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Antiviral activities of two nucleos(t)ide analogs against vaccinia, mpox, and cowpox viruses in primary human fibroblasts

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ABSTRACT

Many poxviruses are significant human and animal pathogens, including viruses that cause smallpox and mpox (formerly monkeypox). Identifying novel and potent antiviral compounds is critical to successful drug development targeting poxviruses. Here we tested two compounds, nucleoside trifluridine, and nucleotide adefovir dipivoxil, for antiviral activities against vaccinia virus (VACV), mpox virus (MPXV), and cowpox virus (CPXV) in physiologically relevant primary human fibroblasts. Both compounds potently inhibited the replication of VACV, CPXV, and MPXV (MA001 2022 isolate) in plaque assays. In our recently developed assay based on a recombinant VACV expressing secreted Gaussia luciferase, they both exhibited high potency in inhibiting VACV replication with EC_{50}s in the low nanomolar range. In addition, both trifluridine and adefovir dipivoxil inhibited VACV DNA replication and downstream viral gene expression. Our results characterized trifluridine and adefovir dipivoxil as strong poxvirus antiviral compounds and further validate the VACV Gaussia luciferase assay as a highly efficient and reliable reporter tool for identifying poxvirus inhibitors. Given that both compounds are FDA-approved drugs, and trifluridine is already used to treat ocular vaccinia, further development of trifluridine and adefovir dipivoxil holds great promise in treating poxvirus infections, including mpox.

1. Introduction

The family *Poxviridae* comprises 22 genera with 83 species based on the 2021 International Committee on Taxonomy of Viruses (ICTV) release. These viruses cause a broad range of human and animal diseases. The *Orthopoxvirus* genus contains 12 known species, including high-consequence human pathogens, such as variola virus that causes smallpox, and mpox (monkeypox) virus (MPXV). Historically, smallpox accounted for the most human deaths among all infectious diseases, claiming an estimated ~300 million lives in the first 80 years of the 20th century alone. Despite its eradication in 1980 (Theves et al. 2016; WHO 2011), the potential re-emergence of smallpox from unsecured stocks or by a synthetic biology approach remains a major national security concern (Noyce et al. 2018; McCarthy 2014), particularly due to the rapid decline of population immunity after the cessation of smallpox vaccination. The loss of cross-protection by smallpox-induced immunity also increases the danger of other orthopoxvirus infections. Consequently, other orthopoxviruses may emerge to pose significant threats to public health (Yang et al. 2021). This is manifested in the ongoing global mpox outbreak, with over 87,000 reported cases in more than 110 countries (~30,000 in the USA, from CDC, 2022 Outbreak Cases & Data, by May 09, 2023). The current mpox outbreak also underlines the pandemic potential of MPXV, of which future outbreaks are expected (Yang 2022; Rothenburg et al., 2022). The current outbreak is caused by MPXV clade II, a less severe/less transmissible clade than clade I that is endemic in central Africa (Likos et al., 2005). Other orthopoxviruses may also emerge to infect humans. For example, animals are believed to have transmitted a novel orthopoxvirus that infected four human individuals in Alaska in recent years (Department of Health and Social service, 2020, 2021; Springer et al., 2017). Such orthopoxviruses may evolve to adapt to human hosts over time and cause more serious concerns.

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FDA has approved two drugs for strategic stockpiling against smallpox: tecovirimat (Yang et al., 2005b) (**1**, Fig. 1) and brincidofovir (Florescu and Keck 2014) (BCV, **2**, Fig. 1), the lipid prodrug of nucleotide analog cidofovir (**3**, Fig. 1). However, the clinical efficacy of BCV against mpox is not promising (Carvalho 2022; Adler et al., 2022), and the clinical use of cidofovir for treating human cytomegalovirus is associated with severe adverse effects (Lea and Bryson 1996; Vandercam et al., 1999; Friedberg 1997) and drug resistance (Chou et al., 1997; Erice et al., 1997; Lurain and Chou 2010). Tecovirimat inhibits viral release by targeting the viral extracellular envelop protein VP37 (Grosenbach et al., 2018; Duraffour et al., 2015; Yang et al., 2005a). Although tecovirimat has shown promising efficacy in some mpox cases (Desai et al., 2022), clinical efficacy data are still very limited (O'Laughlin et al., 2022; Warner et al., 2022). Importantly, tecovirimat has a low barrier to viral resistance (FDA 2021; Yang et al., 2005a), and resistant mutants are expected after extensive use. In fact, resistance during the current mpox outbreak has been documented in severely immunocompromised patients who received prolonged administration of the drug (Alarcon et al., 2023). Therefore, it is critically important to develop new chemical entities against orthopoxviruses to provide valuable leads for rapid and effective countermeasures against re-emerging smallpox, mpox outbreaks, and other emerging orthopoxviruses. While many antiviral candidates against poxviruses have been identified in the past years (Wang et al., 2023; Siegrist and Sassine 2023), the majority of them have not been further characterized.

