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Broad-spectrum non-nucleoside inhibitors of human herpesviruses

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A B S T R A C T

Herpesvirus infections cause considerable morbidity and mortality through lifelong recurrent cycles of lytic and latent infection in several tissues, including the human nervous system. Acyclovir (ACV) and its prodrug, the current antivirals of choice for herpes simplex virus (HSV) and, to some extent, varicella zoster virus (VZV) infections are nucleoside analogues that inhibit viral DNA replication. Rising viral resistance and the need for more effective second-line drugs have motivated searches for additional antiviral agents, particularly non-nucleoside based agents. We evaluated the antiviral activity of five compounds with predicted lysosomotropic activity using conventional and human induced pluripotent stem cell-derived neuronal (iPSC-neurons) cultures. Their potency and toxicity were compared with ACV and the lysosomotropic agents chloroquine and bafilomycin A1. Out of five compounds tested, micromolar concentrations of 30N12, 16F19, and 4F17 showed antiviral activity comparable to ACV (50 μM) during lytic herpes simplex virus type 1 (HSV-1) infections, reduced viral DNA copy number, and reduced selected HSV-1 protein levels. These compounds also inhibited the reactivation of 'quiescent' HSV-1 infection established in iPSC-neurons, but did not inhibit viral entry into host cells. The same compounds had greater potency than ACV against lytic VZV infection; they also inhibited replication of human cytomegalovirus. The anti-herpetic effects of these non-nucleoside agents merit further evaluation *in vivo*.

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1. Introduction

Herpesvirus infections affect the vast majority of adults world-wide, with rates exceeding 95% for human cytomegalovirus (HCMV) and varicella zoster virus (VZV), and up to 60% for herpes simplex virus type I (HSV-1) (Cannon et al., 2010; Xu et al., 2006). These viruses can infect an array of human tissues including the peripheral and/or central nervous system (CNS), causing severe CNS disease (Schmutzhard, 2001; Steiner et al., 2007). Currently, the list of FDA approved antiviral drugs is limited, particularly for HCMV or VZV (Strasfeld and Chou, 2010), moreover, the available drugs can produce serious adverse effects. Acyclovir (ACV) or its prodrug valacyclovir (VACV) are the most widely used agents for HSV-1 and VZV. Both drugs are processed to nucleoside analogues that are selectively activated in virus-infected cells and then block viral DNA replication. Resistance to ACV can develop from mutations in the viral thymidine kinase and/or DNA polymerase (Burrel et al., 2013), with incidence rates of 0.3–7.1% (Stránská et al., 2005; Malvy et al., 2005). Though some new antiherpetic drugs target the viral helicase and primase, viral resistance to these agents has also been reported (Kleymann et al., 2002; Sukla et al., 2010). In addition, nephrotoxicity can occur following prolonged ACV treatment (Izzedine et al., 2005). Moreover, ACV is not

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clinically effective against HCMV. There is thus a compelling need to search for additional drugs to treat herpes virus infections.

Here, we report on novel anti-herpetic compounds that are based on non-nucleoside agents initially proposed to have lysosomotropic activities. Our studies were motivated by earlier reports indicating the efficacy of lysosomotropic drugs like chloroquine (CQ) and bafilomycin A1 (BFLA) in inhibiting HSV-1 infections (Harley et al., 2001). Lysosomotropic agents increase intracellular pH and presumably inhibit viral packaging and maturation through the *trans*-Golgi network, although their precise mechanisms of action against herpesviruses remain unclear (Nieland et al., 2004). In the present study, the *in vitro* efficacy of selected agents for HSV-1, VZV, and CMV infections was tested. We focused particularly on HSV-1, including analyses of lytic infection, as well as a model of 'quiescent' infection in human iPSC-derived neuronal cells that mimics several aspects of HSV-1 latent infections (D'Aiuto et al., 2014). We report on three promising agents that appear to have a broader spectrum of activity compared with ACV.

2. Materials and methods

2.1. Drugs

Compounds 30N12, 16F19, 4F17 (95% purity), 16D20, and 17G7 (90% purity) were purchased from ChemBridge. CQ (⩾98% purity), phosphonoacetic acid (PAA; ⩾98% purity), and BFLA (⩾90% purity) were purchased from Sigma. ACV (⩾98% purity) was purchased from Spectrum Chemical Mfg Corp. The chemical structure of the compounds is depicted in Fig. 1.

2.2. Cell lines and viral constructs

Vero cells were maintained in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS; HyClone) and 5% antibiotic/antimycotic (HyClone). VZV permissive human retinal pigmented epithelial cells (ARPE-19) were maintained in Dulbecco's modified Eagle medium/F12 (DMEM/12) supplemented with 10% FBS (HyClone) and 5% antibiotic/antimycotic (HyClone). Human foreskin fibroblasts (HFF; ATCC, CRL-2088) were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% FBS (HyClone). Human iPSC lines 73-56010-02 and HFF1-S were differentiated into functional neurons (iPSC-neurons) as described (D'Aiuto et al., 2012). All cells were grown at 37 °C, 5% CO_2, and 100% humidity.

