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EVALUATION OF BROAD ANTI-HERPESVIRAL ACTIVITY WITH

α-HYDROXYTROPOLONES

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
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by
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B.Sc., Louisiana State University 2009
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For my tenacious father, resilient mother, and four hysterical siblings.

Thank you for paving the way.

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Whether I am in tears from laughter or sadness, there is always one person who is a phone call or text message away - my loving sister, best friend, and soul mate, Carrie David. You have been by my side for every failure and accomplishment. Your love is unwavering; my life will always be fulfilled with you in it.

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ABSTRACT

Herpesviruses are ubiquitous in animals and cause economic losses concomitant with many diseases, including upper respiratory disease, keratitis, abortion, neonatal death, and neurologic disease. The majority of the domestic animal herpesviruses are within the subfamily *Alphaherpesvirinae*, along with the prototypical human herpes simplex virus 1 (HSV-1). Suppression of HSV-1 replication has been reported with α-hydroxytropolones (αHTs), which are aromatic ring compounds that have broad bioactivity due to potent chelating activity. It is postulated that αHTs inhibit enzymes within the nucleotidyltransferase superfamily (NTS), similarly structured enzymes that require divalent cations for nucleic acid cleavage activity. One potential herpesviral target includes the nuclease of the viral terminase, a highly conserved NTS-like enzyme that cleaves the viral genome for packaging into capsids. Inhibition of the nuclease activity of the viral terminase (pU_L15C) by αHTs previously revealed variable potencies, ranging from negligible to marked. Interestingly, the most potent anti-terminase nuclease αHT compounds had limited effect on inhibiting HSV-1 replication. The aim of this study was to evaluate three different αHT molecules with varying *in vitro* anti-terminase nuclease activity against veterinary herpesviruses (BoHV-1, EHV-1, FHV-1) and HSV-1 to assess for broad inhibitory activity. Additionally, given the discordant potencies between anti-pU_L15C and HSV-1 inhibition, a second objective was to elucidate the mechanism of action of these compounds. The results of this research show that αHTs broadly inhibit herpesviruses, with similar inhibitory effect among HSV-1, BoHV-1, EHV-1, and FHV-1 with IC_{50} values ranging from 30 to ≤ 5 μM. Based on immunoblotting, Southern blotting, and real-time qPCR, the compounds were found to specifically inhibit DNA replication. Thus, αHTs may represent a new class of anti-herpesviral compounds.

CHAPTER 1: INTRODUCTION

The family *Herpesviridae* is composed of double stranded DNA (dsDNA) viruses that are both disseminated in the environment and cause disease in an array of vertebrate and invertebrate hosts. The ubiquity of herpesviruses is largely due to their ability to undergo both a lytic and latent replicative cycle, as latency allows for recurrent infections and transmission. Additionally, some herpesviruses have adapted mechanisms of immune evasion, including the production of homologs to chemokines and ability to spread among contiguous cells without exposure to extracellular antibody [1-3]. These features of latency and immune evasion largely explain the high morbidity of herpesviral infections.

The family is subdivided into three subfamilies based on cytotropism, replication properties, and cytopathogenicity and includes *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae*. Alphaherpesviruses have short replicative cycles, grow rapidly in cell culture, and are neurotropic and cytopathogenic. Important human and veterinary alphaherpesviruses include human simplex virus 1 (HSV-1), bovine herpesviruses 1 (BoHV-1), equine herpesvirus 1 (EHV-1), and feline herpesvirus 1 (FHV-1). Betaherpesviruses cause cytomegaly and have limited host ranges. These viruses are myelotropic with protracted replication and infection, and examples include human cytomegalovirus (HCMV) and suid herpesvirus 2 [4, 5]. Lastly, the gammaherpesviruses exhibit tropism for lymphocytes with variable replicative rates. The gammaherpesviruses have oncogenic potential, as observed with the development of lymphoma in humans infected with human Epstein–Barr virus. Veterinary gammaherpesviruses include bovine herpesvirus 4 and equine herpesvirus 2. Interestingly, there are also a few reports of oncogenic gammaherpesviruses in animals [6-8].

From a veterinary perspective, herpesviruses are a major cause of morbidity, mortality, and economic losses in domestic animal species. Both BoHV-1 and EHV-1 are geographically widespread and have high morbidity in outbreak situations. Major economic losses from both viruses are due to abortions and poor performance in racing horses, and poor growth and milk production in feedlot cattle, and dairy cattle, respectively [9, 10]. The effects of herpesviruses extend into companion animals, as FHV-1 has close to 100% morbidity in felids, particularly in shelter or cattery populations. Persistent infections and recrudescence of disease are common in cats, and management and treatment of severe disease may be costly [11]. Management of veterinary herpesviral infection relies heavily on prevention and control through vaccination, quarantining, and population separation based on disease risk [12]. The efficacy of anti-herpesviral drugs in large animals remains undetermined [13-15]. However, clinical trials assessing the efficacy of oral and ophthalmologic anti-herpesviral therapeutics in cats with FHV-1 revealed improved clinical signs with limited adverse effects [11, 16-18].

BoHV-1 is also known as infectious bovine rhinotracheitis or infectious pustular vulvuovaginitis virus and is a geographically widespread cause of respiratory disease, conjunctivitis, genital lesions, and abortion in the cattle industry. Clinical signs include pyrexia and mucopurulent oculonasal discharge, often with minimal to marked fibrinonecrotic tracheitis. Secondary bacterial infections are common, leading to bronchopneumonia and an increased severity of disease [19]. The virus has a wide host range, infecting cattle, sheep, goats, llamas, swine, and rabbits; however, clinical disease has only been reported in cattle [4]. BoHV-1 is transmitted from (sub)clinically infected cattle or reservoir hosts by aerosolization, contact with mucosal secretions, or indirect contact with fomites. Clinical disease is caused by lytic infection, and as with other alphaherpesviruses, latency can be established in sensory ganglia [4].

Reactivation of BoHV-1 is common in livestock and can be induced by endogenous corticosteroids secondary to the stress of high stocking densities, transport, pregnancy, poor management or diet, or secondary infections [20].

EHV-1 is widely prevalent, primarily causing sporadic or epizootic abortions and neonatal death, upper respiratory tract infections and neurological disease [12]. Viremia depends on the strain of the virus and prior exposure of the host [4]. Two strains of EHV-1 have been described and include the low (V592) and high (Ab4) virulence strains. V592 is commonly associated with abortion, while Ab4 causes neurologic disease. Although there are marked differences in their clinical presentation, the nucleotide variation rate between these two strains is only 0.1%. One important difference between the two strains is a single nucleotide polymorphism in the viral DNA polymerase. A substitution of aspargine (N) to aspartic acid (D) at amino acid position 752 is associated with neuropathogenicity [10, 21, 22]. Aerosolization, ingestion of abortive material, and exposure via fomites are common modes of transmission. The virus invades the epithelial mucosa and infects T-lymphocytes, leading to viremia and widespread endothelial infection. Vascular changes, such as vasculitis, thrombosis, and ischemia, are common. In fact, the pathogenesis of abortion is initiated by these vascular changes, as thrombosis of vessels within the endometrium ultimately leads to infiltration and spread of the virus transplacentally to the fetus. The majority of abortions caused by EHV-1 occur in the last three months of gestation. Viable foals infected in utero usually develop pneumonia, septicemia, and/or encephalitis with eventual death [23].

FHV-1 is a major cause of feline morbidity with an overall low mortality rate. Although all felid species are susceptible, the disease is particularly widespread in domestic cats due to the prominence of multi-cat households and animal shelters. FHV-1 manifests as rhinotracheitis,

conjunctivitis, and ulcerative keratitis; other less common diseases include ulcerative dermatitis and stomatitis, abortion, pneumonia, vasculitis, and multi-organ hemorrhage [24]. In naïve animals, the disease is usually self-limiting; however, lifelong latency with eventual recrudescence of respiratory disease is common. The virus is transmitted through direct contact or aerosolization, allowing invasion of mucosal epithelial cells and eventual lytic replication. Target tissues include the cornea and conjunctival epithelium, due to ideal lower body temperature at these sites. Systemic infection from macrophagic spread to nodal or other target tissues is rare [4].

In addition to their veterinary importance, human herpesviral infections are considered a worldwide pandemic, and the prevalence is increasing [25]. Approximately 90% of the human population is known to be infected with a herpesvirus [26]. Diseases in people with HSV-1 range from mucocutaneous ulcers to blindness and fatal encephalitis, and this range of disease depends largely on immune status. Transmission is through direct contact; as with other herpesviruses, the infection is lifelong and often establishes latency. Current anti-herpesviral treatment aims at controlling infections but does not prevent future outbreaks, and most treatments target the viral DNA polymerase [27]. Additionally, drug-resistant herpesviruses are frequently encountered clinically, particularly among immunocompromised patients [28]. Prevention through vaccine development would ideally control the infection rate. Despite many attempts, however, an effective vaccine has yet to be developed [29]. Thus, there is a great need for novel anti-herpesviral compounds, which ultimately requires an understanding of the viral life cycle.

The herpesvirus virion contains linear dsDNA within the core, which is surrounded by the capsid, tegument, and envelope. Viral entry is initiated through the interaction of surface envelope glycoproteins with cell glycosaminoglycans [30, 31]. The viral envelope fuses with the

host cell membrane, allowing for internalization of the nucleocapsid, which is then transported to nuclear pores by way of microtubular transport. Because the DNA is packaged through a pressurized, ATP-dependent system, destabilization of the capsid by interaction with the nuclear pore complex causes viral DNA to be injected through the nuclear pore into the nucleoplasm. The viral DNA then circularizes and viral transcripts are generated by cellular RNA polymerase II, beginning with transcription of the α (immediate-early) genes. The α proteins derived from translation of these mRNAs then activate β (delayed-early) gene transcription, the products of which are important for DNA replication. Replication of the viral genome occurs through the formation of the replication complex, which is composed of viral DNA polymerase and its processivity subunit, three components of the helicase-primase complex, and single-stranded DNA binding protein [32]. The viral genome is replicated by the rolling circle mechanism, producing a concatemer of DNA that must be cleaved into monomers for packaging into capsids. Cleavage of the concatemeric DNA is proposed to occur through a triplex of proteins, termed the viral terminase. DNA synthesis activates γ (late) gene transcription, involved in capsid production and virion assembly. The filled capsid obtains an envelope by budding from a cellular membrane and egresses out of the cell [32].

Herpesviruses contain many well-conserved enzymes within the nucleotidyltransferase superfamily (NTS), which are enzymes with a similar structure and function that have a ribonuclease H (RNase H)-like fold. RNase H functions to cleave the phosphodiester bonds of RNA that is hybridized to DNA, and examples include the HIV RNase H and HIV integrase [33-36]. In HSV-1, enzymes with similar RNase H-like activity include the single-stranded DNA binding protein, alkaline nuclease, DNA polymerase, and terminase [37-42]. Given the enzymes' similar structure, inhibitors of the HIV RNase and integrase were recently screened for activity

against HSV-1 and HSV-2 [43]. The classes of inhibitors included tropolones, polyoxygenated heterocyles, and hydroxyxanthenones. Tropolones, including natural and synthetic α-hydroxytropolones (αHTs), were found to be potent anti-HSV inhibitors [43, 44]. To further investigate their HSV-1 inhibitory activity, synthetic αHTs were shown to target the nuclease domain of the viral terminase *in vitro*, suggesting anti-cleavage activity [45]. Interestingly, some of the most potent anti-terminase synthetic αHTs exhibited fair to poor HSV-1 replication inhibition, while αHTs that exhibited excellent HSV-1 replication inhibition had poor anti-terminase activity [44, 45].

The aim of this research included testing the activity of three αHTs with varying anti-HSV and *in vitro* anti-terminase potency to important veterinary herpesviruses, including BoHV-1, EHV-1, and FHV-1. Additionally, further exploration into the mechanism of action of αHTs was assessed using HSV-1. The goal was to establish that αHTs have broad herpesviral inhibitory activity at non-toxic concentrations and may serve as a potential veterinary and medical anti-herpesviral therapeutic.

CHAPTER 2: LITERATURE REVIEW

2.1. _Herpesviridae_

2.1.1. The Virion

The fully mature and enveloped virion is the infectious form of a virus. Members of the _Herpesviridae_ family share a common structure of the virion, and virion architecture is considered a criterion for inclusion into the family. The virion is spherical and ~200 nm in diameter, though the size varies among species. The core contains a single copy of linear dsDNA and is surrounded by a capsid, tegument, and lipid envelope. The ~125 nm icosahedral capsid is composed of two shells, consisting of 11 different proteins [46]. A single unique vertex, the portal complex, breaks the symmetry of the icosahedral capsid and is important for DNA packaging [47]. Surrounding the capsid is the proteinaceous tegument, which is important in modulating the host antiviral response and initiating viral transcription. Although mostly unstructured, the tegument exhibits polarity and contains virally encoded proteins. The lipid envelope is studded with glycoproteins, important for viral entry, and is derived from host cytoplasmic membranes [32, 48].

2.1.1.a. The Core and Viral Genome

As the nonchromatinized dsDNA viral genome is large at 125-240 kbp, packaging into the core requires order. The exact arrangement of the viral genome within the capsid has not been elucidated; however, the viral genome is densely accumulated with ~2.6 nm spacing and extends to the inner layer of the capsid shell [49]. Two polyamines, spermine and spermidine, are found in high amounts in the herpesviral capsid and are thought to counteract the strong repulsive forces of the negatively charged phosphate backbone, particularly important as herpesviruses are devoid of histones [50].

The herpesviral genomes are markedly variable and differ in size (125-240 kbp), composition (classes A-E), and G+C content (32-75%), depending on the species (Table 1). However, all herpesviral genomes contain direct or inverted repeats that are involved in intramolecular recombination and encompass the immediate-early genes, packaging signals, and the promoter for latency-associated transcript RNAs [51]. There are five genomic classes, labeled A, B, C, D, and E. Herpesviruses within the *Varicellovirus* genus, including BoHV-1, EHV-1, and FHV-1, fall into Class D. Genus *Simplexivirus*, including HSV-1, has the most complex genomic structure, characterized by class E (Table 1). Both classes D and E contain two unique sequences, a long and short sequence (U_L and U_S, respectively). In class D, the U_S segment is flanked by a repeated terminal sequence with an internal inverted sequence, producing two isomers that differ usually only by the orientation of the U_S [51]. Class E is similar; however, four genomic isomers can be generated, as both the U_L and U_S segments are bracketed by terminal repeats with internal inversions, represented by *ab*-U_L-*b'a'c'*-U_S-*ca* [32].

