ‘>’ indicates that the CC_{50} was not reached and the highest tested drug concentration was used for the calculation. A dash ‘-’ indicates not determined as the compound did not achieve the 50% viral inhibition threshold at 100 μM.
less active in SH-SY5Y cells. Remdesivir was also the most cytotoxic drug in all three human cell types (lowest CC50 values), however, selectivity indexes (SI) remained relatively high (SI > 40) in Huh7.5 cells, with higher CC50 values than previously described in Huh7 cells (Tao et al., 2021). As a phosphoramidate prodrug, remdesivir is efficiently converted into nucleoside monophosphate and subsequently active triphosphate in liver cells (Tao et al., 2021), thus yielding low EC50 values in Huh7.5 cells, as previously described for HCV and SARS-CoV-2 (Ramirez et al., 2016). We found some discrepancies between EC50 values from 14.4 to 50.3 μM in Huh7.5 cells, with EC50 values being consistently lower for TBEV compared to YFV (21.0–29.7 μM versus 35.2–51.8 μM). Compared to our findings, galidesivir was 1.5-fold more potent against TBEV in PS cells (Eyer et al., 2017b), which could suggest enhanced drug metabolism in this animal cell line. For YFV, we found galidesivir EC50 values in Vero E6 cells to be within 2–3.5-fold of those reported previously (Julander et al., 2014; Warren et al., 2014). Of note, it has been suggested that the antiviral effect of galidesivir is generally underrepresented in studies using immortalized cell lines (Warren et al., 2014). Moreover, galidesivir has been shown to provide high protection in YFV-infected hamsters (Julander et al., 2014).

Viruses were differently inhibited by the mutagen nucleotide analogs ribavirin and molnupiravir depending on the cell line. Ribavirin was more active against TBEV than YFV in all cell lines (TBEV EC50 values from 23.8 to 89.2 μM). Molnupiravir was only active against TBEV in Huh7.5 and Daoy cells, with EC50 values of 26.2 and 19.9 μM, respectively, and was only active against YFV in Daoy cells with an EC50 value of 1.35 μM. Ribavirin was previously found ineffective against TBEV in PS and UKE-NB-4 cell lines (Eyer et al., 2015), as well as in rat cerebellum cultures (Lenz et al., 2019). We found no anti-YFV activity of ribavirin in Vero E6 cells at 100 μM, as previously reported with concentrations of 250 μM (Pileggi et al., 2018). However two studies reported anti-YFV activity of ribavirin in Vero cells with EC50 values of 28 and 42.4 μM (Crance et al., 2003; Julander et al., 2009), moreover, ribavirin protected from death in the hamster model (Julander et al., 2009).

4. Discussion

In this study we found antiviral activity against TBEV and YFV of several existing nucs. Importantly, the antiviral activity was cell-type dependent, most likely due to differences in metabolic activation between cell lines as suggested previously (de Freitas et al., 2019; Eyer et al., 2016). We found some discrepancies between EC50 values obtained here and those previously reported, which emphasizes the difficulties of comparing drug activity, due to differences in experimental systems and methodologies.

Compared to previous studies, our work benefits from using human and pathogenesis relevant cell lines and of utilizing equivalent assay conditions permitting a direct head-to-head comparison between TBEV and YFV viruses. Thus, our results highlight the importance of carefully choosing appropriate cell lines, with sufficient metabolic capabilities, when screening nucs for antiviral activity in vitro.

