UNDERSTANDING THE MECHANISM OF ANTIRETROVIRAL NUCLEOSIDE ANALOGS AS INHIBITORS OF EPSTEIN-BARR VIRUS LYTIC DNA REPLICATION

by

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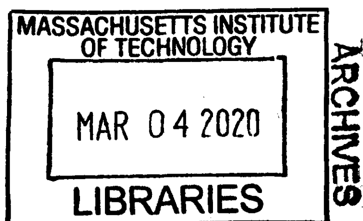

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Abstract

Epstein-Barr virus (EBV) is a human B-cell tropic double-stranded DNA γ-herpesvirus. To date, there are no antiviral agents proven to be clinically effective in the treatment of EBV infection or EBV-associated diseases. In the experiments contained in this thesis, we define the ability of nucleoside/nucleotide analogs licensed for the treatment of HIV to inhibit EBV lytic DNA replication *in vitro*.

Using an established system of EBV lytic DNA replication in HH514-16 cells induced with butyrate, we validate azidothymidine (AZT) as an inhibitor of EBV lytic DNA replication. We further demonstrate that several antiretroviral nucleoside/nucleotide analogs, including stavudine (d4T), abacavir (ABC), tenofovir disoproxil fumarate (TDF) and tenofovir alafenamide (TAF), effectively inhibit EBV DNA replication. Inhibition of DNA replication by these compounds is specific to the lytic cycle, and primarily attributable to effects on the viral DNA polymerase by drug-triphosphates, except in the case of AZT.

We extend studies of the tenofovir prodrugs TDF and TAF to show that these compounds are not only effective, but highly potent inhibitors of EBV lytic DNA replication. TAF has a 35- and 24-fold, and TDF has a 10- and 7-fold lower IC_{50} than acyclovir and penciclovir, respectively. TAF is also twice as potent as the β-herpesviral drug ganciclovir. *In vitro*, the active metabolite of TDF and TAF, tenofovir-diphosphate, is more potent than acyclovir-triphosphate at inhibiting dNTP incorporation into a DNA template by the EBV DNA polymerase. A functional consequence of bypassing viral-dependent drug metabolism is the ability to initiate treatment prior to the viral lytic cycle.

Additionally, we include a clinical case report of a patient with relapsing-remitting multiple sclerosis who experienced symptomatic and radiologic improvement with Combivir (AZT/3TC), and suggest these effects may be mediated via effects on EBV.

These studies highlight the need for further investigation and detailed characterization of antiviral agents for EBV and other herpesviruses. The framework presented in this thesis provides an experimental approach for testing candidate nucleoside/nucleotide analogs and probing their interactions with both cellular and viral targets. This work may inform the selection of drugs for clinical translation in the treatment of EBV infection and EBV-associated diseases.

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CHAPTER 1: BACKGROUND AND SIGNIFICANCE

1.1 Herpesviruses – Overview

1.1.1 Classification of herpesviruses

Herpesviruses are enveloped double-stranded DNA (dsDNA) viruses that are highly disseminated in nature. (1) Nine herpesviruses have been identified as human pathogens – herpes simplex virus 1 (HSV1), herpes simplex virus 2 (HSV2), varicella-zoster virus (VZV), Epstein-Barr virus (EBV), human cytomegalovirus (HCMV), human herpesvirus 6A, 6B, and 7 (HHV6A, HHV6B, and HHV7), and Kaposi's sarcoma-associated herpesvirus (KSHV). (1) A list of human diseases associated with these viruses are listed in Table 1.1. All members of the human *Herpesviridae* family share several key features – namely, they encode a large number of viral enzymes involved in DNA biosynthesis, they perform viral replication in the host cell nucleus, and they can exist in both a latent and lytic phase. (1) However, human herpesviruses also differ among themselves concerning host range, cell tropism, and the fate of infected cells. Based on the variation in these biological properties, human herpesviruses are historically categorized intro three subfamilies: α, β, and γ. (2) First, α-herpesviruses (HSV1, HSV2, and VZV) are distinguished by a highly variable host range, the rapid spread of infection with lysis of infected cells *in vitro*,

and establishment of latency in sensory ganglia *in vivo*. (2) On the other hand, β-herpesviruses (HCMV, HHV6A/B, and HHV7) have a restricted host range, spread slowly *in vitro*, demonstrate a cytopathic effect (cytomegalia), and can establish latency in many different cell types *in vivo*. (2) Finally, γ-herpesviruses (EBV, KSHV) also have narrow host restriction like β-herpesviruses but infect lymphocytes (either B-cells or T-cells), replicate in lymphoblastoid cells *in vitro*, and primarily establish latency in lymphoid tissues *in vivo*. (2)

Table 1.1: Disease associations of human herpesviruses

Herpesvirus	Disease Associations	Subfamily	Group
HSV-1	orolabial herpes, genital herpes; keratoconjunctivitis; encephalitis	α	E
HSV-2	genital herpes	α	E
VZV	chicken pox, shingles	α	D
EBV	infectious mononucleosis, Burkitt lymphoma, Hodgkin lymphoma, nasopharyngeal cancer, gastric cancer, T-cell lymphomas, multiple sclerosis, systemic lupus erythematosus	γ	C
HCMV	IM-like illness, congenital infection, immunocompromised-related diseases (esophagitis, retinitis)	β	E
HHV6A	IM-like illness	β	A
HHV6B	IM-like illness	β	A
HHV7	IM-like illness	β	A
KSHV	Kaposi sarcoma, Castleman disease	γ	C

1.1.2 Structure of herpesviruses

Members of the human *Herpesviridae* family produce mature virions (~120nm-diameter) that are enveloped and contain encapsidated dsDNA genomes. (3) Each icosahedral core contains a single copy of the viral genome arranged as a torus. (4) Viral genes with restricted expression during the lytic phase encode components of the nucleocapsid, viral envelope, and tegument (defined as the proteins between the envelope and capsid). (5) Proteomic studies have demonstrated that virions also contain an array of host proteins, including structural and chaperone proteins like actin, tubulin, HSP70, and HSP90. (6) These may play a role in facilitating viral infection and replication.