Table 1. Genus, Species, and Genome Properties of Applicable α-Herpesviruses

Genus	Species	Genome Class	Genome Size (kbp)	GC Content (%)	ORF Number	Reference
Simplexvirus	HSV-1	E	152	68	84	[52, 53]
Varicellovirus	BoHV-1	D	136	72	73	[54, 55]
	EHV-1	D	150	56	76	[23, 56]
	FHV-1	D	136	45	78	[57]

Conserved across the three subfamilies of *Herpesviridae* are 41 core genes, which can be further grouped into 6 gene blocks based on gene order and polarity. Included in the list are those genes required for DNA replication (e.g., helicase/primase complex, DNA polymerase, single stranded DNA binding protein) and packaging (e.g., terminase binding protein, terminase complex). The majority of genes within subfamilies are homologous; however, minor discrepancies have been documented, as with BoHV-1 and HSV-1 [54, 58]. As HSV-1 is the prototypical herpesvirus, HSV-1 gene and protein names will be used, and the analogous counterparts of the veterinary herpesviruses will be detailed in Table 2.

Table 2. Comparative Analysis of α-Herpesvirus Genes

Name	HSV-1 Gene (ORF Location)	BoHV-1 Gene (ORF Location)	EHV-1 Gene (ORF Location)	FHV-1 Gene (ORF Location)
DNA polymerase	U_L30 (62807-66553)	U_L30 (45238-48978)	ORF30 (51552-55184)	U_L30 (48488-52126)
Processivity factor	U_L42 (93112-94637)	U_L42 (19597-20823)	ORF18 (24479-25696)	U_L42 (23063-24315)
Helicase-primase complex	U_L5 (11754-15132)	U_L5 (93756-96272)	ORF57 (102374-105019)	U_L5 (97406-100776)
	U_L8 (18211-20477)	U_L8 (88485-90766)	ORF54 (97173-99323)	U_L8 (91995-94350)
	U_L52 (109049-113449)	U_L52 (4013-7237)	ORF7 (7056-10301)	U_L52 (3441-9151)
ssDNA binding protein	U_L29 (58410-62054)	U_L29 (49233-52844)	ORF31 (55453-59082)	U_L29 (52392-56039)
Major capsid protein	U_L19 (35024-40529)	U_L19 (69737-73894)	ORF42 (77703-81833)	U_L19 (73018-77193)
Portal vertex	U_L6 (15131-18041)	U_L6 (91640-93706)	ORF56 (100129-102390)	U_L6 (94383-97330)
Terminase complex	U_L15 (29021-34826)	U_L15 (75077-80655)	ORF44 (83148-84319)	U_L15 (78283-83970)
	U_L28 (53059-58160)	U_L28 (53058-55538)	ORF32 (59243-61570)	U_L28 (56050-61141)
	U_L33 (69161-70943)	U_L33 (40769-42450)	ORF27 (48369-48857)	U_L33 (44481-45832)
Reference	NC_001806.2	[54, 55]	[56]	NC_013590.2

2.1.1.b. <u>The Capsid</u>

The architecture and assembly process of the two-shelled capsid is comparable among *Herpesviridae*. The outer component of the capsid is composed of 955 copies of major capsid protein, which forms 150 hexons and 11 pentons [46, 59]. In HSV-1, the major capsid protein is VP5, which is encoded by U_L19. Each hexon contains six molecules of VP5 and six molecules of the "ring" protein, VP26, creating the twenty sides of the icosahedron. The function of VP26 has not fully been elucidated; it has been suggested this protein helps to link the capsid and tegument [60]. Each penton is made up of five copies of VP5, forming 11 of the 12 vertices of the icosahedron. Linking the pentons and hexons in groups of three are the triplex proteins, composed of two molecules of VP23 and one molecule of VP19C [61]. The last vertex is composed of the portal complex, a cylindrical structure composed of 12 copies of pU_L6, which is important for DNA transport. Structural analysis has shown that the portal is 16.5 nm in diameter and 9 nm in length and is markedly similar to the portal vertex in bacteriophages [62]. The inner portion of the capsid contains over 1,000 copies of scaffold proteins [46]. Although encoding genes and proteins are likely different, capsid architecture is likely similar with other herpesviruses.

2.1.2. <u>Viral Gene Expression and DNA Replication</u>

Herpesviral genes can be divided into three groups, immediate-early (α), early (β), and late (γ), based on temporal properties and dependency on protein synthesis. The α genes are expressed independently of protein synthesis, and their products are necessary for synthesis of the β and γ proteins. Synthesis of α proteins peak 2 to 4 hours post-infection. Transcription of the β genes requires the presence of two of the five α proteins. β proteins peak 5 to 7 hours post-infection and include the enzymes necessary for DNA replication. Viral DNA replication occurs

shortly after β gene transcription, detectable 3 to 15 hours post infection. The γ genes produce structural proteins for progeny and their optimal transcription is dependent on DNA synthesis [37].

In HSV-1, α gene de-repression occurs through the interaction of tegument protein VP16, host cell factor 1 (HCF-1), octamer-binding protein 1 (Oct-1), and lysine-specific demethylase 1 (LSD1). Upon release from the tegument, VP16 binds to HCF-1, a regulatory cell cycle host protein. In the nucleus, the VP16-HCF-1 complex binds to Oct 1, which recognizes a consensus sequence called the octamer motif in viral DNA. Interestingly, the octamer motif is present in both cellular and viral enhancers. To ensure specificity of VP16-HCF-1 complex binding to the viral enhancer, a conformational change ensues when Oct-1 binds to GARAT elements, sequences present in the viral enhancer. Lastly, LSD1 recruitment to the complex ultimately results in demethylation of an inhibitor present on the α promoter, allowing α gene transcription [63]. Thus, these four molecules form a complex that de-represses immediate-early promoters and initiates transcription of the five immediate-early genes (i.e., α4, α27, α47, α0, and α22).

The broad functions of the α proteins are two-fold, triggering expression of β genes and providing negative feedback on α gene expression. Important α proteins include α4 protein (ICP4), α0 protein (ICP0), and α27 protein (ICP27). ICP4 interacts with transcription factors on viral promoters for post- α gene transcription and represses its own expression. ICP27 enables β protein expression and translation of viral mRNAs, particularly mRNAs of β gene products required for DNA synthesis [64, 65]. Lastly, ICP0 is a transcriptional activation protein that contains ubiquitin ligase domains, which degrade nuclear proteins required for the formation of replication compartments as well as cellular proteins that function in antiviral defense [66, 67].

Expression of seven β genes are required for DNA replication, and these genes encode viral DNA polymerase (U_L30) and the processivity factor (U_L42), an *ori*-binding protein (U_L9), single-stranded DNA binding protein (U_L29), and the heterotrimeric helicase-primase complex (U_L5, U_L8, and U_L52). These essential proteins localize to the replication compartments [66]. Upon entry into the nucleus, the linear viral dsDNA circularizes, likely through either recombination or the activity of DNA ligase IV/XRCC4 which forms endless genomes [37, 68]. After circularization, DNA replication likely occurs in two steps: bidirectional theta-type replication, which is origin site dependent, followed by a switch to rolling circle replication. The mechanism that causes the switch from theta-type to rolling circle replication is currently unknown [69].

HSV-1 contains three origins of DNA replication, oriL located in the U_L region and two copies of oriS located in the repeat region flanking U_S. Initiation of theta DNA replication begins with *ori*-binding protein (OBP), a DNA helicase with a C-terminal domain that has sequence-specific binding to origins of replication. Specifically, OBP recognizes the sequence, TTCGCAC, which is highly conserved among alphaherpesviruses [70]. Binding of OBP to the *ori* initiates strand unwinding, which induces the formation of a stable hairpin [70]. Single-stranded DNA binding protein (ICP8) facilitates OBP by stimulating the helicase activity as well as binding preferentially to single-stranded DNA [32, 71]. ICP8 interacts with an alkaline nuclease, encoded by U_L12, to promote strand exchange and reannealing of complementary strands. Both ICP8 and U_L12 are predicted to have an RNase-like fold. ICP8 is also involved in regulation of γ gene expression [37, 38, 40, 42, 72]. Binding of the OBP and ICP8 to DNA recruit the remaining five required β proteins.

The replisome includes helicase-primase complex, viral DNA polymerase and its

processivity protein, and ICP8. The helicase-primase trimer contains the U_L5 helicase, the U_L52

primase, and the U_L8 accessory protein. The helicase-primase dimer is enzymatically functional

without the accessory protein, but requires the protein for localization to the nucleus [73]. The

complex likely unwinds short oligonucleotides annealed to single-stranded DNA. As the enzyme

has 5' to 3' directionality, the complex is likely functional along the lagging strand at the

replication fork [37]. Viral DNA polymerase is a heterodimer, composed of U_L30 enzyme, a

polymerase with intrinsic 3' to 5' exonuclease activity, and the U_L42 processivity protein. The

U_L30 polymerase is 25% identical to DNA polymerase δ from *Saccharomyces cerevisiae* and

contains three sequence motifs that are homologous to the exonuclease sequences of *E. coli*

DNA polymerase [37, 73]. Interestingly, HSV-1 DNA polymerase has also been shown to

degrade the RNA strand of RNA:DNA hybrid substrates, indicating RNase activity [37, 41]. As

the name implies, the U_L42 processivity protein increases the catalyzing ability of DNA

polymerase activity. Together, the HSV-1 U_L30-U_L42 complex exhibits an average elongation

rate of 44 nucleotides/second [73]. Theta-type replication yields intermediate circular DNA that

then switches to origin site independent rolling circle replication. Rolling circle replication

begins with a nick of one of the circular strands. The 3' end of the nicked strand serves as a

primer for unidirectional synthesis, using the unnicked strand as a template. Ultimately,

concatemers of DNA are generated that are cleaved and packaged into capsids.

Initiation of DNA replication promotes expression of γ genes, and transcription of γ

genes occurs within the replication compartments. The γ genes encode structural proteins that are

important for assembly of infectious progeny. There are two classes of γ genes, which are

separated based on their dependency for DNA synthesis. The γ1 ("leaky-late") genes are

expressed optimally after the onset of DNA synthesis, while expression of the γ2 ("true" late) genes require DNA synthesis. The majority of the γ genes encode capsid, tegument, and envelope proteins (e.g., U_L19, which encodes VP5, and U_L25).

2.1.3. Capsid Assembly

Within replication compartments around the time of DNA synthesis, capsid assembly occurs. The capsid proteins localize to the nucleus, but the important capsid proteins, VP5, VP26, and VP23 are unable to localize to the nucleus individually. VP5 is likely carried to the nucleus by interacting with VP22a (ICP35), the major component of the inner shell, in the cytoplasm. VP23 requires expression of VP19C in order to enter the nucleus, and lastly, VP26 migrates to the nucleus only when complexed to VP5-VP19C or VP22a [32]. This strict requirement for nuclear entry serves as a control mechanism for capsid assembly. Additionally, the binding of VP23 to VP19C ensures the correct number of proteins for triplex formation [46].

Capsid assembly begins with the formation of the portal vertex, which is composed of 12 copies of pU_L6. These 12 proteins assemble into a ring, requiring a leucine zipper and disulfide bonding for stability [74, 75]. Once the portal vertex is formed, the hexons, pentons, and triplexes are added, forming the spherical procapsid. Initiating capsid assembly with the portal vertex ensures that each capsid contains the pore, which is essential for production of infectious progeny. Capsid maturation is dependent on a protease, located at the N terminus of the scaffold protein encoded by U_L26. Maturation of the capsid, from a less-stable spherical procapsid to icosahedral capsid, begins with cleavage and subsequent activation of the protease. Three types of capsids can mature from procapsids, including types A, B, and C, so named based on their separation via sucrose gradient centrifugation. Due to an abortive packaging event, A capsids lack both the inner scaffold and DNA. A capsids rarely occur in wild-type infections. B capsids

resemble procapsids as the scaffold is retained; DNA fails to insert due to defective packaging. The B capsids are referred to as dead-end products and represent ~20-30% of capsids seen in cells infected with wild-type virus [46]. C capsids contain viral genomes and develop into infectious virions [46, 76].

2.1.4. <u>Encapsidation</u>

Encapsidation is the process of insertion and cleavage of concatameric viral DNA into unit-length monomers. The process is energy-dependent and requires seven gene products, including those of U_L6, U_L15, U_L17, U_L25, U_L28, U_L32, and U_L33 [32]. Specifically, the long component terminus of the genome is cleaved then inserted into the capsid portal followed by a subsequent cleavage event of the short component terminus [32]. A complex of two capsid-associated proteins have been observed on all mature capsid types. This complex is named the capsid vertex-specific complex (CVSC) and contains pU_L17 and pU_L25 [77]. The function of this complex is associated with DNA cleavage and packaging. pU_L17 likely allows the capsid to be competent for DNA packaging [78]. The function for pU_L25 is specific to cleavage of the short component terminus, as U_L25-null mutants result in normal cleavage of the L terminus with a truncated S terminus [76, 79]. Other suggested roles for pU_L25 include structural support for the capsid or assisting in DNA retention in the capsid [80, 81].

The terminase is a multienzyme complex, composed of gene products from U_L15, U_L28, and U_L33, and serves as a molecular motor to both drive dsDNA into the capsid and cleave the concatamer. Herpesviral terminases are similar to bacteriophage T4 terminases in that one gene (U_L15) encodes a canonical Walker box, indicative of ATPase activity [46, 82]. The terminase must dock to the capsid, presumably adjacent to the portal vertex. dsDNA is pumped into the capsid through hydrolysis of ATP and is scanned by the terminase for specific cleavage sites.

Recall that the HSV-1 genome falls in the class E category, where both the unique sequences are flanked by inverted repeats, represented by *ab*-U_L-*b'a'c'*-U_S-*ca*. The sequences required for genome maturation (*cis*-acting sequences) are present in the *a* terminal repeat, which contains its own unique sequences (U_c and U_b) that are bracketed by a series of direct repeats (DR). The signal for cleavage occurs upon terminase recognition of the two *packaging* sequences, *pac1* and *pac2*, which are located within U_c and U_b of the *a* terminal sequence. The specific site of DNA cleavage is within one of the DR regions. The *pac1* sequence is specifically recognized by the product of U_L28, which is bound to U_L15 and stabilized by U_L33 [83]. The terminase complex thus has both DNA-binding and nuclease capacity provided by the functions of pU_L28 and pU_L15, respectively.

