Broad anti-herpesviral activity of α-hydroxytropolones

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ABSTRACT

Herpesviruses are ubiquitous in animals and cause economic losses concomitant with many diseases. Most of the domestic animal herpesviruses are within the subfamily *Alphaherpesvirinae*, which includes human herpes simplex virus 1 (HSV-1). Suppression of HSV-1 replication has been reported with α-hydroxytropolones (αHTs), aromatic ring compounds that have broad bioactivity due to potent chelating activity. It is postulated that αHTs inhibit enzymes within the nucleotidyltransferase superfamily (NTS). These enzymes require divalent cations for nucleic acid cleavage activity. Potential targets include the nuclease component of the herpesvirus terminase (pU$_L$15C), a highly conserved NTS-like enzyme that cleaves viral DNA into genomic lengths prior to packaging into capsids. Inhibition of pU$_L$15C activity in biochemical assays by various αHTs previously revealed a spectrum of potencies. Interestingly, the most potent anti-pU$_L$15C αHT inhibited HSV-1 replication to a limited extent in cell culture. The aim of this study was to evaluate three different αHT molecules with varying biochemical anti-pU$_L$15C activity for a capacity to inhibit replication of veterinary herpesviruses (BoHV-1, EHV-1, and FHV-1) and HSV-1. Given the known discordant potencies between anti-pU$_L$15C and HSV-1 replication inhibition, a second objective was to elucidate the mechanism of action of these compounds. The results show that αHTs broadly inhibit herpesviruses, with similar inhibitory effect against HSV-1, BoHV-1, EHV-1, and FHV-1. Based on immunoblotting, Southern blotting, and real-time qPCR, the compounds were found to specifically inhibit viral DNA replication. Thus, αHTs represent a new class of broadly active anti-herpesviral compounds with potential veterinary applications.

1. Introduction

Herpesviruses are a major cause of morbidity, mortality, and economic loss in domestic animal species. Bovine herpesvirus 1 (BoHV-1) is a geographically widespread cause of respiratory disease, conjunctivitis, genital lesions, and abortion in cattle (Biswas et al., 2013). Similarly, equine herpesvirus 1 (EHV-1) is widely prevalent, primarily causing sporadic or epizootic abortions and neonatal death, upper respiratory tract infections and neurological disease in horses (Dunowska, 2014). The effects of herpesviruses extend into companion animals, as disease induced by feline herpesvirus 1 (FHV-1) approaches 100% morbidity in felids, particularly in shelter or cattery populations (Gould, 2011). In addition to their veterinary importance, human herpesvirus infections are considered a worldwide pandemic, and the prevalence is increasing (Koelle and Corey, 2008). Management of veterinary herpesvirus infection usually relies on prevention and control

(Dunowska, 2014). For example, clinical trials assessing the efficacy of oral and ophthalmologic anti-FHV-1 therapy in cats with FHV-1 revealed improved clinical signs with limited adverse effects (Fontenelle et al., 2008; Gould, 2011; Thomasy et al., 2011; Thomasy et al., 2016). In people, most anti-herpesviral therapeutics target the viral DNA polymerase, in which acquired mutations and subsequent viral resistance are a major limitation, especially in patients undergoing prolonged therapy (Collins and Darby, 1991; Gable et al., 2014; Krawczyk et al., 2013). Thus, a great need exists for novel anti-herpesviral compounds in both medical and veterinary medicine.