Vaccinia virus (VACV) is the prototype poxvirus and is a closely related surrogate to study highly pathogenic poxviruses (e.g., mpox and variola viruses) due to their >95% genome identity (Hendrickson et al., 2010). We previously developed a VACV-Gaussia luciferase reporter assay and screened a library comprising FDA-approved antiviral drugs and Selleck bioactives (Peng et al., 2020). Of the many hits identified, we have further characterized the antiviral activities of two nucleos(t)ide analogs: trifluridine and adefovir dipivoxil. They significantly inhibited VACV, cowpox virus (CPXV), and MPXV replication in physiologically relevant primary human foreskin fibroblasts (HFFs) without discernible cytotoxicity. Mechanistically, they both target the DNA replication stage of viral infection. Our findings reveal two strong candidates suitable for further development as antivirals against orthopoxviruses.

2. Materials and methods

2.1. Viruses and cells

Primary Human Foreskin Fibroblasts (HFFs) were obtained from Dr. Nicholas Wallace at Kansas State University. HFFs and E6 cells (ATCC-CRL-1586) were cultured in Dulbecco's Minimal Essential Medium (DMEM; Fisher Scientific). BS-C-1 cells (ATCC-CCL26) were cultured in Eagle's Minimum Essential Medium (EMEM). The EMEM or DMEM was supplemented with 10% fetal bovine serum (FBS: VWR), L-glutamine (2 mM, VWR), streptomycin (100 μg/mL, VWR), and penicillin (100 units/mL, VWR). Cells were cultured in an incubator with 5% CO_2 at 37 °C.

Vaccinia virus Western Reserve (WR, ATCC VR-1354) strain was propagated and purified by ultracentrifugation onto a sucrose cushion as described previously (Earl et al., 2001). MPXV-WA 2003–044 (Weiner et al., 2019), an MPXV-MA001 2022 isolate (GenBank: ON563414.3), CPXV (strain Brighton Red) were utilized in this study. Recombinant VACV expressing Gaussia luciferase under VACV early, intermediate, or late promoter vEGluc, vIGluc, and vLGluc were described previously (Pant et al. 2019). Preparation and infection of VACV and MPXV were carried out as described previously (Cotter et al., 2017; Hughes et al., 2017).

2.2. Titration of VACV, cowpox virus (CPXV) and MPXV by plaque assay

Titration of VACV, CPXV, and MPXV by plaque assay was carried out as described previously (Cotter et al., 2017). BS-C-1 (for VACV and CPXV) and E6 (for MPXV) cells were cultured in 6- or 12-well plates, infected with diluted virus samples, and incubated in culture medium (VACV and CPXV, EMEM, 2.5% FBS; MPXV, DMEM, 2% FBS) and 0.5–1% methylcellulose for 48 h–96 h. Cells were stained with 0.1% crystal violet for 15 min, followed by washing with water, and the number of plaques was counted. For MPXV, the staining solution contained >12% formalin.

2.3. Chemicals

Cytarabine (AraC) was purchased from Sigma-Aldrich. Brincidofovir (BCV), trifluridine, and adefovir dipivoxil were purchased from TargetMol.

2.4. Cell viability assay

Cell viability was measured by trypan blue staining or MTT assay. For the trypan blue staining assay, cells were cultured in the presence of DMSO or a specific compound at a desired concentration. Cells were then examined using trypan-blue exclusion as described elsewhere (Cao et al., 2017). The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide] assay was performed using an MTT assay kit (Cayman Chemical). Cells in 96-well plates were treated with DMSO or desired chemical inhibitors at different concentrations and incubated for the designated time. Ten μL of MTT reagent were added to each well and cells were incubated for 3 h. A 100 μL crystal resolving solution was added to each well and absorbance at 570 nm was measured using the citation 5 imaging reader (UV light) (Biotek) for each well after 18 h of incubation at 37 °C.

2.5. Determination of half maximal effective concentration (EC_{50})

HFFs were cultured in 96-well plates. The cells were infected with vLGluc at a multiplicity of infection (MOI) of 0.01 in the presence of DMSO or specific compound at a series of concentrations. Gluc activities were measured at 24 hpi. The EC_{50} was calculated using the following

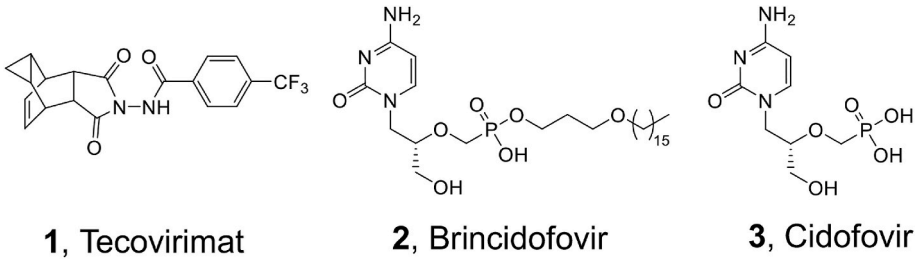


Fig. 1. Structures of FDA-approved smallpox drugs. Tecovirimat (**1**) inhibits viral release by targeting the envelop protein VP37. Brincidofovir (**2**) is the prodrug of Cidofovir (**3**) which targets viral DNA polymerase.

equation: log (inhibitor) vs. normalized response – variable slope in GraphPad Prism software (version 9.5.0).

2.6. Luciferase assay

Gaussia luciferase activities in culture medium were measured using a Pierce Gaussia Luciferase Flash Assay Kit (Thermo Scientific) using a GloMax Luminometer (Promega) according to the manufacturer's instructions.