The C-terminal nuclease domain of pU_L15 (pU_L15C) resembles an RNase H-like domain and has recently been crystallized [39]. The crystal structure is packed as a trimer of three identical molecules with a central seven-stranded β-sheet surrounded by six α-helices and four loops. Although the amino acid sequence identity is poor between pU_L15C and RNase H, the structure of pU_L15C is markedly similar and superimposable to enzymes in the NTS. The active site is located at the central β-sheet and contains several acidic residues. Consistent with NTS enzymes, these residues generate marked negative charges allowing for cation-mediated DNA binding and cleavage. As proof of concept, the nuclease activity of pU_L15C is potentiated by Mg^{2+} and inhibited by the chelator EDTA [39]. In order to simulate the binding of dsDNA to pU_L15C, a model was constructed using an RNAse H1 template. Based on this model, the loop structures make contact with dsDNA which is reinforced by binding of positively charged residues to the DNA phosphate backbone. This binding likely allows for proper orientation of the β-sheet active site for cleavage activity [39].

Upon binding of dsDNA and recognition of the cleavage signal, the pU_L15C component of the terminase makes an initial cut in the long terminus followed by subsequent cleavage of the short terminus, freeing a monomer of DNA from the concatemer. Viral DNA is then inserted into a procapsid through the portal vertex by conversion of chemical energy from ATP hydrolysis [32]. As DNA becomes packaged into the procapsid, the internal capsid scaffold is removed and the outer shell angularizes from a spherical to icosahedral shape, generating a mature C capsid and sealing DNA inside [46, 84]. Envelopment and viral egress follow, resulting in formation of infectious viral progeny.

2.1.5. Differences in BoHV-1 and EHV-1 Virion Structure and Life Cycle

2.1.5.a. BoHV-1

The structural composition of the BoHV-1 virion is similar to HSV-1. VP5, which is conserved across the alphaherpesviruses, is an essential gene in BoHV-1 and also serves as the major capsid protein [54, 85]. The BoHV-1 tegument is composed mostly of VP8 [86]. The majority of BoHV-1 genes are homologous to HSV-1; however, there are four unique BoHV-1 ORFs, Circ, $U_L0.5$, $U_L3.5$, and US1.5 [55]. Three of these unique ORFs are essential in BoHV-1 [54]. As previously reported in Table 2, many genes encoding important enzymes for DNA synthesis and packaging are well-conserved. Transcription of α proteins is also initiated by VP16 in BoHV-1, but through different cellular targets [87]. There are two origins of replication in BoHV-1, which are present within the inverted repeat region of U_S [54]. DNA replication and packaging is largely similar to HSV-1 [88, 89].

2.1.5.b. EHV-1

Five unique genes with no known homologs to other herpesviruses are encoded by the EHV-1 genome. These include ORFs 1, 2, 67, 71, and 75; the roles of these genes has yet to be

understood [23]. EHV-1 is unique in that the virus encodes only one α gene, ORF 64, which is essential for viral replication [90]. The homolog to HSV-1 VP16 is a late protein product of ORF12, ETIF, which also serves to transactivate the immediate-early promoter [56, 91]. EHV-1 possesses three *ori*, and DNA replication, cleavaging, and packaging are thought to be similar to HSV-1 [56].

2.2. The Pathway to Drug Discovery

Development of novel therapeutics is expensive and time-consuming, as the process can take approximately 10-15 years and cost $800 million to $2 billion [92, 93]. Additionally, the process is overwhelmingly inefficient as only 10% of researched drugs become approved by the FDA [94]. The traditional approach to drug development is separated into clinical phases. In the preclinical phase, the drug is tested in cell culture and/or animal models to assess biological activity and safety. This phase takes an average of 5 years and costs over 100 million dollars, with only 5-20 compounds passing to phase 1. A phase 1 clinical trial includes a small number of healthy and/or diseased individuals to study the safety and maximum tolerated dose of a compound. Phase 2 trials usually have larger numbers of human subjects and are designed to test pharmacokinetics and pharmacodynamics. To assess efficacy and further evaluate safety, phase 3 trials are performed, involving a target population [95]. The three clinical phases each take an average of 2 years; cost can vary from $30 to 100 million with the larger, later phases (phase 3) being the most expensive. An average of 3 compounds are approved to each subsequent clinical phase. Following a successful phase 3 clinical trial, a selected compound is reviewed by the FDA. If approval is granted, a fourth confirmatory trial, also called a surveillance study, may be mandated by the FDA for a minimum of 2 years or may be requested by the pharmaceutical company for further evaluation of pharmacokinetics, efficacy, and safety [93].

2.3.   Current and Developing Treatments for Herpesviruses

2.3.1.   Inhibition of DNA Polymerase

To date, nearly all anti-herpesviruses available on the market target the viral DNA polymerase. The only exception includes fomivirsen, which is an antisense oligonucleotide for the treatment of HCMV retinitis [27]. Nucleoside analogues are a specific class of anti-herpesviral therapy that become incorporated into DNA and thus inhibit DNA replication. Six of the nucleoside analogues are guanosine analogues, including acyclovir and ganciclovir. These compounds rely on both a viral and host kinase, as two phosphorylation events are required for their activation. Their dependency on viral kinases results in varying affinities in different herpesvirus systems; for example, acyclovir has a higher affinity for α-herpesviral thymidine kinases compared to ganciclovir and is thus a better target for HSV-1 [27]. Viral resistance is a major limitation of nucleoside analogues and is frequently caused by acquired mutations in the viral thymidine kinase gene and/or mutations in DNA polymerase [96, 97]. Viral resistance occurs more often in immunocompromised patients that are on long-term therapy, and cross-resistance with other kinase-dependent nucleoside analogues often renders this class of therapeutics ineffective. Additionally, side effects secondary to long-term use of guanosine analogues include nephro- and neurotoxicity and have been linked with inhibition of mitochondrial DNA polymerase [98, 99]. Kinase-independent drugs include cidofovir and foscarnet. Cidofovir is a cytidine analogue that solely requires host kinases for activation. Foscarnet specifically prevents chain elongation of DNA polymerase by binding to the pyrophosphate binding site [27]. Although these compounds confer less resistance, both drugs must be administered intravenously and are linked with severe adverse effects, like

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myelosuppression and nephrotoxicity [27]. Due to the rise in viral resistance, development of anti-herpetic therapeutics with different mechanisms of action is in dire need.

2.3.2. Inhibition of Helicase-Primase Complex

In 2002, Bayer AG developed pritelivir, a compound that inhibits the helicase primase complex. Pritelivir specifically binds both U_L5 and U_L52, inhibits their respective DNA helicase and RNA polymerase activities, and ultimately ceases DNA synthesis. More importantly, this compound does not require a phosphorylation activation step by viral or cellular kinases [100]. In 2010, the efficacy of pritelivir was assessed in people with genital lesions caused by HSV-2; pritelivir reduced both viral shedding time and days with genital lesions. The results were confirmed by an additional study in 2013 comparing the effects of pritelivir to valacyclovir, a nucleoside analogue and prodrug to acyclovir; pritelivir significantly decreased HSV-2 shedding and lesions compared to valacyclovir [101, 102]. A clinical trial evaluating the efficacy of pritelivir against HSV-1 is underway (ClinicalTrials.gov Identifier: NCT02871492). More recently, another helicase-primase inhibitor known as ASP2151 has been shown to inhibit HSV-1, HSV-2, and VZV and is more potent than acyclovir [103-105]. Several clinical trials assessing ASP2151 in herpesviruses have been completed; results of these studies have yet to be posted (ClinicalTrials.gov).

2.3.3. Inhibition of the Portal Vertex

A thiourea compound, identified as WAY-150138, is a potent HSV-1 inhibitor that inhibits the formation of the portal vertex. In the presence of WAY-150138, viral DNA cleavage is blocked with subsequent build-up of B capsids [106]. Escape mutants specifically revealed single point mutations in the U_L6 gene, and it has been suggested that this compound prevents

the incorporation of U_L6 into capsids [107]. Current research regarding WAY-150138 is lacking, which may be due to the compound's poor bioavailability [27, 108].

2.3.4. Inhibition of the Terminase

Currently, anti-terminase drugs include a few compounds that inhibit HCMV with limited activity against alphaherpesviruses. These compounds are within three classes, benzimidazole ribonucleosides (TCRB and BDCRB), sulfonamides (BAY 38-4766), and dihydro-quinazolines (Letermovir/AIC246) [27]. Resistance mutants of the benzimidazole ribonucleosides and BAY 38-4766 contain mutations in genes that encode HCMV terminase components (U_L56 and U_L89). Current research regarding these three compounds is lacking. However, letermovir has shown promising results against HCMV with an impressive EC_{50} of 4-5 nM. Letermovir escape mutants, which have been mapped to pU_L56, are susceptible to other classes of anti-terminases suggesting a separate mechanism of action [109, 110]. Interestingly, this compound was found to be ineffective in vitro for rodent (mouse and guinea pig) cytomegalovirus strains [111]. A phase III trial evaluating letermovir for the prevention of HCMV has been completed without published results (ClinicalTrials.gov Identifier: NCT02137772).

2.4. Nucleotidyltransferase Superfamily

The nucleotidyltransferase superfamily is a group of enzymes that share a similar structure and function. The enzymes transfer nucleoside monophosphate from nucleoside triphosphate to a nucleic acid or protein hydroxyl group, and function in DNA repair, RNA editing and polyadenylation, and chromatin remodeling activities [112]. These proteins usually contain a central β-sheet surrounded by four α-helices [112]. This conserved motif, particularly near the active sites, successfully predicts that these enzymes depend on divalent cations for function [35,

39, 113, 114]. Select examples of NTS enzymes include *E coli* RNase H1 and 2, human RNase H1 and 2, human DNA polymerase, HIV RNase H, and HIV integrase [34, 36, 112, 115-118]. As previously mentioned, herpesviruses have a few enzymes with RNase H-like folds, including ssDNA bp (ICP8), alkaline nuclease, DNA polymerase, and pU_L15 of the viral terminase [37-39, 42]. Development of HIV RNase H and integrase inhibitors has been of recent interest, including compounds that interfere with metal chelation of the active site [119-122]. Given the structural homology of these enzymes, use of anti-RNase H drugs against herpesviruses has been explored; the results of these studies will be discussed in the subsequent section [38, 43-45].

2.5.   Inhibition of HSV-1 by NTS Inhibitors

Select NTS inhibitors, including compounds within the tropolone, polyoxygenated heterocycles, and hydryoxyxanthenone classes, were recently screened against HSV-1, HSV-2, HCMV, and acyclovir-resistant mutants of HSV-1 and HSV-2. Several compounds were found to inhibit viral replication at low drug concentrations with minimal cytotoxicity; the majority of the effective compounds were within the tropolone family, specifically the αHT subclass [64]. These tropolones had 50% effective concentration values ranging between 0.35 and 1.94 μM against HSV-1 and minimal toxicity [43]. Based on the results of time-to-addition assays, the inhibitory effect of one representative αHT compound (β-thujaplicinol) occurred early in viral replication as the inhibitory effect decreased when application of the compound was delayed beyond 2 hours. To assess whether this class of compounds required viral thymidine kinase phosphorylation for activation, β-thujaplicinol was screened against acyclovir-resistant mutants of HSV-1 and HSV-2; replication of both strains of mutants was suppressed. These compounds were thus considered to be exciting new herpesviral inhibitors [43].

2.5.1. α-Hydroxytropolones

αHTs are within the troponoid family, a chemical group of stable non-benzenoid seven-membered ring compounds that exist in nature (e.g., fungal products, wood of cedar). αHTs have been of much historical and recent interest due to their broad bioactivities [123]. The most studied αHT, β-thujaplicinol was discovered from the western red cedar tree, *Thuja plicata*, due to its natural fungicidal activity [124]. Aside from antiviral properties, extensive research with αHTs has revealed that these compounds also contain antifungal, antibacterial, antimalarial, and anticancer properties. The chemical structure of αHTs consists of three oxygen atoms that create a potent negative charge at physiological pH, explaining their potent metal chelating ability [124]. It has recently become possible to synthesize αHTs with chemical modifications that may potentiate their activity or eliminate cytotoxic effects [124].

2.5.2. Inhibition of HSV-1 by α-Hydroxytropolones

It is postulated that the inhibitory effect of αHTs on HSV-1 is due to suppression of enzymes with RNase H-like domains, particularly through chelation of required divalent cations. One possible enzyme in HSV-1 requiring such cations is the nuclease active site of one terminase component, pU_L15, which is highly conserved among all herpesviruses. To further investigate inhibition of the viral terminase, the nuclease activity was assessed in the presence of several synthetic β-thujaplicinol derivatives through the analysis of pU_L15C-mediated hydrolysis of short DNA duplexes using dual-probe fluoresecence [45]. Twenty-one synthetic αHTs with different chemical substitutions at positions 3 and/or 4 of the seven-membered ring were monitored for pU_L15C inhibition. The compounds had variable potencies, with IC_{50} values ranging from 0.14 to 49 μM; compounds with smaller ester and ketone substitutions were more potent than those with larger or aromatic modifications [124].

Comparing the *in vitro* anti-terminase and *in vivo* anti-HSV activities of synthetic αHTs reveals an indirect relationship. The more potent anti-terminase αHTs have poor to fair anti-HSV suppression, as exemplified by compound CM1012-6a with an IC_{50} value of 180 nM against the terminase nuclease domain but no effect against HSV-1 at 5 μM [44, 45]. Alternatively, potent anti-HSV αHTs have poor to fair anti-terminase activity. The IC_{50} value of compound RM-YM-3-0613 is ~50 μM against the viral terminase, but was found to be a potent anti-HSV compound with an EC_{50} of 180 nM [44, 45]. Although αHTs were found to have anti-terminase activity *in vitro*, based on the discordant anti-HSV activity, other essential viral enzymes are likely being targeted.

CHAPTER 3: MATERIALS AND METHODS

3.1. Compound Selection

Three αHTs, numbered 106, 111, and 115, were synthesized and provided by RP Murelli; the compounds were synthesized from kojic acid as previously described [125-127]. The chemical structures of each compound and previously reported anti-herpesviral activity are shown in Figure 1 and Table 3, respectively. All compounds were dissolved in DMSO, aliquoted, and stored at -20°C.