Herpesviruses encode several well-conserved enzymes within the nucleotidyltransferase superfamily (NTS) that contain a ribonuclease H (RNase H)-like fold. In HSV-1, these enzymes include the single stranded DNA binding protein (ICP8), alkaline nuclease (pU$_L$12), DNA polymerase, and the nuclease of the viral terminase (pU$_L$15C) (Boehmer and Lehman, 1997; Bryant et al., 2012; Liu et al., 2006; Schumacher

et al., 2012; Selvarajan Sigamani et al., 2013; Yan et al., 2014). Given the enzymes' similar structure, inhibitors of the HIV RNase and integrase were recently screened for activity against HSV-1 and HSV-2 (Tavis et al., 2014). Tropolones, most notably natural and synthetic α-hydroxytropolones (αHTs), were found to be potent anti-HSV inhibitors (Ireland et al., 2016; Tavis et al., 2014). While tropolones are well known metalloenzyme inhibitors due to the high negative charge character on both the carbonyl and adjacent oxygen at physiological pH, αHTs provide an additional contiguous oxygen that makes them particularly good inhibitors of dinuclear metalloenzymes (Bentley, 2008; Piettre et al., 1997). To investigate the nature of αHT HSV-1 inhibitory activity, one specific HSV-1 NTS-like enzyme, pU$_L$15C of the viral terminase, was assessed in the presence of several synthetic β-thujaplicinol derivatives through the analysis of pU$_L$15C-mediated hydrolysis of short DNA duplexes using dual-probe fluorescence (Masaoka et al., 2016). Interestingly, some of the most potent pU$_L$15C synthetic αHTs inhibited HSV-1 replication only modestly, while those that strongly inhibited HSV-1 replication had poor anti-terminase activity (Ireland et al., 2016; Masaoka et al., 2016). It follows that although αHTs have anti-pU$_L$15C activity in biochemical assays, other essential viral activities are likely responsible for their antiviral effects.

Using αHTs with varying anti-HSV replication and biochemical anti-terminase potency, the goals of this research were to determine whether αHTs have broad herpesviral inhibitory activity against important veterinary herpesviruses, including BoHV-1, EHV-1, and FHV-1, and to determine the mechanism of action of these αHTs using HSV-1 as a model.

2. Materials and methods

2.1. Compound selection

Three αHTs, numbered 106, 111, and 115, were synthesized from kojic acid as previously described (Hirsch et al., 2014; Meck et al., 2012; Williams et al., 2013). The chemical structure of each compound is detailed in Fig. 1. The 50% inhibitory activities (IC$_{50}$) against pU$_L$15C for the three synthetic αHTs have been previously measured and include 0.18 μM for αHT-106, 5.6 μM for αHT-111, and 49.1 μM for αHT-115 (Ireland et al., 2016; Masaoka et al., 2016). Similarly, the *in vitro* anti-HSV potencies of the three synthetic αHTs were previously determined. Full suppression of HSV-1 was noted at 50 μM for αHT-106 and at 5 μM for αHT-111. The 50% effective concentration (EC$_{50}$) against HSV-1 was 0.18 μM for αHT-115 (Ireland et al., 2016; Masaoka et al., 2016). Lastly, the 50% cytotoxic concentration (CC$_{50}$) for two of the compounds, determined in Vero cells, was previously calculated as > 50 μM for αHT-111 and > 100 μM for αHT-115 (Ireland et al., 2016; Masaoka et al., 2016). All compounds were dissolved in DMSO, aliquoted, and stored at −20 °C.

2.2. Cells and viruses

African green monkey kidney epithelial cells (CV-1) and bovine kidney epithelial cells (MDBK) were maintained in DMEM growth medium containing 10% newborn calf serum and 100 IU/ml penicillin-0.1 mg/mL streptomycin. Crandell feline kidney epithelial cells (CRFK)

Fig. 1. The chemical structure of the three synthetic αHTs compounds used in this study.

were maintained in DMEM growth medium containing 10% fetal bovine serum and 100 IU/ml penicillin-0.1 mg/mL streptomycin. The following strains of virus were used: HSV-1 wild-type strain F, U$_L$15-null mutant virus (Baines et al., 1997), BoHV-1 Cooper strain, EHV-1 strain 10N0148, and FHV-1 strain FH2CS. Virus stocks were grown and titered by plaque assay on permissive cell monolayers. These included CV-1 for HSV-1, MDBK for BoHV-1 and EHV-1, and CRFK for FHV-1. All virus and cell stocks were stored in −80 °C.