Firefly luciferase activities for MPXV experiments were measured using an ENSPIRE plate reader (PerkinElmer, Waltham, MA) using the Luciferase Assay System (Promega, Madison, WI, United States) according to the manufacturer's instructions.

2.7. Quantitative real-time PCR

Total DNA was extracted using EZNA Blood DNA Kit. Relative viral DNA levels were quantified by CFX96 real-time PCR instrument (Bio-Rad, Hercules, CA) using All-in-oneTM 2 × qPCR mix (GeneCopoeia) with specific VACV primers against the C11 gene. 18 S rRNA gene primers were used as the internal reference.

2.8. Statistical analysis

All data were represented as the means of at least three independent experiments. Student's T-test was used to assess for significant difference between the two means with $P \leq 0.05$.

3. Results

3.1. Trifluridine and adefovir dipivoxil potently inhibit VACV and CPXV replication in primary HFFs

We have previously screened focused compound libraries for VACV inhibitors using a reporter VACV expressing Gaussia luciferase (Gluc) (vLGluc) in transformed HeLa cells, and have identified a number of strong hits (Peng et al., 2020), including several nucleos(t)ide analogs. To further confirm the antiviral effects of trifluridine (**4**, Fig. 2A) and adefovir dipivoxil (**5**, Fig. 2A), we tested their effects on VACV replication in primary HFFs by plaque assay, the gold standard method of infectious viral yield measurement. It is worth noting that dermal fibroblasts are physiologically relevant to orthopoxvirus infection as they are among the major cell types in poxvirus infection and dissemination (Lum et al., 2022). Under a multiplicity of infection (MOI) of 0.01 and 48 h infection incubation time, trifluridine and adefovir dipivoxil strongly suppressed VACV yield by ~9500- and 4500-fold, respectively, at 10 μM (Fig. 2B), without reducing the viability of HFFs (Fig. 2C). As a positive control, cytarabine (AraC, **6**, Fig. 2A), a well-studied compound that blocks poxvirus genome replication, also strongly suppressed VACV replication in HFFs by 24,000-fold (Fig. 2B and C). Trifluridine and adefovir dipivoxil also significantly suppressed CPXV replication at 10 μM in HFFs (Fig. 2D).

We next determined the half-maximal effective concentration (EC_{50}) of trifluridine and adefovir dipivoxil in inhibiting VACV replication in HFFs using the vLGluc. The vLGluc is a recombinant VACV expressing Gluc under a viral late promoter used in our initial screening of VACV inhibitors (Peng et al., 2020; Pant et al. 2019). We first evaluated if this assay is suitable for measuring VACV inhibitor's EC_{50} using BCV (Chan-Tack et al., 2021). With an MOI of 0.01 and 24 h infection incubation time, the EC_{50} of BCV was determined to be ~38 nM in HFFs (Fig. 3A and C), which is similar to reported values (Lanier et al., 2010; JW Huggins et al., 2002), suggesting that vLGluc is suitable for EC_{50} measurement. Using the same MOI and incubation time, we determined the EC_{50}s of trifluridine (EC_{50} = 138 nM) and adefovir dipivoxil (EC_{50} = 302 nM) (Fig. 3A and C). The EC_{50} of AraC was also determined (EC_{50} = 123 nM) (Fig. 3A). Remarkably, both trifluridine and adefovir dipivoxil, as well as the positive control compounds BCV and AraC, caused no significant cytotoxicity in HFFs at high concentrations ($CC_{50} > 250$ μM for trifluridine, AraC, and adefovir dipivoxil, $CC_{50} > 50$ μM for BCV) as measured in an MTT assay (Fig. 3B and C).

Together, the above results established that trifluridine and adefovir dipivoxil inhibit VACV with EC_{50}s at low nM and low cytotoxic effects in HFFs. Our results also further validated the Gluc expressing reporter VACV (vLGluc) as a valuable tool in poxvirus inhibitor identification and characterization.

3.2. Trifluridine and adefovir dipivoxil inhibit VACV genome replication

Poxvirus replication is divided into the following steps: entry, early gene expression, uncoating, DNA replication, intermediate gene