3.2. 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) 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 viruses were used: HSV-1 wild-type strain F, BoHV-1 wild-type Cooper strain, EHV-1 strain 10N0148, and FHV-1 strain FH2CS. Virus stocks were grown and titered in permissive cell lines, including 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.

3.3. 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 hours). The following concentrations of each αHT compound were used: 100 μM, 125 μM, 150 μM, and 175 μM. These concentrations were added to 1 mL of growth medium 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 dilution percentage served as the negative control. After

24 hour 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 hour

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 log (inhibitor)-versus- response

algorithm, was employed to determine the 50% cytotoxic concentration.

3.4. HSV-1, BoHV-1, EHV-1, and FHV-1 Replication Inhibition Assays

HSV-1, BoHV-1, and FHV-1 were added to a permissive cell line (CV-1, MDBK, and

CRFK, respectively) at a multiplicity of infection (MOI) of 0.01 for 1 hour. EHV-1 was added

to a permissive cell line (MDBK) at MOI 0.1 for 1 hour. After 1 hour viral adsorption, the virus-

inoculated medium was removed. The cells were washed once with PBS and replaced with

DMEM containing either DMSO (at the highest dilution percent) or various concentrations of

αHT ([10 µM to 50 µM] for compounds 106 and 111 and [2 to 10 µM] for compound 115) for an

additional 47 hours. At 48 hours post-infection, the cells were assessed by phase-contrast

microscopy for cytopathic effect or toxicity. 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. The 50% inhibitory concentration values were

calculated using GraphPad Prism. The concentrations of each compound were log transformed,

and the inhibitory activity was normalized with the bottom value set to 0. Non-linear regression,

using a log (inhibitor)-versus-normalized response algorithm was employed to determine 50% inhibitory concentration.

3.5. Transmission Electron Microscopy

BoHV-1 was added at MOI 1.0 to a ~90% confluent monolayer of MDBK cells in a 6-well plate for 1 hour incubation. After 1 hour, the virus-inoculum was removed and replaced with DMEM containing one of the following: DMSO (at the highest dilution percent), 50 μM αHT-106, or 8 μM αHT-115 for an additional 13 hour incubation. The cells were then fixed, dehydrated, embedded, and thin sections were visualized by transmission electron microscopy as previously described [128].

3.6. Immunoblotting

CV1 cells in 6-well plates were infected for 1 hour with HSV-1 at an MOI of 5.0; virus-inoculated medium was removed and replaced with DMEM containing either DMSO (at the highest dilution percent), or DMSO solublized 50 μM αHT-106, 30 μM αHT-111, or 8 μM αHT-115 for an additional 5 or 17 hours. The cells were then washed with PBS and solubilized in SDS sample buffer. The solubilized proteins were separated on 12% SDS polyacrylamide gels and transferred to nitrocellulose membranes. The 6 hour samples were probed with a monocolonal antibody (mAb) to ICP 27 (diluted 1:1,000 in PBS plus 1% BSA), while the 18 hour samples were probed with a mAb directed against glycoprotein C (gC) that was diluted 1:2,000 in PBS with 1% BSA. The bound antibodies were detected by incubating with anti-mouse immunoglobulin conjugated with horseradish peroxidase (HRP) and visualized by enhanced chemiluminescence (ECL; Amersham). For a loading control, the blot was stripped and reprobed with anti-actin antibodies (diluted 1:1000) and developed similarly.

3.7.   Real-Time Quantitative PCR

CV1 cells were infected with HSV-1(F) at an MOI of 5.0. After 1 hour incubation, the virus inoculated medium was removed and replaced with a DMSO vehicle control, 50 μM αHT-106, 30 μM αHT-111, or 8 μM αHT-115. The samples were incubated for an additional 12 hours until DNA extraction (DNeasy). Target primers for UL51 (forward: 5'-GCC AGT CGT TCT AGG TTC AC-3' and reverse: 5'-GTT AAC GCG CTA CTT CCC G-3') were used to measure DNA replication, and the assay was performed with SYBR green (Thermo Scientific, Pittsburgh, PA) according to the manufacturer's directions. Briefly, reactions were performed with a volume of 20 μl, consisting of 50 ng of DNA template, 100 nM of each primer, 7.6 μl H_2O, and 10 μl SYBR green mix. The thermal cycling protocol included an initial denaturation for 3 min at 95°C and 35 cycles consisting of a denaturation step at 95°C for 10 s and an annealing step at 60°C for 30 s. Each sample was analyzed in triplicate, and average threshold cycle (CT) values were used for further analysis.

3.8.   Southern Blotting

Five 100-mm plates of CV1 cells, one of which was pre-treated with 300 μg/mL of phosphonoacetic acid (PAA; Sigma Aldrich) for 30 minutes, were infected with HSV-1 (F) at an MOI of 5.0; one 100-mm plate was infected with U_L15-null virus at an MOI of 5.0. After the 1 hour incubation, virus inoculation media was removed and replaced with DMSO vehicle control (HSV-1 and U_L15-null), 300 μg/mL PAA, 50 μM αHT-106, 30 μM αHT-111, or 8 μM αHT-115. After an additional 17 hour incubation, the medium was removed and the cells were washed with phosphate-buffered saline (PBS), scraped, and pelleted by centrifugation at 1,000 x g for 5 minutes. The cells were lysed, and total cellular DNA was extracted as described previously described [129]. Total DNA (~10 μg) was digested with BamHI, separated on 0.8% agarose gel

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via electrophoresis, followed by denaturization in 1 M NaOH, neutralization in Tris buffer. The

denatured DNA was transferred to a nylon membrane by capillary action, and the transferred

DNA was hybridized with [^{32}P]dCTP-labeled BamHI P fragment of HSV-1(F) DNA. The bound

probe was visualized by exposure to X-ray film with intensifying screens at −80°C.

CHAPTER 4: RESULTS

4.1. Compound selection

Three synthetic αHTs were selected that have varying potencies against the nuclease domain of the viral terminase as determined by an *in vitro* dual-probe fluorescence assay [45]. The anti-HSV activity of these three αHTs was also previously evaluated [44]. The *in vitro* anti-terminase and *in vivo* anti-HSV potencies of the compounds were found to be discordant. The chemical structure and pharmacologic properties of the three selected compounds are detailed in Figure 1 and Table 3, respectively.
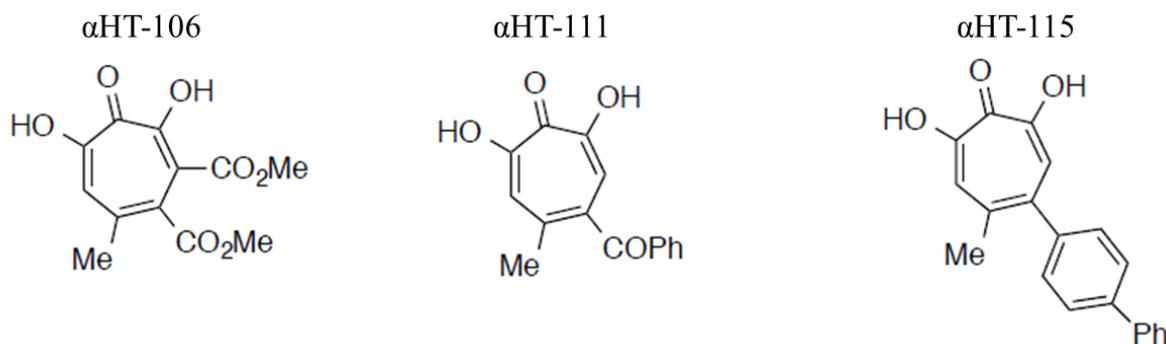
Figure 1. The chemical structure of the three synthetic αHTs compounds used in this study.

Table 3. The pharmacologic properties of the three synthetic αHTs used in this study.

Synthetic αHT	Compound Name	Anti-pU_L15C IC$_{50}$ (μM)	Anti-HSV-1 activity (μM)	CC50 (μM)[1]	Reference
106	CM1012-6a	0.18	Full suppression at 50	NA	[44, 45]
111	CM1012-6f	5.6	Full suppression at 5	>50	[44, 45]
115	RM-YM-3-0613	49.1	EC$_{50}$ = 0.18	>100	[44, 45]

[1]CC$_{50}$ determined in Vero cells.

4.2. αHTs are minimally toxic in mammalian cell lines

The cytotoxicity of the three αHTs was assessed on MDBK cells using a cell proliferation assay. The three compounds showed minimal cytotoxicity at 24 hours with 50% cytotoxic concentration values exceeding 100 μM (Table 4).

Table 4. CC_{50} values of αHT-106, -111, and -115 on MDBK cells.

Compound	CC_{50} (μM)
αHT-106	144
αHT-111	129
αHT-115	187

4.3. αHTs broadly suppress herpesviral replication inhibition

Replication inhibition activity of αHTs was initially assessed with BoHV-1 at concentrations of 0.1, 1, 10 and 50 μM for αHT-106, -111 and -115. The viral titers, determined by plaque assay, were then compared to the DMSO-treated control. Complete inhibition was noted between concentrations of 10 and 50 μM for αHT-106 and -111 and between 1 and 10 μM for αHT-115, indicating increased potency of αHT-115. Given this initial data, replication inhibition was then assessed for HSV-1, BoHV-1, EHV-1, and FHV-1 using 10 and 50 μM of αHT-106 and -111 and 2 and 10 μM of αHT-115. For each virus, minimal suppression (an average of one $\log_{10}$ decrease) of viral replication was observed at the low αHT concentration (2 and 10 μM) with marked suppression (an average of five $\log_{10}$ decrease) at the higher αHT concentrations (10 and 50 μM) (Figure 2).
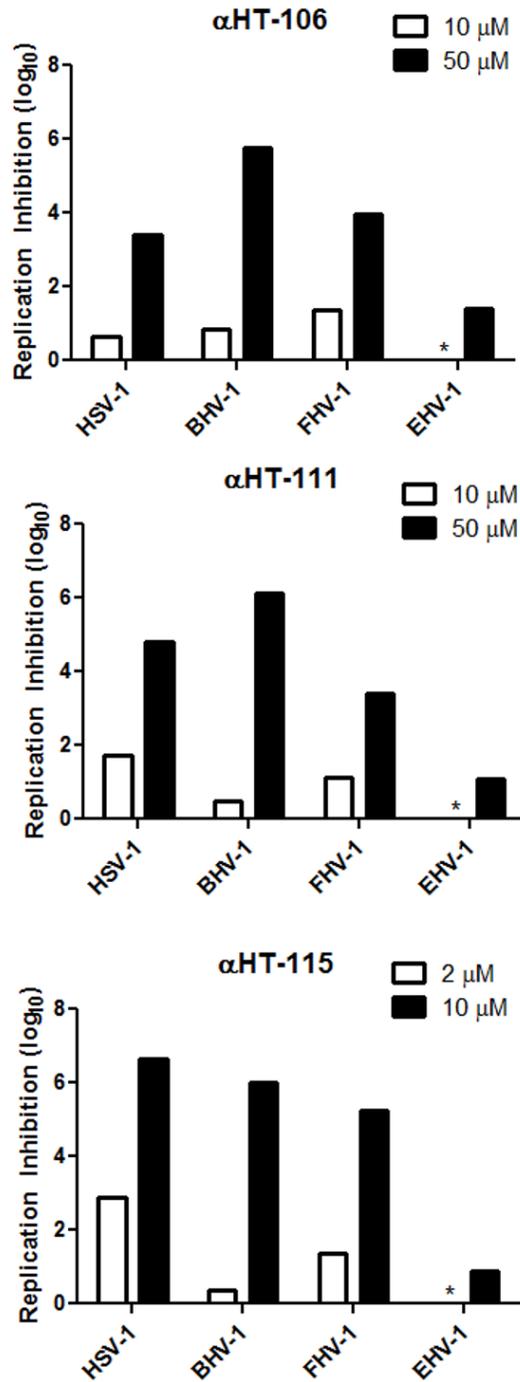
Figure 2. Replication inhibition of HSV-1, BoHV-1, EHV-1, and FHV-1 by αHTs. Relative to the control, there is marked suppression of viral replication at the higher concentrations 10 (αHT-115) and 50 μM (αHT-106 and -111) and minimal inhibition at the lower concentrations 2 (αHT-115) and 10 μM (αHT-106 and -111). The asterisks indicate that the titer is equivalent to the control.

4.4. αHT-115 has more potent anti-herpesviral activity than αHT-106 and -111

In order to calculate the 50% inhibitory concentration of each compound, additional

replication inhibition assays were performed with αHT concentrations of 10, 20, 30, 40, and 50

μM (αHT-106 and -111) and 2, 4, 6, 8, 10 μM (αHT-115). The 50% inhibitory concentration

values were similar between αHT-106 and -111 at around 20 μM. In contrast, αHT-115 was

markedly more potent with an IC_{50} ranging between 1.5 and 5 μM. Thus, αHT-115 has more

potent anti-herpesviral replication inhibition activity than αHT-106 and -111.

Table 5. IC_{50} values of αHT-106, -111, and -115 with HSV-1, BoHV-1, FHV-1, and EHV-1.
The asterisk indicates partial IC_{50} calculations.

		Titer (Log_{10})		
	μM	HSV-1	BoHV-1	FHV-1	EHV-1
αHT-106	0	6.98	8.59	5.97	4.06
	10	6.34	7.76	4.60	4.59
	20	5.32	6.63	3.20	4.60
	30	4.62	5.34	2.00	3.48
	40	4.36	3.96	1.48	3.24
	50	3.58	2.83	2.00	2.64
	IC_{50} μM	20	26	15	30

	μM	HSV-1	BoHV-1	FHV-1	EHV-1
αHT-111	0	6.28	6.12	6.18	4.02
	10	4.54	5.65	5.04	4.13
	20	2.00	2.70	3.38	3.66
	30	2.73	0.00	3.69	3.20
	40	1.00	0.00	3.00	3.19
	50	1.48	0.00	2.84	2.92
	IC_{50} μM	13	20	13	23

	μM	HSV-1	BoHV-1	FHV-1	EHV-1
αHT-115	0	7.00	6.00	5.23	3.70
	2	4.00	5.64	3.85	4.20
	4	2.30	3.88	3.41	3.80
	6	2.30	2.78	3.08	3.10
	8	0.00	0.00	-	2.60
	10	0.00	0.00	-	2.80
	IC_{50} μM	2.2	5.0	1.5*	5.0

Table 6. Therapeutic indices for αHTs against HSV-1, BoHV-1, FHV-1, and EHV-1.