2.3. Cytotoxicity assay

One mammalian cell line, MDBK cells, served as a representative sample. MDBK cells were seeded into 12-well plates and incubated in DMEM growth medium as previously described until 80% confluency was reached (∼24 h). αHT compounds were added to 1 ml of growth medium to achieve final concentrations of 100 μM, 125 μM, 150 μM, and 175 μM and applied to the cells in duplicate. The cytotoxic agent sodium azide (Sigma-Aldrich) was added to one well (in duplicate) at 300 μM to serve as a positive control. DMSO at the highest concentration served as the negative control. After 24-h incubation, 200 μl of a tetrazolium compound [3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] and an electron coupling reagent (phenazine ethosulfate; PES) (Promega) were added to each well with an additional 2-h incubation. The absorbance at 490 nm was recorded and the survival rate was calculated, using the following equation: Absorbance $_{sample}$/Absorbance $_{control}$ x 100. The 50% cytotoxic concentration values were calculated using GraphPad Prism. The concentrations of the compounds were log transformed. Non-linear regression, via the four-parameter variable-response log(inhibitor)-versus-response algorithm, was employed to determine the 50% cytotoxic concentration.

2.4. HSV-1, boHV-1, EHV-1, and FHV-1 replication inhibition assays

HSV-1, BoHV-1, and FHV-1 were added to an appropriate permissive cell line (CV-1, MDBK, and CRFK, respectively) at a multiplicity of infection (MOI) of 0.01 for 1 h. EHV-1 was added to a permissive cell line (MDBK) at MOI 0.1 for 1 h. After a 1-h adsorption period, the virus-inoculated medium was removed and the cells were washed once with PBS. Medium was replaced with DMEM containing either DMSO (at the highest concentration) or various concentrations of αHT (10 μM to 50 μM for compounds 106 and 111 or 2 to 10 μM for compound 115) for an additional 47 h. At 48 h post-infection, the cells were assessed by phase-contrast microscopy for cytopathic effect. Samples were frozen at −80 °C, thawed, removed from the dish by scraping, and sonicated to release intracellular virus. Infectious virus was measured by plaque assay on permissive cells, performed once at each concentration for each virus. The activity of the compounds was compared by assessing the degree of viral replication inhibition at 10 μM for the three αHTs.

2.5. Transmission electron microscopy (TEM)

BoHV-1 was added at an MOI of 1.0 PFU/cell to a ∼90% confluent monolayer of MDBK cells in a 6-well plate. After 1 h incubation, the virus-inoculum was removed and replaced with DMEM containing one of the following: DMSO (at the highest concentration), 50 μM αHT-106, or 8 μM αHT-115 for an additional 13-h incubation. Fixative was then added to each sample to a final concentration of 2% formaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer pH of 7.4. After 10 min, the cells were removed by scraping, pelleted by centrifugation, and subjected to another round of fixation, resuspension, and centrifugation. The pellet was mixed with an equal amount of 3% agarose and once solidified, cut into cubes and placed in 0.1 M phosphate buffer pH 7.4. The sample was washed with 0.1 M phosphate buffer and 0.08 M glycine for 15 min, followed by fixation in 2% osmium tetroxide in 0.1 M phosphate 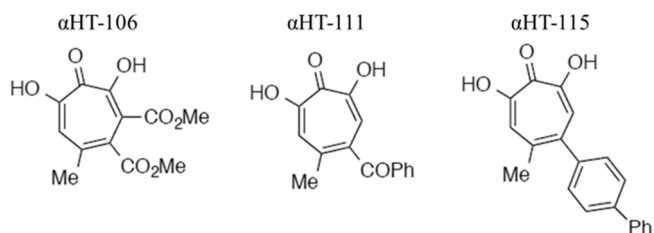buffer pH 7.4 in the dark for 1 h. The samples were