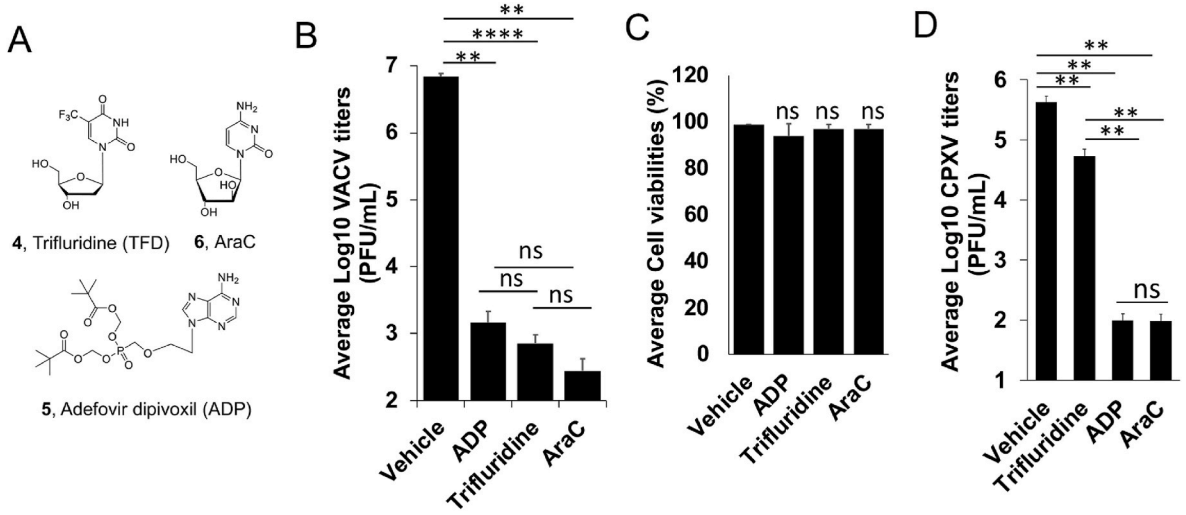
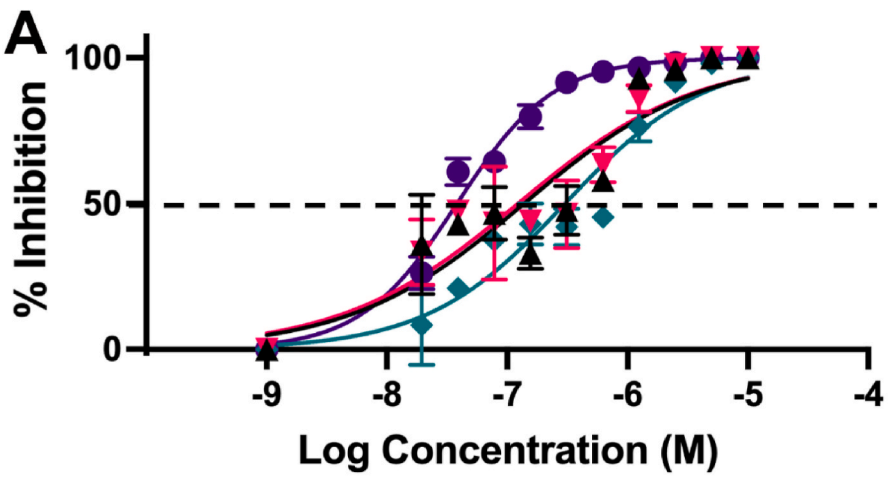
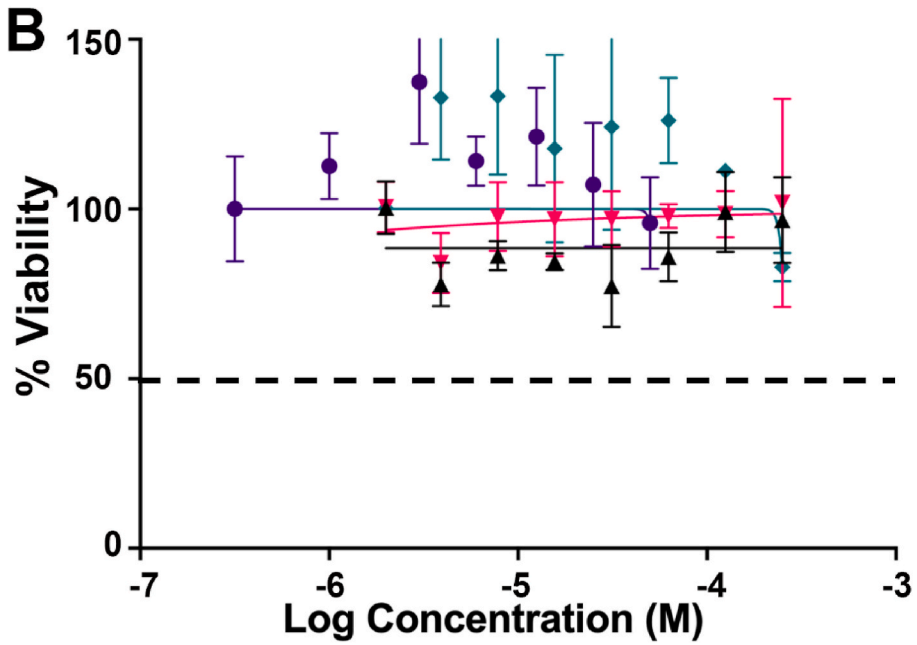


Fig. 2. Inhibition of VACV and CPXV replication by trifluridine and Adefovir dipivoxil (ADP) by plaque assay. (A) Structures of trifluridine (TFD, **4**), adefovir dipivoxil (ADP, **5**), and AraC (**6**). (**B**) HFFs were infected with VACV at an MOI of 0.01 and treated with indicated compounds at 10 μM for 48 h. Viral yields were titrated using a plaque assay using BS-C-1 cells. (**C**) HFF cell viability was measured 48 h after the cells were treated with indicated compounds at 10 μM. (**D**) HFFs were infected with CPXV at an MOI of 0.01 and treated with indicated compounds at 10 μM for 48 h. Viral yields were titrated using a plaque assay using BS-C-1 cells. The plotted values represent the means of at least three repeats. Error bars represent standard deviation. **0.001<p ≤ 0.01; ****0.00001<p ≤ 0.0001; ns, not significant.