Remdesivir, sofosbuvir and uprifosbuvir were highly active against both viruses in Huh7.5 cells. Uprifosbuvir exhibited the highest SI in both Huh7.5 and Daoy cells. Anti-YFV activity of sofosbuvir has been shown in Huh7 cells with comparable EC50 values (de Freitas et al., 2019), and sofosbuvir has shown anti-Zika virus activity in vitro in neuronal stem cells (Bullard-Feibelman et al., 2017) and in Huh7 cells (Bullard-Feibelman et al., 2017; Mumtaz et al., 2017). More importantly, sofosbuvir protected against vertical transmission and neural disease from Zika virus in different animal models suggesting that it can reach the brain at therapeutic concentrations (Bullard-Feibelman et al., 2017; Ferreira et al., 2017; Gardinali et al., 2020). Thus, it would be interesting to evaluate the efficacy of sofosbuvir and uprifosbuvir using animal models of TBEV neuropathogenesis. Proof-of-concept of the clinical value of sofosbuvir was reported for two YFV infected patients with acute liver disease (Mendes et al., 2019) and a randomized clinical trial has been initiated in Brazil (Piqueiredo-Mello et al., 2019). In light of the better in vitro activity of uprifosbuvir, and since this drug already underwent clinical phase III studies for the treatment of HCV (Gane et al., 2017), it would be relevant to investigate its therapeutic value for YFV in clinical trials. Based on reported maximum plasma concentrations (Cmax) in humans of 7.3 μM for remdesivir (Humeniuk et al., 2020), 4.3 μM for uprifosbuvir (Merck Sharp & Dohme LLC, 2018), and 1.2 μM for sofosbuvir (Babusic et al., 2018), therapeutic concentrations of all three drugs could likely be achieved. Moreover, due to accumulation in the liver, the effective intracellular nuc concentrations are higher (Babusic et al., 2018). Using a comparable assay,
sofosbuvir EC$_{50}$ values of up to 0.9 μM can be routinely obtained for HCV in HuH7.5 cells (Ramirez et al., 2016), thus, within 5-fold of the highest EC$_{50}$ value obtained here. However, to better evaluate the clinical relevance of sofosbuvir, uprifosbuvir and remdesivir, a comparative analysis of their triphosphate levels in primary liver and primary neural cells versus the transformed cells used in this study would be worthwhile. Likewise, in vivo studies could be conducted to establish the efficacy of the different compounds in preventing disease. Lack of potent antiviral activity in vitro does not preclude antiviral activity in vivo as exemplified with favipiravir, which was found to significantly improve disease parameters in YFV-infected hamsters, despite showing very modest antiviral activity in cell culture (Julanler et al., 2009).

Since the structurally closely related sofosbuvir has shown therapeutic value in animal models of Zika disease (Bullard-Feibelman et al., 2017), uprifosbuvir might also be able to cross the blood-brain barrier and therefore be relevant for the treatment of TBEV. The high potency remdesivir might also be relevant for treatment of TBEV, as it can accumulate in the brain of rhesus monkeys (Warren et al., 2016). However remdesivir has been associated to liver injury in a few COVID-19 patients (Zhai et al., 2021), which could be a hindrance for YFV treatment.

The diagnosis of orthoflaviviruses infections can be challenging as disease symptoms from different viruses overlap in endemic areas, thus in the clinic, having compounds that could be used as broad-spectrum panorthoflavivirus inhibitors would represent a great advance in the field of antiviral therapy. Previous studies have found that sofosbuvir can also inhibit dengue (Gan et al., 2018), West Nile (Dragoni et al., 2020) and Zika viruses in various cell lines or animal models (Bullard-Feibelman et al., 2017; Ferreira et al., 2017; Gardinali et al., 2020; Muntaz et al., 2017). Thus, with the exceptional anti-TBEV and anti-YFV activity of sofosbuvir and of its closely related compound uprifosbuvir reported here, sofosbuvir-like compounds represent relevant panorthoflavivirus antivirals with potential for clinical use.

5. Conclusions

Among all nucs tested we found the best drug candidates against TBEV and YFV to be remdesivir, sofosbuvir and uprifosbuvir. These compounds, originally found to inhibit HCV, and sofosbuvir and remdesivir currently used to treat chronic hepatitis C and COVID-19, respectively, could easily be repurposed for their use against TBEV or YFV infection. Their translational relevance should therefore be investigated further in clinical trials.

Author contributions

Alejandro Binderup: Conceptualization, Investigation, Methodology, Validation, Formal analysis, Writing – original draft, Writing – review & editing, Andrea Galli: Methodology, Software, Writing – review & editing, Nicolas Fossat: Methodology, Writing – review & editing, Carlota Fernandez-Antunez: Methodology, Writing – review & editing, Lotte S. Mikkelsen: Investigation, Writing – review & editing, Lizardo Rene Rivera-Rangel: Methodology, Writing – review & editing, Troels K. H. Scheel: Methodology, Writing – review & editing, Ulrik Fahnøe: Methodology, Writing – review & editing, Jens Bukh: Writing – review & editing, Supervision, Project administration, Funding acquisition, Santeshary Ramirez: Conceptualization, Investigation, Methodology, Validation, Formal analysis, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virology.2023.06.002.

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