Compound	HSV-1	BoHV-1	FHV-1	EHV-1
αHT-106	7.2	5.5	9.6	4.8
αHT-111	9.9	6.5	9.9	5.6
αHT-115	85.0	37.4	124.7	37.4

Based on the therapeutic indices (Table 6), αHT-115 had the highest therapeutic range, particularly for HSV-1 and FHV-1, while the indices of αHT-106 and αHT-111 were at least six-fold lower (Table 6).

4.5. αHTs decrease production of infectious progeny

Transmission electron microscopy (TEM) was employed to assess virion development in the presence of αHTs. MDBK cells were infected with BoHV-1, in the presence of a DMSO vehicle control, 50 μM αHT-106, or 8 μM αHT-115. Given that αHT-111 is considered an intermediate anti-herpesviral and anti-terminase compound, this compound was excluded. Numerous virions and C capsids were observed in the DMSO control BoHV-1 sample (Figure 3). Application of both αHT-106 and -115 resulted in a marked decrease in extracellular viral particles and intranuclear capsids (Figure 4 and 5). A very rare small cluster of intranuclear electron-dense round structures was observed in αHT-115 treated cells and is of unknown significance (Figure 6). The decrease in virion number further supports the compounds' anti-herpesviral activity.
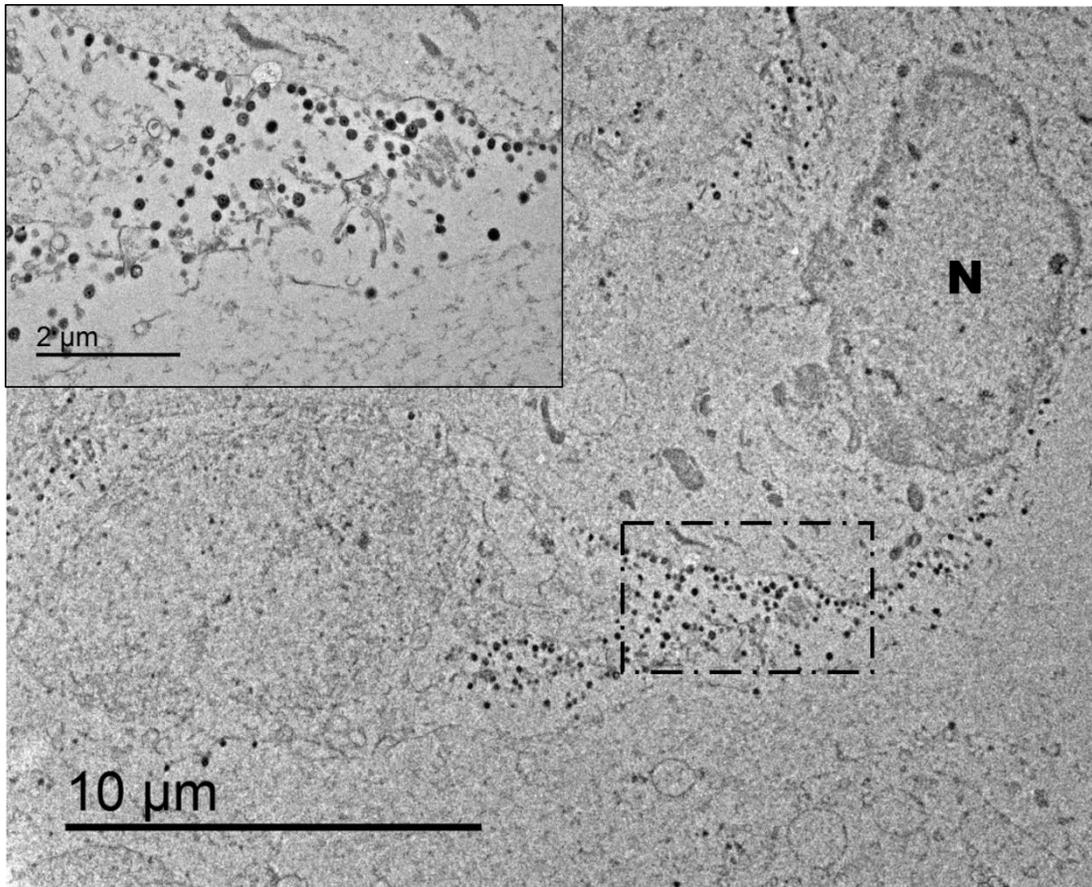
Figure 3. TEM of MDBK cells inoculated with BoHV-1 and treated with DMSO control. Note the myriad extracellular virions, more clearly viewed on higher magnification (*inset*). N, nucleus.
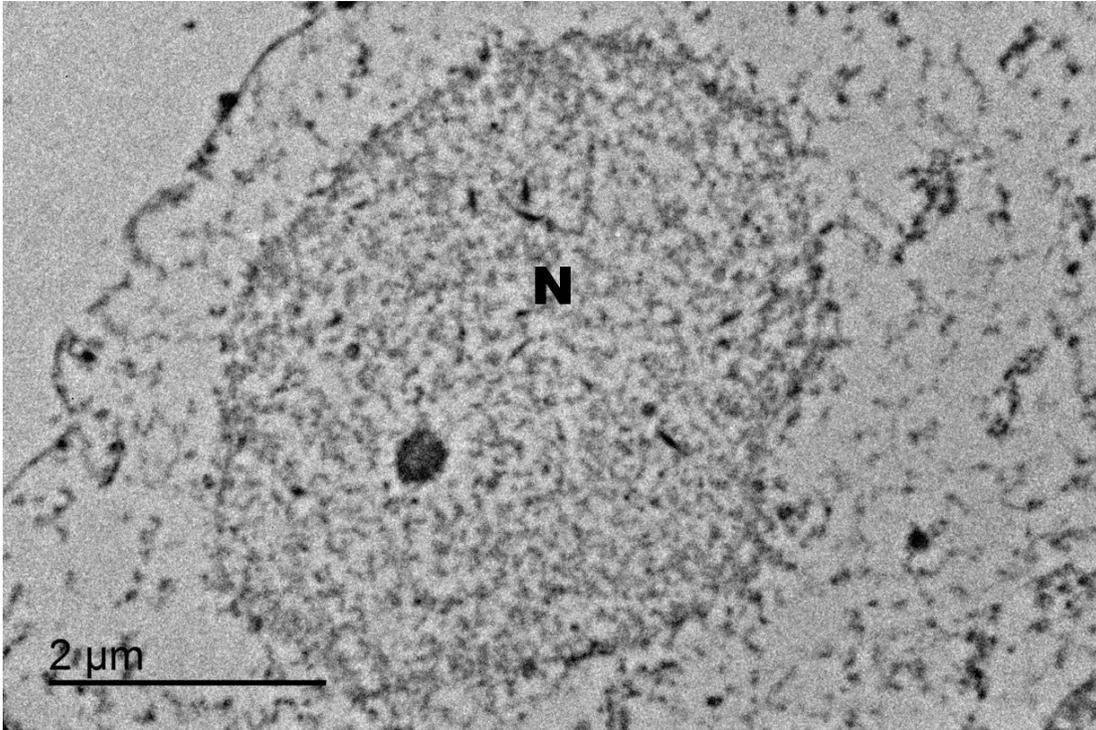
Figure 4. TEM of MDBK cells inoculated with BoHV-1 and treated with 50 µM αHT-106. No virions or capsids are observed. N, nucleus.
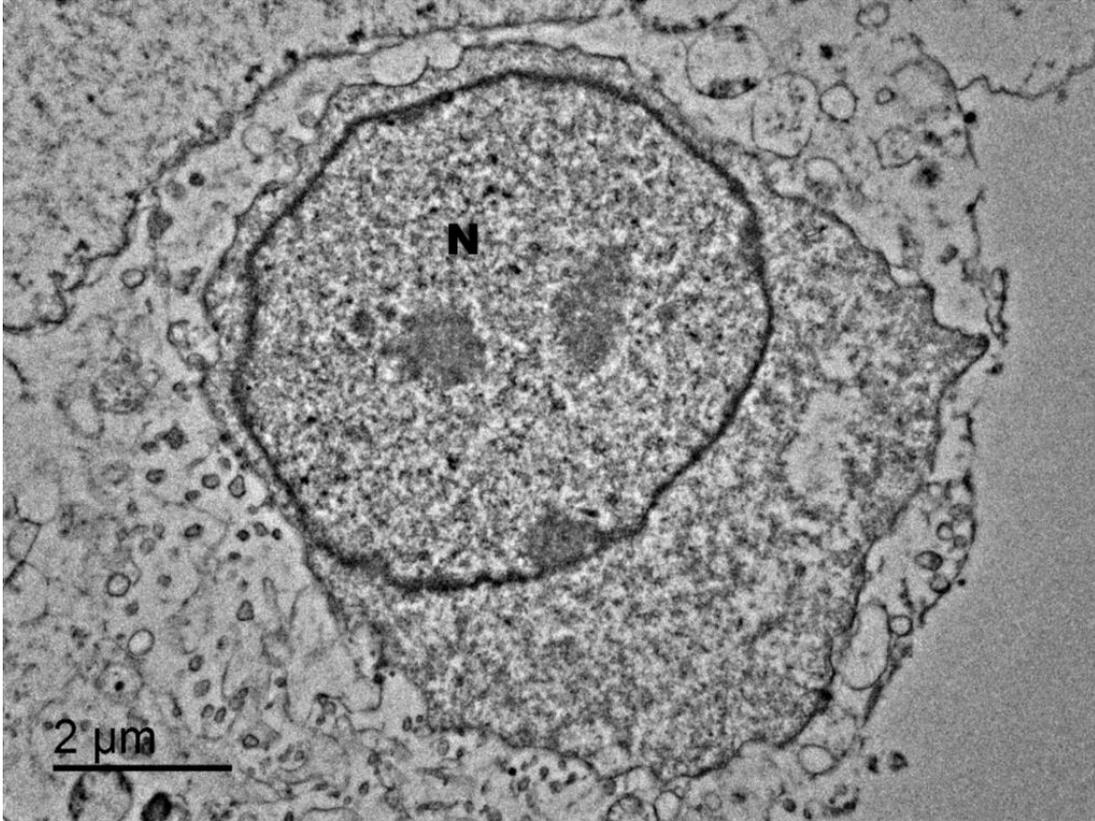
Figure 5. TEM of MDBK cells inoculated with BoHV-1 and treated with 8 μM αHT-115. No virions or capsids are observed. Three large, round electron dense structures represent nucleoli. N, nucleus.
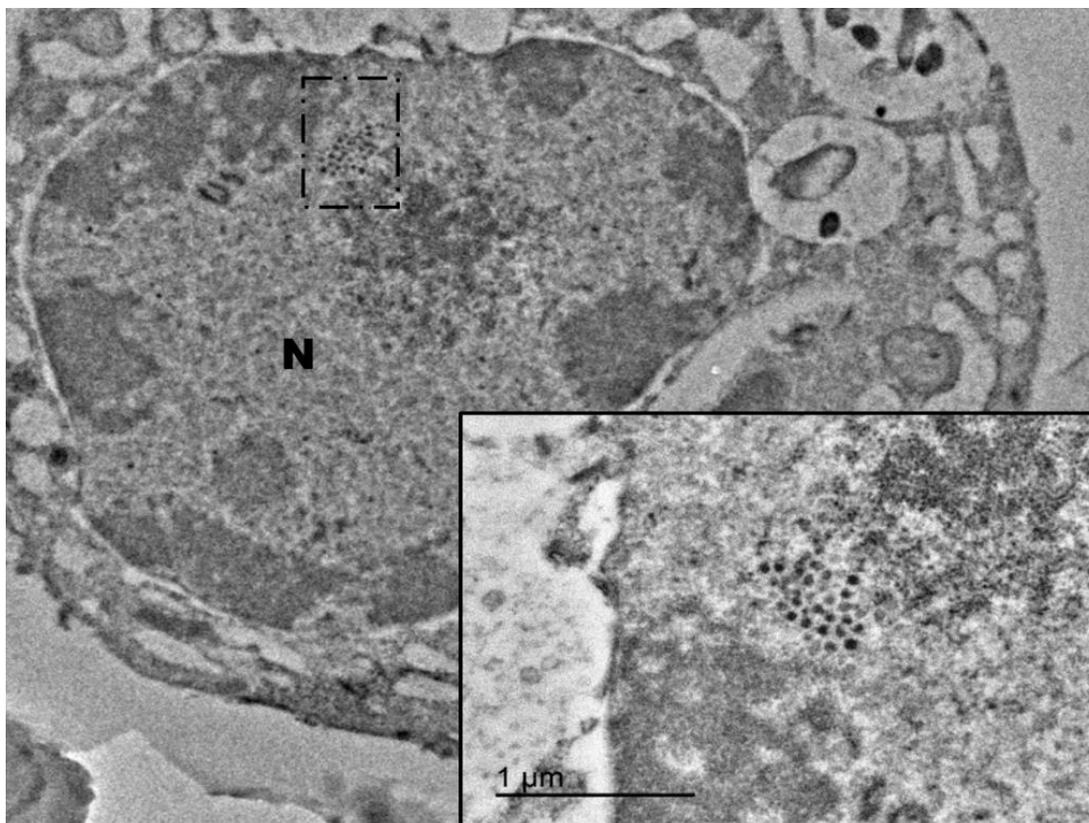
Figure 6. TEM of MDBK cells inoculated with BoHV-1 and treated with 8 μM αHT-115. Clusters of round electron dense structures were very rarely present intranuclearly (*inset*). N, nucleus.