C

Compound	EC_{50} (μM)	CC_{50} (μM)
Trifluridine	0.1382	>250
AraC	0.1229	>250
ADP	0.302	>250
Brincidofovir	0.0382	>50

Fig. 3. Measurement of EC_{50} and CC_{50} of indicated compounds in HFFs. (A) HFFs were infected with vLGluc at an MOI of 0.01 and treated with indicated individual compounds at a series of concentrations (or vehicle DMSO) for 24 h. Gluc activities were measured to determine the EC_{50}. **(B)** HFFs cell viability was determined by an MTT assay after incubation with indicated compounds at a series of concentrations for 24 h. **(C)** EC_{50} and CC_{50} of the compounds in A and B are shown. The plotted values represent the means of at least three repeats. Error bars represent standard deviation.

expression, late gene expression, and post-gene expression events such as viral morphogenesis, assembly, and spreading (Moss, 2013). Nucleos (t)ide analogs, e.g., trifluridine and adefovir dipivoxil, presumably inhibit VACV replication at the DNA replication stage. Subsequently, the post-DNA replication gene expression should also be affected due to the excessive need for RNA synthesis. To test this hypothesis, we first used recombinant VACVs with stage-specific Gluc reporter genes. In addition to the VACV encoding Gluc under the late F17R promoter (vLGluc), two other recombinant VACVs were also used: in one, the Gluc gene is under the control of the VACV early C11R (vEGluc) promoter, and in the other, it is under the control of the G8R intermediate (vIGluc) promoter (Pant et al. 2019). The C11R, G8R, and F17R genes are well-characterized, exclusively early, intermediate, and late VACV genes, respectively, and their promoters can be used to effectively distinguish stages of VACV gene expression (Yang et al. 2010, 2011). Neither trifluridine nor adefovir dipivoxil affected the Gluc expression under the VACV early C11R promoter (Fig. 4A), while they both strongly inhibited Gluc expression under intermediate G8R and late F17R promoters (Fig. 4B and C). The trends were similar to the AraC treatment (Fig. 4A–C).

As poxvirus intermediate and late gene expression depend on viral genomic DNA replication, we examined VACV DNA levels in the presence or absence of individual compounds. We found that trifluridine, adefovir dipivoxil, and the positive control AraC strongly reduced viral DNA levels by 32- to 114-fold (Fig. 4D), respectively. Together, these

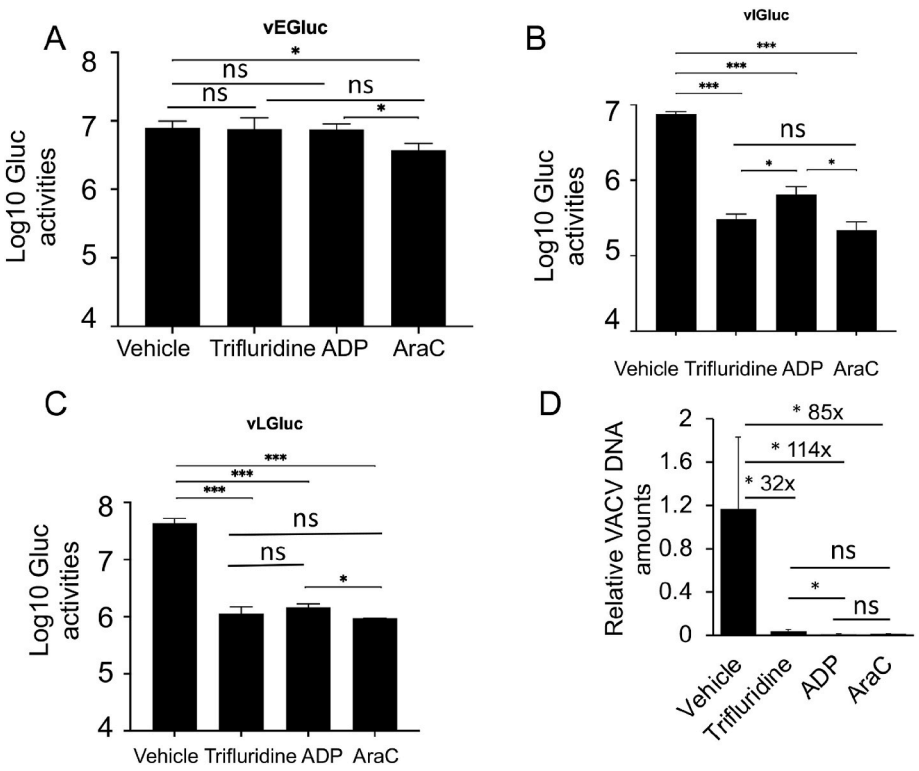


Fig. 4. Trifluridine and adefovir dipivoxil (ADP) suppress VACV DNA replication and post-replicative gene expression but not early gene expression. (A–C) HFFs were infected with vEGluc (**A**), vIGluc (**B**), and vLGluc (**C**) at an MOI of 2 and treated with indicated compounds at 10 μM, respectively, or vehicle DMSO. Gluc activities were measured at 4 h (vEGluc), 8 h (vIGluc), and 8 h (vLGluc), respectively. **(D)** HFFs were infected with VACV at an MOI of 2 in the presence of Indicated compounds at 10 μM for 8 h. Relative amounts of Viral DNA were determined by real-time PCR using VACV-specific primers. The plotted values represent the means of at least three repeats. Error bars represent standard deviation. *0.01<p ≤ 0.05; ***0.0001<p ≤ 0.001; ns, not significant. DMSO was used as the vehicle for the compounds.

results confirmed that trifluridine and adefovir dipivoxil function to restrict VACV DNA synthesis.