The HSV-1 strain was based on the KOS virus (VR-1493; ATCC). This recombinant virus expresses the enhanced green fluorescent protein (EGFP) from the ICP0 promoter, and the monomeric red fluorescent protein (RFP) from the glycoprotein C promoter (Ramachandran et al., 2008). The VZV construct, derived from an infectious Bacterial Artificial Chromosome of the whole VZV genome of the Parent of Oka strain, was engineered to express the luciferase reporter as a fusion to the VZV ORF9 protein, as described recently (Bayer et al., 2015). The human cytomegalovirus (HCMV) recombinant virus, derived from the Towne strain, was engineered to express the luciferase under the control of the viral pp28 gene promoter (He et al., 2011).

2.3. Viral infections

To test the effects of the compounds during acute HSV-1 infection, cell-free virus was adsorbed onto cells for 2 hours (h) at a multiplicity of infection (MOI) of 1.0 for Vero cells and 0.3 for iPSC-neurons. The inocula were removed, cells were washed with PBS, and medium was exchanged. Compounds 30N12, 16F19, 4F17, 16D20, and 17G7 (Nieland et al., 2004) were added 2 h post infection (hpi) at the concentrations indicated. Parallel cultures were pretreated for 24 h with CQ (50 μM) or BFLA (0.1 μM; (Joubert et al., 2009). As positive controls, cultures were pretreated with ACV (50 μM; D'Aiuto et al., 2014) for 24 h or pretreated with PAA, a HSV-1 DNA polymerase inhibitor (300 μg/ml; Blaho et al., 1993) for 1 h prior to the infection.

We tested effects on HSV-1 attachment to the host cell membrane and host cell entry using a published protocol (Krepstakies et al., 2012; Supplementary Fig. S6).

To test the effect of the compounds on the HSV-1 reactivation, quiescence was established using (E)-5-(2-bromovinyl)-2′-deoxyuridine + interferon alpha (5BVdU + IFN-α; 30 μM, 125 U/ml, respectively) for 7 days as reported (D'Aiuto et al., 2014). After this period, the quiescence-inducers were withdrawn, and iPSC-neurons were cultured for 48 h in the presence or absence of the test compounds. HSV-1 infection was then reactivated by treatment with sodium butyrate (NaB; 5 mM) for 5 days (D'Aiuto et al., 2014). The number of cells expressing EGFP and RFP was determined by flow cytometry (FC) on Fortessa FACS analyzer (Becton Dickinson).

Inhibition of VZV was assessed on ARPE-19 cells, inoculated with 1000 PFU/well of a cell-associated high titer VZV stock for 2 h at 37 °C, in which >70% of cells were infected and infectious. Small molecules were added in the same manner as just detailed, and luciferase activities were assayed 48 hpi.

Inhibition of HCMV was evaluated by cell-free virus infection adsorbed on HFF cells for 90 min at MOI of 1.0, at which point, small molecule inhibitors were added in a manner as just detailed. Ganciclovir (5 μM) was used as control in all experiments. Luciferase activities were assayed 72 hpi.

2.4. qPCR assays for viral DNA

The EGFP sequence from the genetically engineered HSV-1 was utilized as the target for copy number analysis. Copy number qPCR was performed as previously reported (Kaufman et al., 2005), employing an EGFP plasmid standard curve, using 5 ng total DNA with biological and technical triplicates, along with corresponding non-template controls. The probe master mix had final concentrations of 18 μM EGFP forward primer (5′-ccacatgaagcagcacgactt-3′), 18 μM EGFP reverse primer (5′-ggtgcgctcctggacgta-3′), and 5 μM probe (5′-6FAM-ttcaagtccgccatgcccgaa-TAMRA-3′). The reaction conditions were 95 °C for 3 min followed by 45 cycles of 95 °C for 15 s and 55 °C for 30 s, and finished with 72 °C for 30 s. All qPCRs were conducted on a 7900HT Fast Real-Time PCR System (Applied Biosystems) and analyzed with manufacturer's software SDS 2.4.

2.5. Cytotoxicity assay

The viability of iPSC-neurons following exposure for 24 h at different concentrations of compounds 30N12, 16F19, and 4F17 was assayed by FC using the LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (Life Technologies). The cytotoxicity analysis was also performed in ARPE-19 cells as described for the iPSC-neurons, but the cells were incubated with the drugs for 48 h instead of 24 h. The drug-induced cytotoxicity in HFF cells used for HCMV infection was estimated using CellTiter-Glo Assay Kit following incubation with the drugs for 72 h.

2.6. Statistical analyses

Statistical analyses were conducted using the open source software R statistical package version 3.0.1, IBM SPSS v21, GraphPad Prism v5.02, and SigmaPlot. The drug concentration that reduced the number of fluorescent cells in cultures infected with HSV-1 or VZV by 50% (IC50) was estimated using the *drc* package in R

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