4.6. αHTs inhibit expression of true late (γ2) proteins

To further elucidate the mechanism of action of αHTs, immunoblotting was performed to assess immediate-early (α, represented by ICP27) and late (γ2, represented by gC) protein expression in the presence of αHTs. CV1 cells were inoculated with wild-type HSV-1 and treated with a DMSO vehicle control, 50 μM αHT-106, 30 μM αHT-111, or 8 μM αHT-115. No change in immediate-early expression was noted between the DMSO control or drug treated samples (Figure 7A). However, compared to the wild-type HSV-1 control, there was decreased gC expression in the αHT-treated samples. The decrease in protein expression was more

marked in cells treated with αHT-115 and αHT-111 than αHT-106, and thus mirrored the relative

antiviral potencies of these compounds (Figure 7B). These findings indicate that the effects on

protein expression occurred between the expression of immediate-early proteins and that of late

proteins. Such a timeframe is consistent with the onset of viral DNA replication.
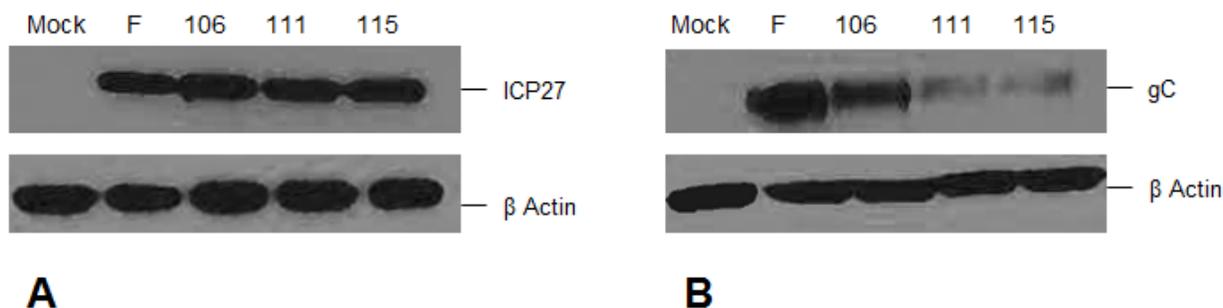
Figure 7. Immunoblotting of α (A) and γ (B) protein in αHT-treated HSV-1 samples.
(A). There is no change in immediate-early protein expression compared to the HSV-1 wild-type
control. β-actin, loading control. (B). There is decreased late protein expression in the αHT-
treated samples relative to the wild-type HSV-1 control, and the effect is potentiated by the
increased potency of the compounds. β-actin, loading control.

4.7. αHTs inhibit DNA replication

To evaluate whether HTs affected DNA replication, HSV-1 viral DNA produced in the

presence or absence of drug was measured by qPCR using U_L51 specific probe and primers.

Significant decreases in HSV-1 viral load were observed in αHT-treated samples (50 μM αHT-

106, 30 μM αHT-111, or 8 μM αHT-115) compared to wild-type HSV-1 control ($p < 0.05$)

(Figure 8). Specifically, there was ~72 to 93% reduction in the amount of U_L51 target sequences

in αHT-treated samples compared to the DMSO treated control. Based on Tukey's post-hoc

analysis, no significant differences were observed between samples treated with different drugs at the concentrations tested. Nevertheless, these data indicate that αHTs inhibit HSV-1 DNA replication.
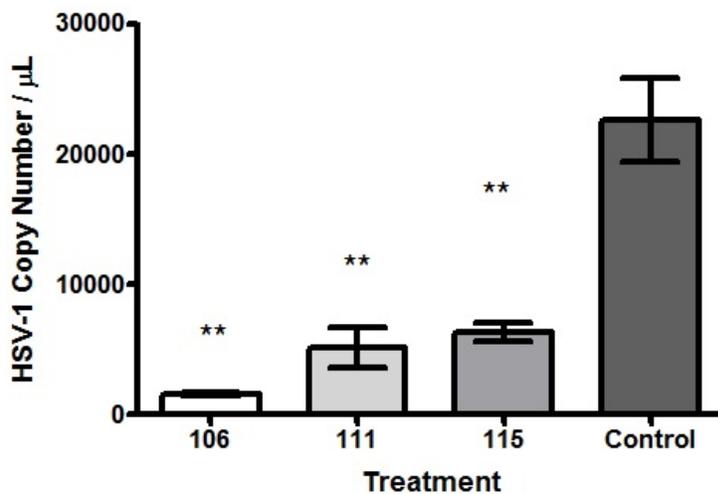
Figure 8. Real-time qPCR of viral DNA in wild-type HSV-1 control and αHT-treated samples. The decrease in HSV-1 DNA copy number is significant, as determined by one-way ANOVA and Tukey's post-hoc analysis (*, $p < 0.05$). No significant differences were observed between treated samples (50 μM αHT-106, 30 μM αHT-111, or 8 μM αHT-115).

4.8.   Attempted assessment of viral cleavage activity in the presence of αHT

Previous research has shown that the αHTs, inhibit nuclease activity of pU$_L$15C to varying extents using an *in vitro* dual-probe fluorescence assay. Specifically, αHT-106 was found to be more potent than αHT-111, and the effect of αHT-115 was negligible (Table 3) [45]. Although the results of immunoblotting and qPCR shown above indicated inhibition of viral DNA

replication, a concurrent effect on cleavage via inhibition of the viral terminase could not be excluded.

To assess DNA cleavage in the presence and absence of drug treatment, CV1 cells were infected with HSV-1(F) and U_L15-null. HSV-1(F) infected CV1 cells in the presence of PAA (a known viral DNA synthesis inhibitor), αHT-106, αHT-111, αHT-115, or a DMSO vehicle-control. Viral DNA was digested with a restriction enzyme (BamHI), separated electrophoretically, and probed with a radiolabeled terminus of the short component (P fragment). In Figure 9, the junctional S-P fragments can be observed in all samples. HSV-1(F) generated a broad P fragment. In the PAA, sample, low levels of DNA were detected and likely represented DNA from virions adsorbed to the cells (input DNA). As expected, the U_L15-null virus failed to generate a short genomic segment. DNA levels in cells infected in the presence of the three αHTs were comparable to the PAA control, suggesting that the drugs conferred a marked inhibition of viral DNA replication.. However, the levels of DNA in cells treated with the αHTs were too low to permit meaningful comparison between levels of terminal fragments and the junction fragments, which is a metric necessary to assess DNA cleavage *in vivo*. Thus, the ability to verify the previous *in vitro* findings of anti-terminase activity of αHTs was precluded *in vivo* by their profound inhibitory activity of viral DNA replication.
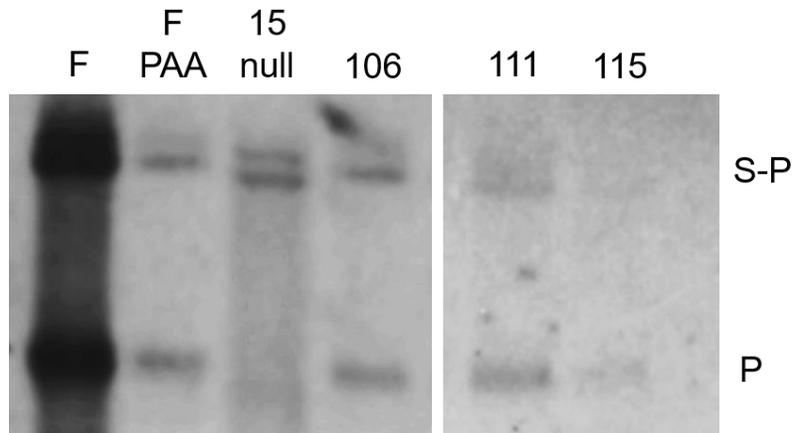
Figure 9. Fluorographic image of viral DNA probed with radiolabeled terminus of the short component (P fragment).
CV1 cells were infected with HSV-1(F) or U_L15-null. The HSV-1(F) infected samples were treated with PAA, αHT-106, αHT-111, αHT-115 or DMSO vehicle-control. The positions of the S-P junctional fragment and BamHI P fragment are indicated. Decreased bands in the junctional and short fragments are noted in the αHT-treated samples and are comparable in level to that of the PAA-treated sample.

CHAPTER 5: DISCUSSION

The presented research shows that αHTs are broad anti-herpesviral compounds that act primarily at the level of viral DNA synthesis. αHTs were previously documented to inhibit HSV-1 and -2 replication *in vivo* and postulated targets include viral proteins with an RNase H-like domain [43, 44]. In HSV-1, these targets include DNA polymerase (pU$_L$30), single-stranded DNA binding protein (ICP8, pU$_L$29), alkaline nuclease (pU$_L$12) and the nuclease domain of the viral terminase (pU$_L$15C) [37-42].

αHTs were previously found to inhibit the nuclease domain of the viral terminase *in vitro* via a dual-probe fluorescence assay. Twenty-one synthetic αHTs were screened and variable potencies were documented, and the potencies were found to negatively correlate to the bulkiness of their modified side chain [45]. Interestingly, the αHTs with potent inhibition of viral replication were relatively poor inhibitors of pU$_L$15C nuclease activity suggesting other HSV targets were being inhibited [44, 45].

The three αHTs selected for this research had variable *in vitro* potencies against pU$_L$15C, ranging from negligible (αHT-115), intermediate (αHT-111), to marked (αHT-106). These compounds were screened for activity against three important veterinary herpesviruses, BoHV-1, EHV-1, and FHV-1 and were also screened for activity against HSV-1 as a control. Each compound was found to be non-toxic with a 50% cytotoxic concentration value >100 μM, based on a cell proliferation assay performed on MDBK cells. The results of the replication inhibition assays were comparable across the different herpesviruses for each compound. Specifically, the 50% inhibitory concentration values ranged from 15-30 μM for αHT-106, 13-23 μM for αHT-111, and 1.5-5 μM for αHT-115 for the different viruses.

The relative potencies of these three αHTs are comparable to the previous observations against HSV-1 and -2 [44]. αHT-115 had the widest therapeutic range, with indices that ranged from 37 to 124. In contrast, the therapeutic indices for αHT-106 and αHT-111 were narrow, ranging from 4.8 to 9.6 and 5.6 to 9.9, respectively. Given there are no other differences between these compounds we conclude that αHT-115's biaryl side chain optimized its inhibitory effect [44]. It is unclear why the addition of a biaryl side chain enhances anti-viral activity. Previous evaluation of synthetic αHT activity against Hepatitis B Viral RNase H showed markedly decreased to diminished effect as the modified side chains became longer [130]. However, similar to the research presented here, a previous study found that the relative potencies of two synthetic αHT compounds with biaryl side chains against HSV-1 and -2 were greater compared to αHT with alternate (including smaller) chemical modifications [44]. Thus, it is possible the addition of a biaryl appendage either enhances the binding with an enzyme's active site or negates unfavorable interactions.

To further elucidate the mechanism of action of αHTs against herpesviruses, the effect of αHTs against HSV-1 was assessed with transmission electron microscopy, immunoblotting, real-time qPCR, and southern blotting. The results of these findings were consistent with inhibition of viral DNA replication. Specifically, production of capsids and infectious progeny depend upon optimal DNA synthesis, explaining the decreased virions and capsids in the αHT-treated samples relative to the DMSO-treated control as viewed by electron microscopy. Similarly, despite similar levels of immediate-early protein production in the presence of the compounds, we observed decreased DNA replication dependent true-late (γ_2) protein expression in the αHT-treated samples. The results of qPCR confirmed HSV-1 DNA replication inhibition by αHTs, as there was an approximately 72-93% decrease in U_L51 target sequences in cells treated with αHT

compared to the wild-type control. Although the profound decrease in viral DNA synthesis precluded assessment of viral DNA cleavage we cannot exclude an additional effect on terminase activity of these compounds *in vivo*.

Taken together, these data show that αHTs are broad, non-toxic compounds that primarily target herpesviral DNA replication. As αHTs have potent chelating ability due to the arrangement of three oxygen compounds, inhibition of cation-dependent enzyme(s) is likely [123]. As previously discussed, enzymes with RNase H-like domains require metal binding in their active sites. HSV-1 enzymes that function in DNA replication with RNase H activity include DNA polymerase (pU_L30), single-stranded DNA binding protein (ICP8, pU_L29), and the alkaline nuclease (pU_L12) [37, 38, 40-42]. Due to their importance, these enzymes are highly conserved across *Herpesviridae*, a conclusion that is supported by the broad effects of αHTs presented in this study.

Although, αHTs were previously reported to have anti-terminase activity, inhibition of viral DNA cleavage could not be confirmed. The previous *in vitro* study assessing the nuclease activity of pU_L15 was performed with the addition of short DNA duplexes, thereby countering the inhibitory effect on DNA replication by the compounds [45]. In this study, evaluation of cleavage activity was precluded by the inability of herpesviruses to synthesize sufficient DNA in the presence of αHTs. Although not verified in this study, a concurrent inhibitory effect on the nuclease domain of the viral terminase or other RNase H-like HSV-1 enzyme is possible.

It is unclear why the compounds showed similar, but reverse potencies with the *in vitro* anti-pU_L15C assay and *in vivo* HSV-1 replication inhibition. As the *in vitro* anti-pU_L15C activities were performed with supplemented DNA, it is possible that αHTs with different side chains have alternate anti-herpesviral effects. Of the three αHTs, αHT-106 had diverging results

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with greater γ_2 protein expression and larger suppression of DNA copy number relative to αHT-111 and -115. Although Tukey's post-hoc analysis did not yield a statistically significant difference between the αHT-treated samples, the possibility of additional targets by αHT-106 cannot be excluded. Nonetheless, although some αHT compounds have negligible potency against pU_L15C based on a dual-probe fluorescent assay (i.e., αHT-115), the effect of this compound against HSV-1 replication *in vivo* was impressive with IC_{50} ranging from 1.5-5 μM, which falls into the ideal potency range for pharmaceutical development [131].

The activity of αHTs, specifically αHT-115, appears to rival those anti-herpesviral compounds currently available on the market. Although not directly compared in this study, the reported IC_{50} concentrations of acyclovir against HSV-1 range between 0.08 to 55 μM [132]. Additionally, as previous research has shown that αHTs suppress replication of acyclovir-resistant HSV-1 and -2 mutants, these compounds appear to have a different mechanism of action than acyclovir and do not require phosphorylation by viral thymidine kinase for activation [43]. Kinase-independence is a potentially important attribute of these compounds, as viral resistance to nucleoside analogues is thought to occur through acquired mutations in the viral thymidine kinase. However, as mutations in the DNA polymerase have also been documented, determination of the exact mechanism of action of these compounds is critical [96, 97].