3.3. Trifluridine and adefovir dipivoxil significantly inhibit MPXV replication in primary HFFs

We used two methods to examine the effects of trifluridine and adefovir dipivoxil on MPXV replication. In one method, we used a WA strain MPXV-USA-2003-044 expressing firefly luciferase (Fluc) under a viral early/late promoter (luc + MPXV) gene as the reporter. We observed that trifluridine and adefovir dipivoxil strongly inhibited MPXV replication with similar potency to AraC (Fig. 5A). We also tested the inhibitory effects on an MPXV-MA001 2022 isolate by plaque assay and found that both trifluridine and adefovir dipivoxil significantly suppressed MPXV replication (Fig. 5B).

4. Discussion

Nucleos(t)ide analogs represent a main class of antiviral drugs (De Clercq and Li 2016; Jordheim et al., 2013), as exemplified by various herpesvirus inhibitors (Sadowski et al., 2021), a large panel of nucleos(t)ide reverse transcriptase inhibitors against HIV (Holec et al., 2017), hepatitis B virus (HBV) (Tavakolpour et al., 2018), hepatitis C virus (HCV) inhibitors (Sofia et al., 2017), and recently, SARS-CoV-2 (Beigel et al., 2020; Yu and Chang 2022; Xie et al., 2021; Zhang et al., 2021). Mechanistically, most nucleoside analogs act as chain terminators. Per this mechanism, the analogs are intracellularly converted to the active triphosphate (TP) form via monophosphate (MP) and diphosphate (DP) intermediates by the host or virally-encoded kinases. The TPs then compete against endogenous nucleoside triphosphates (NTPs) for incorporation by the viral polymerase. Once incorporated, these analogs act as chain terminators to stall viral genome replication. In cases where

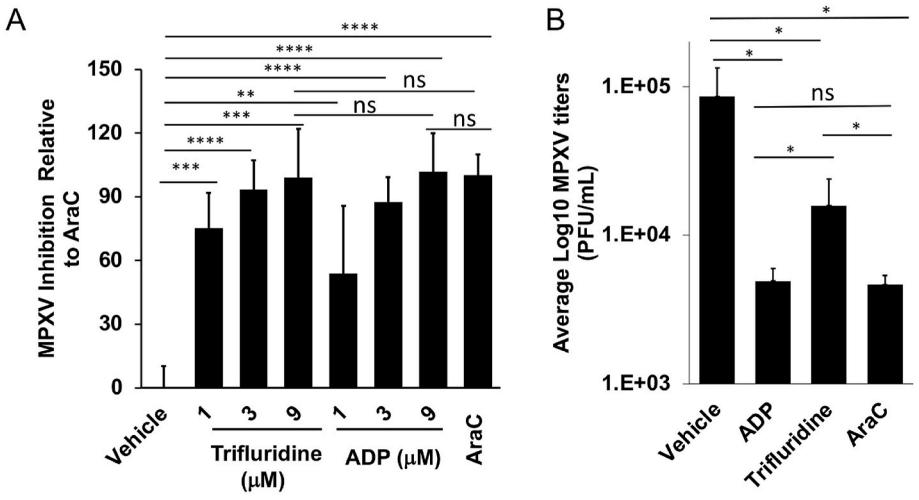


Fig. 5. Inhibition of MPXV replication by trifluridine and Adefovir dipivoxil (ADP). (A) HFFs were infected with MPXV-WA-2003-Fluc (luc + MPXV) under an early/late promoter (MOI = 0.01) and treated with indicated compounds at the indicated concentration for 24 h. Firefly luciferase activities were measured. The inhibition by AraC was normalized to 100. **(B)** MPXV-MA001 2022 isolate was added to cells at an MOI of 2 for 1 h. The virus was removed, cells were washed with PBS, and trifluridine or adefovir dipivoxil was added at 10 μM. Cells were harvested 24 h post-infection. AraC treatment was used as the positive control. Viral yields were titrated using a plaque assay on E6 cells. The plotted values represent the means of at least three repeats. Error bars represent standard deviation. *p ≤ 0.05; **0.001<p ≤ 0.01; ***0.0001<p ≤ 0.001; ****0.00001<p ≤ 0.0001; ns, not significant. DMSO was used as the vehicle for the compounds.

the intracellular conversion into MP, which is typically the rate-limiting step of nucleoside drug bioactivation, is inefficient, the MP is chemically installed to bypass kinase functions, constituting a mechanistically distinct nucleotide drug family. The FDA-approved smallpox drug BCV is a prodrug of the nucleotide drug cidofovir, which belongs to the acyclic nucleoside phosphonate (ANP) (De Clercq and Holy 2005) sub-class. Important antiviral drugs of this sub-class also include the reverse transcriptase inhibitors tenofovir (Naesens et al., 1998; Lyseng-Williamson et al. 2005), for treating HIV and HBV, and adefovir (Naesens et al., 1997; Dando and Plosker 2003), an HBV drug, typically administered in an ester prodrug form to overcome the low cell permeability. The two drugs characterized in this study, trifluridine, and adefovir dipivoxil, represent these two pharmacologically distinct classes of nucleos(t)ide drugs.