Although anti-viral therapy is of limited in use in veterinary medicine, research has shown promising response with nucleoside analogue therapy in FHV-1 infected cats [16, 17, 24]. Specifically, IC_{50}s for the following compounds were calculated *in vitro* against FHV-1: 4.3 μM for idoxuridine, 5.2 μM for ganciclovir, 11 μM for cidofovir, 58 μM for acyclovir, and 233 μM for foscarnet [133]. Thus, αHT-115, which has a partial IC_{50} of 1.5 against FHV-1, may serve as a promising compound for use in FHV-1 infected cats.

One limitation of this research includes the exclusive use of alphaherpesviruses. However, suppression of viral replication by αHTs with HCMV, a betaherpesvirus, has previously been performed, and the inhibition by αHTs was comparative to HSV-1 [43]. Given the well-conserved nature of core genes, it is likely that these compounds would inhibit herpesviruses in each subfamily.

In conclusion, αHTs inhibit DNA replication and may comprise effective therapeutic agents against different species of herpesviruses. As certain synthetic αHT compounds have higher therapeutic indices compared to others, further optimization of therapeutic activity by alteration of the chemical side chains is worth testing. Additional studies are recommended to further evaluate the efficacy of these compounds, including the genomic evaluation of escape mutants to specifically identify the viral target(s).

REFERENCES

1.   Lodmell, D.L., et al., *Prevention of cell-to-cell spread of herpes simplex virus by leukocytes.* J Exp Med, 1973. **137**(3): p. 706-20.

2.   *Herpesvirales*, in *Fenner's Veterinary Virology*, J.N. MacLachlan and E.J. Dubovi, Editors. 2017, Academic Press: Boston. p. 189-216.

3.   Alcami, A., *Viral mimicry of cytokines, chemokines and their receptors.* Nat Rev Immunol, 2003. **3**(1): p. 36-50.

4.   *Pharynx, Larynx, and Trachea*, in *Jubb, Kennedy & Palmer's Pathology of Domestic Animals*. 2007, Elsevier: Edinburgh. p. 537-540.

5.   Mocarski, E., *Betaherpesviral genes and their functions* in *Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis.* . 2007, Cambridge University Press: Cambridge.

6.   King, D.P., et al., *Otarine herpesvirus-1: a novel gammaherpesvirus associated with urogenital carcinoma in California sea lions (Zalophus californianus).* Veterinary Microbiology, 2002. **86**(1–2): p. 131-137.

7.   Huang, S.-H., et al., *Evidence of an oncogenic Gammaherpesvirus in domestic Dogs.* Virology, 2012. **427**(2): p. 107-117.

8.   Ackermann, M., *Pathogenesis of gammaherpesvirus infections.* Vet Microbiol, 2006. **113**(3-4): p. 211-22.

9.   Graham, D.A., *Bovine herpes virus-1 (BoHV-1) in cattle–a review with emphasis on reproductive impacts and the emergence of infection in Ireland and the United Kingdom.* Irish Veterinary Journal, 2013. **66**(1): p. 15-15.

10.   Lunn, D.P., et al., *Equine Herpesvirus-1 Consensus Statement.* Journal of Veterinary Internal Medicine, 2009. **23**(3): p. 450-461.

11.   Gould, D., *Feline Herpesvirus-1.* Journal of Feline Medicine and Surgery, 2011. **13**(5): p. 333-346.

12.   Dunowska, M., *A review of equid herpesvirus 1 for the veterinary practitioner. Part A: clinical presentation, diagnosis and treatment.* N Z Vet J, 2014. **62**(4): p. 171-8.

13.   Friday, P.A., et al., *Ataxia and paresis with equine herpesvirus type 1 infection in a herd of riding school horses.* J Vet Intern Med, 2000. **14**(2): p. 197-201.

14.   Henninger, R.W., et al., *Outbreak of Neurologic Disease Caused by Equine Herpesvirus-1 at a University Equestrian Center.* Journal of Veterinary Internal Medicine, 2007. **21**(1): p. 157-165.

15. Murray, M.J., et al., *Neonatal equine herpesvirus type 1 infection on a thoroughbred breeding farm.* J Vet Intern Med, 1998. **12**(1): p. 36-41.

16. Thomasy, S.M., et al., *Evaluation of orally administered famciclovir in cats experimentally infected with feline herpesvirus type-1.* Am J Vet Res, 2011. **72**(1): p. 85-95.

17. Thomasy, S.M., et al., *Oral administration of famciclovir for treatment of spontaneous ocular, respiratory, or dermatologic disease attributed to feline herpesvirus type 1: 59 cases (2006–2013).* Journal of the American Veterinary Medical Association, 2016. **249**(5): p. 526-538.

18. Fontenelle, J.P., et al., *Effect of topical ophthalmic application of cidofovir on experimentally induced primary ocular feline herpesvirus-1 infection in cats.* Am J Vet Res, 2008. **69**(2): p. 289-93.

19. Biswas, S., et al., *Bovine herpesvirus-1 (BHV-1) - a re-emerging concern in livestock: a revisit to its biology, epidemiology, diagnosis, and prophylaxis.* Vet Q, 2013. **33**(2): p. 68-81.

20. Turin, L., S. Russo, and G. Poli, *BHV-1: new molecular approaches to control a common and widespread infection.* Mol Med, 1999. **5**(5): p. 261-84.

21. Nugent, J., et al., *Analysis of equid herpesvirus 1 strain variation reveals a point mutation of the DNA polymerase strongly associated with neuropathogenic versus nonneuropathogenic disease outbreaks.* J Virol, 2006. **80**(8): p. 4047-60.

22. Dunowska, M., *A review of equid herpesvirus 1 for the veterinary practitioner. Part B: pathogenesis and epidemiology.* N Z Vet J, 2014. **62**(4): p. 179-88.

23. Slater, J., *Equine herpesviruses.* Equine Infectious Diseases, ed. D. Sellon and M. Long. 2007, St. Louis, Missouri: Saunders Elsevier.

24. Thiry, E., et al., *Feline herpesvirus infection. ABCD guidelines on prevention and management.* J Feline Med Surg, 2009. **11**(7): p. 547-55.

25. Koelle, D.M. and L. Corey, *Herpes Simplex: Insights on Pathogenesis and Possible Vaccines.* Annual Review of Medicine, 2008. **59**(1): p. 381-395.

26. Wald A, C.L., *Persistence in the population: epidemiology, transmission. In: Arvin A, Campadelli-Fiume G, Mocarski E, et al., editors. Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis.* 2007, Cambridge: Cambridge University Press.

27. Gable, J.E., T.M. Acker, and C.S. Craik, *Current and potential treatments for ubiquitous but neglected herpesvirus infections.* Chem Rev, 2014. **114**(22): p. 11382-412.

28.   Gilbert, C., J. Bestman-Smith, and G. Boivin, *Resistance of herpesviruses to antiviral drugs: clinical impacts and molecular mechanisms.* Drug Resist Updat, 2002. **5**(2): p. 88-114.

29.   Alami Chentoufi, A., et al., *Towards a Rational Design of an Asymptomatic Clinical Herpes Vaccine: The Old, the New, and the Unknown.* Clinical and Developmental Immunology, 2012. **2012**: p. 16.

30.   Herold, B., et al., *Glycoprotein C of herpes simplex virus type 1 plays a principal role in the adsorption of virus to cells and in infectivity.* Journal of Virology, 1991. **65**(3): p. 1090-1098.

31.   Campadelli-Fiume, G. and L. Menotti, *Entry of alphaherpesviruses into the cell*, in *Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis.* 2007, Cambridge University Press: Cambridge.

32.   Fields, B.N., Knipe, D. M., Howley, P. M., & Griffin, D. E., *Fields virology.* 2001, Philadelphia: Lippincott Williams & Wilkins.

33.   Tadokoro, T. and S. Kanaya, *Ribonuclease H: molecular diversities, substrate binding domains, and catalytic mechanism of the prokaryotic enzymes.* FEBS Journal, 2009. **276**(6): p. 1482-1493.

34.   Nowotny, M., *Retroviral integrase superfamily: the structural perspective.* EMBO Rep, 2009. **10**(2): p. 144-51.

35.   Nowotny, M., et al., *Crystal structures of RNase H bound to an RNA/DNA hybrid: substrate specificity and metal-dependent catalysis.* Cell, 2005. **121**(7): p. 1005-16.

36.   Dyda, F., et al., *Crystal structure of the catalytic domain of HIV-1 integrase: similarity to other polynucleotidyl transferases.* Science, 1994. **266**(5193): p. 1981-6.

37.   Boehmer, P.E. and I.R. Lehman, *Herpes simplex virus DNA replication.* Annu Rev Biochem, 1997. **66**: p. 347-84.

38.   Yan, Z., et al., *HIV integrase inhibitors block replication of alpha-, beta-, and gammaherpesviruses.* MBio, 2014. **5**(4): p. e01318-14.

39.   Selvarajan Sigamani, S., et al., *The structure of the herpes simplex virus DNA-packaging terminase pUL15 nuclease domain suggests an evolutionary lineage among eukaryotic and prokaryotic viruses.* J Virol, 2013. **87**(12): p. 7140-8.

40.   Bryant, K.F., et al., *Identification of a divalent metal cation binding site in herpes simplex virus 1 (HSV-1) ICP8 required for HSV replication.* J Virol, 2012. **86**(12): p. 6825-34.

41.   Liu, S., et al., *Crystal structure of the herpes simplex virus 1 DNA polymerase.* J Biol Chem, 2006. **281**(26): p. 18193-200.

42. Schumacher, A.J., et al., *The HSV-1 Exonuclease, UL12, Stimulates Recombination by a Single Strand Annealing Mechanism.* PLOS Pathogens, 2012. **8**(8): p. e1002862.

43. Tavis, J.E., et al., *Inhibitors of nucleotidyltransferase superfamily enzymes suppress herpes simplex virus replication.* Antimicrob Agents Chemother, 2014. **58**(12): p. 7451-61.

44. Ireland, P.J., et al., *Synthetic alpha-Hydroxytropolones Inhibit Replication of Wild-Type and Acyclovir-Resistant Herpes Simplex Viruses.* Antimicrob Agents Chemother, 2016. **60**(4): p. 2140-9.

45. Masaoka, T., et al., *Characterization of the C-Terminal Nuclease Domain of Herpes Simplex Virus pUL15 as a Target of Nucleotidyltransferase Inhibitors.* Biochemistry, 2016. **55**(5): p. 809-19.

46. Baines, J.D., *Herpes simplex virus capsid assembly and DNA packaging: a present and future antiviral drug target.* Trends Microbiol, 2011. **19**(12): p. 606-13.

47. Owen, D.J., C.M. Crump, and S.C. Graham, *Tegument Assembly and Secondary Envelopment of Alphaherpesviruses.* Viruses, 2015. **7**(9): p. 5084-114.

48. Liu F, Z.Z., *Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis.* Comparative virion structures of human herpesviruses., ed. C.-F.G. Arvin A, Mocarski E, et al., editors. . 2007, Cambridge: Cambridge University Press.

49. Booy, F.P., et al., *Liquid-crystalline, phage-like packing of encapsidated DNA in herpes simplex virus.* Cell, 1991. **64**(5): p. 1007-15.

50. Gibson, W. and B. Roizman, *Compartmentalization of spermine and spermidine in the herpes simplex virion.* Proc Natl Acad Sci U S A, 1971. **68**(11): p. 2818-21.

51. Thiry, E., et al., *Recombination in alphaherpesviruses.* Rev Med Virol, 2005. **15**(2): p. 89-103.

52. Argnani, R., et al., *Replication-competent herpes simplex vectors: design and applications.* Gene Ther, 0000. **12**(S1): p. S170-S177.

53. Brown, J.C., *High G+C Content of Herpes Simplex Virus DNA: Proposed Role in Protection Against Retrotransposon Insertion.* Open Biochem J, 2007. **1**: p. 33-42.

54. Robinson, K.E., et al., *The essential and non-essential genes of Bovine herpesvirus 1.* J Gen Virol, 2008. **89**(Pt 11): p. 2851-63.

55. Schwyzer, M. and M. Ackermann, *Molecular virology of ruminant herpesviruses.* Vet Microbiol, 1996. **53**(1-2): p. 17-29.

56. Telford, E.A., et al., *The DNA sequence of equine herpesvirus-1.* Virology, 1992. **189**(1): p. 304-16.

57.   Maes, R., *Felid Herpesvirus Type 1 Infection in Cats: A Natural Host Model for Alphaherpesvirus Pathogenesis.* ISRN Veterinary Science, 2012. **2012**: p. 14.

58.   Roizman, B., *The function of herpes simplex virus genes: a primer for genetic engineering of novel vectors.* Proc Natl Acad Sci U S A, 1996. **93**(21): p. 11307-12.

59.   Huet, A., et al., *Extensive subunit contacts underpin herpesvirus capsid stability and interior-to-exterior allostery.* Nat Struct Mol Biol, 2016. **23**(6): p. 531-9.

60.   Booy, F.P., et al., *Finding a needle in a haystack: detection of a small protein (the 12-kDa VP26) in a large complex (the 200-MDa capsid of herpes simplex virus).* Proc Natl Acad Sci U S A, 1994. **91**(12): p. 5652-6.

61.   Okoye, M.E., et al., *Functional Analysis of the Triplex Proteins (VP19C and VP23) of Herpes Simplex Virus Type 1.* J Virol, 2006. **80**(2): p. 929-40.

62.   Brown, J.C. and W.W. Newcomb, *Herpesvirus capsid assembly: insights from structural analysis.* Curr Opin Virol, 2011. **1**(2): p. 142-9.

63.   Liang, Y., et al., *Inhibition of the histone demethylase LSD1 blocks alpha-herpesvirus lytic replication and reactivation from latency.* Nat Med, 2009. **15**(11): p. 1312-7.

64.   Fontaine-Rodriguez, E.C. and D.M. Knipe, *Herpes Simplex Virus ICP27 Increases Translation of a Subset of Viral Late mRNAs.* Journal of Virology, 2008. **82**(7): p. 3538-3545.

65.   Uprichard, S.L. and D.M. Knipe, *Herpes simplex ICP27 mutant viruses exhibit reduced expression of specific DNA replication genes.* J Virol, 1996. **70**(3): p. 1969-80.

66.   Livingston, C.M., et al., *Oligomerization of ICP4 and rearrangement of heat shock proteins may be important for herpes simplex virus type 1 prereplicative site formation.* J Virol, 2008. **82**(13): p. 6324-36.

67.   Gu, H., Y. Zheng, and B. Roizman, *Interaction of herpes simplex virus ICP0 with ND10 bodies: a sequential process of adhesion, fusion, and retention.* J Virol, 2013. **87**(18): p. 10244-54.