As an antiviral drug, trifluridine has long been approved for topically treating herpes simplex virus (HSV) infection of the eyes (keratoconjunctivitis) (Carmine et al., 1982). Interestingly, trifluridine has also been used to treat eye infections of VACV in humans and has been tested in rabbits for VACV keratitis (Parkhurst et al. 1976; Altmann et al., 2011), and mpox during the 2022 outbreak (Perzia et al., 2023). In addition, trifluridine is approved as a systemic drug to treat colorectal and gastric cancers. However, since trifluridine is highly labile toward degradation by thymidine phosphorylase (TP), it is used along with a TP inhibitor tipiracil in a combination setting for systemic cancer treatment (Burness and Duggan 2016). We report here its low nM potency inhibiting VACV infection in HFFs, and strong antiviral effect on MPXV replication.

Mechanistically, trifluridine can act as a chain terminator or antimetabolite (Fig. 6A), both requiring the intracellular conversion to trifluridine-MP (TFD-TP) (**7**, Fig. 6A) by thymidine kinase (TK). Under the chain termination mechanism, TFD-MP is further phosphorylated to TFD-TP (**8**, Fig. 6A) by thymidine monophosphate kinase (TMPK) and thymidine diphosphate kinase (TDPK). As a thymidine analog, TP (8, Fig. 6A) competes against the endogenous dTTP for incorporation by the viral DNA polymerase, leading to the termination of viral DNA. It is noteworthy that orthopoxviruses encode both TK and TMPK required for phosphorylating synthetic nucleoside analogs (Paoletti and Moss 1972; Kit et al. 1963; Moss, 2013), with substrate specificity partially overlapping with that of cellular kinases (Topalis et al., 2005; Caillat et al., 2008). Multiple lines of evidence suggest that in orthopoxvirus-infected