68.   Muylaert, I. and P. Elias, *Knockdown of DNA ligase IV/XRCC4 by RNA interference inhibits herpes simplex virus type I DNA replication.* J Biol Chem, 2007. **282**(15): p. 10865-72.

69.   Lehman, I.R. and P.E. Boehmer, *Replication of Herpes Simplex Virus DNA.* Journal of Biological Chemistry, 1999. **274**(40): p. 28059-28062.

70.   Olsson, M., et al., *Stepwise evolution of the herpes simplex virus origin binding protein and origin of replication.* J Biol Chem, 2009. **284**(24): p. 16246-55.

71.   Boehmer, P.E., *The herpes simplex virus type-1 single-strand DNA-binding protein, ICP8, increases the processivity of the UL9 protein DNA helicase.* J Biol Chem, 1998. **273**(5): p. 2676-83.

72.   Balasubramanian, N., et al., *Physical interaction between the herpes simplex virus type 1 exonuclease, UL12, and the DNA double-strand break-sensing MRN complex.* J Virol, 2010. **84**(24): p. 12504-14.

73.   Muylaert, I., K.W. Tang, and P. Elias, *Replication and recombination of herpes simplex virus DNA.* J Biol Chem, 2011. **286**(18): p. 15619-24.

74.   Nellissery, J.K., et al., *A putative leucine zipper within the herpes simplex virus type 1 UL6 protein is required for portal ring formation.* J Virol, 2007. **81**(17): p. 8868-77.

75.   Albright, B.S., et al., *Disulfide bond formation in the herpes simplex virus 1 UL6 protein is required for portal ring formation and genome encapsidation.* J Virol, 2011. **85**(17): p. 8616-24.

76.   Cockrell, S.K., et al., *Role of the UL25 Protein in Herpes Simplex Virus DNA Encapsidation.* Journal of Virology, 2009. **83**(1): p. 47-57.

77.   Yang, K., et al., *Association of herpes simplex virus pUL31 with capsid vertices and components of the capsid vertex-specific complex.* J Virol, 2014. **88**(7): p. 3815-25.

78.   Toropova, K., et al., *The herpes simplex virus 1 UL17 protein is the second constituent of the capsid vertex-specific component required for DNA packaging and retention.* J Virol, 2011. **85**(15): p. 7513-22.

79.   McNab, A.R., et al., *The Product of the Herpes Simplex Virus Type 1 UL25 Gene Is Required for Encapsidation but Not for Cleavage of Replicated Viral DNA.* Journal of Virology, 1998. **72**(2): p. 1060-1070.

80.   Bowman, B.R., et al., *Structural Characterization of the UL25 DNA-Packaging Protein from Herpes Simplex Virus Type 1.* Journal of Virology, 2006. **80**(5): p. 2309-2317.

81.   Thurlow, J.K., et al., *Herpes simplex virus type 1 DNA-packaging protein UL17 is required for efficient binding of UL25 to capsids.* J Virol, 2006. **80**(5): p. 2118-26.

82.   Mitchell, M.S., et al., *Sequence analysis of bacteriophage T4 DNA packaging/terminase genes 16 and 17 reveals a common ATPase center in the large subunit of viral terminases.* Nucleic Acids Research, 2002. **30**(18): p. 4009-4021.

83.   Adelman, K., B. Salmon, and J.D. Baines, *Herpes simplex virus DNA packaging sequences adopt novel structures that are specifically recognized by a component of the cleavage and packaging machinery.* Proc Natl Acad Sci U S A, 2001. **98**(6): p. 3086-91.

84.   Trus, B.L., et al., *The herpes simplex virus procapsid: structure, conformational changes upon maturation, and roles of the triplex proteins VP19c and VP23 in assembly.* J Mol Biol, 1996. **263**(3): p. 447-62.

85.   Levings, R.L. and J.A. Roth, *Immunity to bovine herpesvirus 1: I. Viral lifecycle and innate immunity.* Anim Health Res Rev, 2013. **14**(1): p. 88-102.

86.   Carpenter, D.E. and V. Misra, *The most abundant protein in bovine herpes 1 virions is a homologue of herpes simplex virus type 1 UL47.* J Gen Virol, 1991. **72 (Pt 12)**: p. 3077-84.

87.   Misra, V., et al., *The bovine herpesvirus alpha gene trans-inducing factor activates transcription by mechanisms different from those of its herpes simplex virus type 1 counterpart VP16.* J Virol, 1995. **69**(9): p. 5209-16.

88.   Hammerschmidt, W., H. Ludwig, and H.J. Buhk, *Specificity of cleavage in replicative-form DNA of bovine herpesvirus 1.* Journal of Virology, 1988. **62**(4): p. 1355-1363.

89.   Muylkens, B., et al., *Bovine herpesvirus 1 infection and infectious bovine rhinotracheitis.* Veterinary Research, 2007. **38**(2): p. 181-209.

90.   Kim, S.K., et al., *Interaction of the equine herpesvirus 1 EICP0 protein with the immediate-early (IE) protein, TFIIB, and TBP may mediate the antagonism between the IE and EICP0 proteins.* J Virol, 2003. **77**(4): p. 2675-85.

91.   von Einem, J., et al., *The alpha-TIF (VP16) homologue (ETIF) of equine herpesvirus 1 is essential for secondary envelopment and virus egress.* J Virol, 2006. **80**(6): p. 2609-20.

92.   Orloff, J., et al., *The future of drug development: advancing clinical trial design.* Nat Rev Drug Discov, 2009. **8**(12): p. 949-957.

93.   Ciociola, A.A., L.B. Cohen, and P. Kulkarni, *How drugs are developed and approved by the FDA: current process and future directions.* Am J Gastroenterol, 2014. **109**(5): p. 620-3.

94.   Mullard, A., *Parsing clinical success rates.* Nat Rev Drug Discov, 2016. **15**(7): p. 447-447.

95.   Umscheid, C.A., D.J. Margolis, and C.E. Grossman, *Key Concepts of Clinical Trials: A Narrative Review.* Postgraduate Medicine, 2011. **123**(5): p. 194-204.

96.   Krawczyk, A., et al., *Overcoming drug-resistant herpes simplex virus (HSV) infection by a humanized antibody.* Proceedings of the National Academy of Sciences, 2013. **110**(17): p. 6760-6765.

97.   Collins, P. and G. Darby, *Laboratory studies of herpes simplex virus strains resistant to acyclovir.* Reviews in Medical Virology, 1991. **1**(1): p. 19-28.

98.   Hanes, J.W., et al., *Enzymatic therapeutic index of acyclovir. Viral versus human polymerase gamma specificity.* J Biol Chem, 2007. **282**(34): p. 25159-67.

99.   Berdis, A.J., *DNA Polymerases as Therapeutic Targets.* Biochemistry, 2008. **47**(32): p. 8253-60.

100.   Kleymann, G., et al., *New helicase-primase inhibitors as drug candidates for the treatment of herpes simplex disease.* Nat Med, 2002. **8**(4): p. 392-8.

101.   Wald , A., et al., *Helicase–Primase Inhibitor Pritelivir for HSV-2 Infection.* New England Journal of Medicine, 2014. **370**(3): p. 201-210.

102.   Wald, A., et al., *Effect of pritelivir compared with valacyclovir on genital hsv-2 shedding in patients with frequent recurrences: A randomized clinical trial.* JAMA, 2016. **316**(23): p. 2495-2503.

103.   Chono, K., et al., *ASP2151, a novel helicase–primase inhibitor, possesses antiviral activity against varicella–zoster virus and herpes simplex virus types 1 and 2.* Journal of Antimicrobial Chemotherapy, 2010. **65**(8): p. 1733-1741.

104.   Chono, K., et al., *Synergistic activity of amenamevir (ASP2151) with nucleoside analogs against herpes simplex virus types 1 and 2 and varicella-zoster virus.* Antiviral Res, 2013. **97**(2): p. 154-60.

105.   Yajima, M., et al., *Profile of anti-herpetic action of ASP2151 (amenamevir) as a helicase-primase inhibitor.* Antiviral Res, 2017. **139**: p. 95-101.

106.   van Zeijl, M., et al., *Novel class of thiourea compounds that inhibit herpes simplex virus type 1 DNA cleavage and encapsidation: resistance maps to the UL6 gene.* J Virol, 2000. **74**(19): p. 9054-61.

107.   Newcomb, W.W. and J.C. Brown, *Inhibition of herpes simplex virus replication by WAY-150138: assembly of capsids depleted of the portal and terminase proteins involved in DNA encapsidation.* J Virol, 2002. **76**(19): p. 10084-8.

108.   Bloom, J.D., et al., *Thiourea inhibitors of herpes viruses. Part 2: N-Benzyl-N'-arylthiourea inhibitors of CMV.* Bioorg Med Chem Lett, 2004. **14**(13): p. 3401-6.

109.   Goldner, T., et al., *Geno- and Phenotypic Characterization of Human Cytomegalovirus Mutants Selected In Vitro after Letermovir (AIC246) Exposure.* Antimicrobial Agents and Chemotherapy, 2014. **58**(1): p. 610-613.

110.   Goldner, T., et al., *The novel anticytomegalovirus compound AIC246 (Letermovir) inhibits human cytomegalovirus replication through a specific antiviral mechanism that involves the viral terminase.* J Virol, 2011. **85**(20): p. 10884-93.

111.   Lischka, P., et al., *In Vitro and In Vivo Activities of the Novel Anticytomegalovirus Compound AIC246.* Antimicrobial Agents and Chemotherapy, 2010. **54**(3): p. 1290-1297.

112.   Kuchta, K., et al., *Comprehensive classification of nucleotidyltransferase fold proteins: identification of novel families and their representatives in human.* Nucleic Acids Res, 2009. **37**(22): p. 7701-14.

113.   Yang, W. and T.A. Steitz, *Recombining the structures of HIV integrase, RuvC and RNase H.* Structure. **3**(2): p. 131-134.

114.   Klumpp, K., et al., *Two-metal ion mechanism of RNA cleavage by HIV RNase H and mechanism-based design of selective HIV RNase H inhibitors.* Nucleic Acids Research, 2003. **31**(23): p. 6852-6859.

115.   Frank, P., et al., *Cloning, subcellular localization and functional expression of human RNase HII.* Biol Chem, 1998. **379**(12): p. 1407-12.

116.   Katayanagi, K., et al., *Three-dimensional structure of ribonuclease H from E. coli.* Nature, 1990. **347**(6290): p. 306-9.

117.   Lai, L., et al., *Crystal structure of archaeal RNase HII: a homologue of human major RNase H.* Structure, 2000. **8**(8): p. 897-904.

118.   Lima, W.F., H. Wu, and S.T. Crooke, *Human RNases H.* Methods Enzymol, 2001. **341**: p. 430-40.

119.   Himmel, D.M., et al., *Structure of HIV-1 reverse transcriptase with the inhibitor beta-Thujaplicinol bound at the RNase H active site.* Structure, 2009. **17**(12): p. 1625-35.

120.   Billamboz, M., et al., *Magnesium chelating 2-hydroxyisoquinoline-1,3(2H,4H)-diones, as inhibitors of HIV-1 integrase and/or the HIV-1 reverse transcriptase ribonuclease H domain: discovery of a novel selective inhibitor of the ribonuclease H function.* J Med Chem, 2011. **54**(6): p. 1812-24.

121.   Kirschberg, T.A., et al., *RNase H active site inhibitors of human immunodeficiency virus type 1 reverse transcriptase: design, biochemical activity, and structural information.* J Med Chem, 2009. **52**(19): p. 5781-4.

122.   Agrawal, A., et al., *Probing chelation motifs in HIV integrase inhibitors.* Proc Natl Acad Sci U S A, 2012. **109**(7): p. 2251-6.

123.   Meck, C., et al., *The biology and synthesis of alpha-hydroxytropolones.* MedChemComm, 2014. **5**(7): p. 842-852.

124.   Bentley, R., *A fresh look at natural tropolonoids.* Natural Product Reports, 2008. **25**: p. 118-138.

125. Hirsch, D.R., et al., *Inhibition of the ANT(2")-Ia resistance enzyme and rescue of aminoglycoside antibiotic activity by synthetic alpha-hydroxytropolones.* Bioorg Med Chem Lett, 2014. **24**(21): p. 4943-7.

126. Meck, C., N. Mohd, and R.P. Murelli, *An Oxidopyrylium Cyclization/Ring-Opening Route to Polysubstituted α-Hydroxytropolones.* Organic Letters, 2012. **14**(23): p. 5988-5991.

127. Williams, Y.D., et al., *Triflic Acid-Mediated Rearrangements of 3-Methoxy-8-oxabicyclo[3.2.1]octa-3,6-dien-2-ones: Synthesis of Methoxytropolones and Furans.* The Journal of Organic Chemistry, 2013. **78**(23): p. 11707-11713.

128. Thompkins, K., et al., *Temperature Sensitivity and Cell Division Defects in an Escherichia coli Strain with Mutations in yghB and yqjA, Encoding Related and Conserved Inner Membrane Proteins.* Journal of Bacteriology, 2008. **190**(13): p. 4489-4500.

129. Yang, K., E.G. Wills, and J.D. Baines, *A Mutation in U(L)15 of Herpes Simplex Virus 1 That Reduces Packaging of Cleaved Genomes.* Journal of Virology, 2011. **85**(22): p. 11972-11980.

130. Lu, G., et al., *Hydroxylated Tropolones Inhibit Hepatitis B Virus Replication by Blocking Viral Ribonuclease H Activity.* Antimicrobial Agents and Chemotherapy, 2015. **59**(2): p. 1070-1079.

131. Hughes, J.P., et al., *Principles of early drug discovery.* British Journal of Pharmacology, 2011. **162**(6): p. 1239-1249.

132. *ZOVIRAX® [package insert]. DSM Pharmaceuticals, Inc., Greenville, NC; November 2003.* [cited 2017; Available from: https://www.accessdata.fda.gov/drugsatfda_docs/label/2004/18603slr027_zovirax_lbl.pdf

133. Maggs, D.J. and H.E. Clarke, *In vitro efficacy of ganciclovir, cidofovir, penciclovir, foscarnet, idoxuridine, and acyclovir against feline herpesvirus type-1.* Am J Vet Res, 2004. **65**(4): p. 399-403.

VITA

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