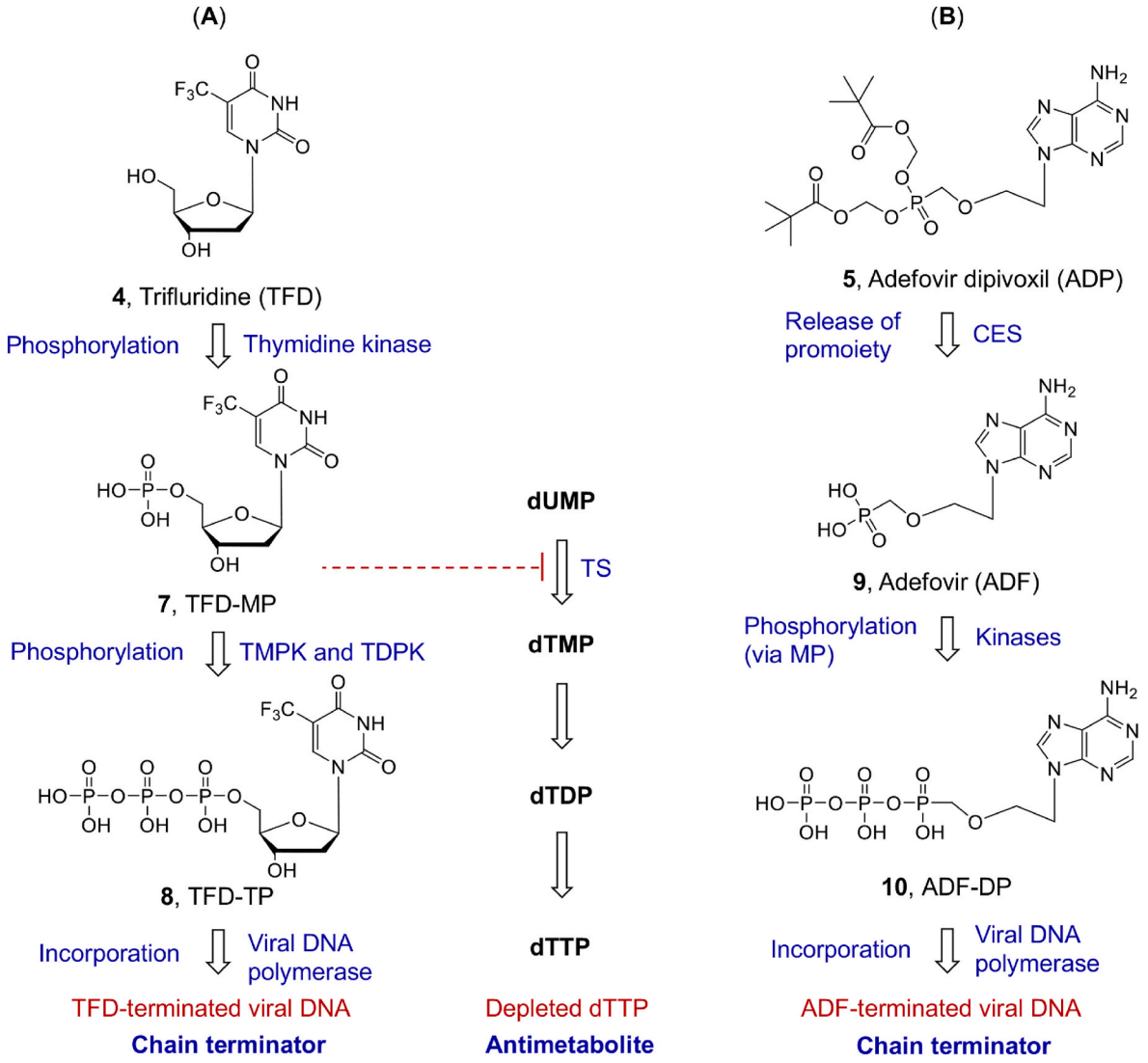


Fig. 6. Mechanisms of action of nucleoside analog trifluridine (TFD, 4) and nucleotide analog prodrug adefovir dipivoxil (ADP, 5). (A) TFD is intracellularly converted into TFD-MP by cellular or viral thymidine kinase, followed by two additional phosphorylation steps to yield TFD-TP. The incorporation of TFD-TP by viral DNA polymerase terminates viral DNA (chain terminator). Alternatively, TFD-MP inhibits thymidylate synthase (TS) to stall the conversion of dUMP to dTMP, ultimately depleting the cellular dTTP pool (antimetabolite); **(B)** Ester prodrug ADP is intracellularly converted into adefovir first under the action of carboxylesterase (CES). The subsequent successive phosphorylation produces the active ADF-DP. When incorporated, ADF-DP terminates the viral DNA (chain terminator).

cells, C5-modified nucleosides, trifluridine included, are likely phosphorylated by the viral kinases (Prichard et al., 2007). In cancer cells, trifluridine likely inhibits DNA synthesis via dual mechanisms (Fig. 6A): as a chain terminator; and as a dTTP antimetabolite in the form of TFD-MP (Temmink et al., 2007). Cellular dTTP anabolism critically entails the function of thymidylate synthase (TS), which converts dUMP to dTMP (Fig. 6A). TFD-MP inhibits thymidylate synthase and ultimately leads to the depletion of the cellular dTTP pool.

The other drug studied herein, adefovir dipivoxil (Dando and Plosker 2003), is a prodrug of ANP adefovir for HBV treatment. Upon cellular uptake, adefovir dipivoxil is cleaved by a cellular carboxylesterase (CES) to release the ester promoiety and generate Adefovir (**9**, Fig. 6B). This is followed by two successive phosphorylation by AMP kinase to produce the active adefovir-DP (Naesens et al., 1997) (**10**, Fig. 6B). By competing against cellular dATP, adefovir-DP is incorporated by the viral DNA polymerase, and subsequently causes an obligate chain termination. While adefovir dipivoxil was a hit from previous antiviral screening against poxviruses (Peng et al., 2020; Kern 2003), it has not been used to treat poxvirus infection in humans.

Against VACV and MPXV replication in HFFs, both trifluridine and adefovir dipivoxil showed potent inhibition of VACV with EC_{50}s in the nM range without discernible cytotoxicity (CC_{50} > 250 μM). Interestingly, it has been reported that trifluridine is quite toxic when given systemically due to its effect on cellular DNA synthesis (Lee and Chu 2017). The lack of cytotoxicity in cultured HFFs observed suggests that further characterizations with additional cell lines and possibly *in vivo* testing are needed to better understand the toxic effects of the compound. Nonetheless, these results validate both drugs as viable candidates for further investigation as potential anti-MPXV and other orthopoxvirus drugs. The successful repurposing of adefovir dipivoxil will add to the already approved BCV to further enhance ANP prodrugs as an important drug class for treating poxvirus infections. In addition, nucleoside analog trifluridine as a poxvirus drug candidate will introduce a mechanistically distinct drug class and expand the options for synergistic combination therapies with ANPs or tecovirimat.

In this study, we also validated the utility of the Gluc expression VACV under a late promoter F17R (vLGluc) by measuring Gluc activities in the media to determine EC_{50} of compounds in poxvirus drug research. Because Gluc is secreted into the medium (Tannous and Teng 2011), this assay is a rapid, non-disruptive, and highly simplified VACV replication reporter with an exceptionally high Signal-to-Basal ratio (Peng et al., 2020). This reporter VACV is suitable for high-throughput screening, as shown in our previous study (Peng et al., 2020), which will facilitate our future antiviral research against poxviruses.

5. Conclusion

In summary, we characterized the antiviral profiles of nucleoside analog trifluridine and acyclic nucleoside phosphonate adefovir dipivoxil against VACV, CPXV, and MPXV in primary fibroblasts. Further testing in animal models will determine their *in vivo* anti-MPXV and other orthopoxvirus potential. Chemical modification of the compounds may also improve their potency, pharmacokinetic (PK), and safety profiles.

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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.

Data availability

Data will be made available on request.

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Glossary

VACV	vaccinia virus
MPXV	mpox (monkeypox) virus
CPXV	cowpox virus
HBV	hepatitis B virus
HCV	hepatitis C virus
AraC	cytarabine
BCV	brincidofovir
ADP	adefovir dipivoxil
ADF	adefovir
TFD	trifluridine
TMPK	thymidine monophosphate kinase
TDPK	thymidine diphosphate kinase
TK	thymidine kinase
ANP	acyclic nucleoside phosphonate
NTP	nucleoside triphosphate
Gluc	Gaussia luciferase
EC_{50}	half maximal effective concentration
CC_{50}	50% cytotoxic concentration

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