1.1.3 Genome organization of herpesviruses

Herpesvirus particles contain linear dsDNA genomes. For human herpesviruses, the length of the genome ranges from 125-236 kbp. (1) Following infection, viral genomes circularize in the nucleus and persist as extrachromosomal DNA. (7–9) Multiple copies of both terminal and internal repeat sequences are present in wild-type herpesviral genomes. (2) The location and organization of these repeats divide herpesviruses into six distinct groups – designated A-F. (1) Human herpesviruses are classified as follows: type A (HHV6A/6B and HHV7), type C (EBV and KSHV), type D (VZV), and type E (HSV1/2 and HCMV). (1) Type C herpesviruses, which are

the subject of this thesis, contain short terminal repeats at each end of the linear viral genome in the same orientation (typically 2-5 tandem 0.5-kbp repeats), as well as a variable number of internal repeats that divide the genome into distinct segments. (1, 2)

Of note, extensive passaging in cell culture has led to large deletions in commonly used laboratory strains. For example, the EBV wild-type genome is ~171 kbp in length, but established cell lines that carry the virus as episomes (e.g., P3HR1, B95-8, etc.) are missing segments >5kbp encoding multiple viral genes and regulatory elements. (10, 11) Similar to EBV, HCMV clinical isolates carry at least 19 genes not found in laboratory strains. (12) These mutant variants have been invaluable for mapping viral functions to specific areas of herpesviral genomes.

1.1.4 Regulation of herpesviral gene expression

Herpesviruses contain 70-200 open reading frames. (1) Of these, 41 are considered core protein-coding herpesvirus genes because they are conserved across all subfamilies (α, β, and γ). These genes are critical for nucleotide metabolism, DNA replication, and virion architecture. (1) However, herpesviruses also encode genes unique to a particular subfamily or individual virus. For example, LMP1 and 2 are specific to EBV, a B-cell tropic virus, and function to modulate B-cell signaling. The

expression of viral genes also differs significantly between the latent and lytic cycle. In latency, few viral genes are expressed, primarily restricted to those involved in the maintenance of the viral episome (e.g., EBNA1 for EBV which tethers the episome to host chromosomal DNA). (13) In contrast, nearly all viral genes are robustly expressed during the lytic phase. Most genes are transcribed by the cellular RNA polymerase II except for small transcripts reliant on the cellular RNA polymerase III (e.g., EBERs in EBV which are small non-coding RNAs). (13, 14) Since more than 50% of herpesviral encoded genes are dispensable for viral replication in cells in culture, (1) the function of many viral proteins remains unknown. These are likely important for effective colonization and dissemination *in vivo*. For example, the viral ribonucleotide reductase of HSV1 is dispensable for viral production *in vitro*. (15) However, mutants of this enzyme reduce virulence by 10^6-fold, (16) and are critical for acute viral replication and establishment of latency *in vivo*. (17)

During the lytic cycle, herpesvirus gene expression is temporally regulated by mechanisms that are conserved across subfamilies. (1) The immediate-early genes, expressed first, encode major viral transcription factors (e.g., BZLF1 and BRLF1 for EBV). (18–21) These are followed by the expression of early genes, which are defined by transcription in a manner independent of viral DNA replication, and encode components of the DNA replication machinery. (1, 22) Finally, the

expression of viral late genes is dynamically regulated by the process of continuous DNA synthesis. Late genes mainly encode structural proteins involved in nucleocapsid packaging and assembly, as well as envelope glycoproteins. (1, 22, 23) The transcriptional dependence of structural components on active DNA replication ensures the coordination of viral genome replication with virion assembly. Particularly relevant to the work presented in this thesis, this feature of herpesviruses provides an avenue to reduce viral gene expression in infected cells by targeting viral DNA synthesis. Inhibitors of viral DNA replication are therefore not only useful for preventing infection from spreading to naïve cells but can also affect gene expression in virus-producing cells.

1.1.5 Herpesviral lytic DNA replication machinery

Herpesviruses encode an extensive array of enzymes involved in nucleotide biosynthesis and DNA replication. All human herpesviruses encode a viral ribonucleotide reductase large subunit, uracil DNA glycosylase, and deoxyuridine triphosphatase. (1) Concerning DNA replication, all herpesviruses also carry a viral helicase/primase complex, DNA polymerase catalytic subunit and processivity factor, and ssDNA binding protein. (1) β-herpesviruses differ from α- and γ-herpesviruses because they lack a ribonucleotide reductase small subunit (therefore lack a functional enzyme), (24) and also lack a viral thymidine kinase. (25, 26) The

latter feature has important clinical implications for the metabolism of anti-herpesviral drugs as covered later in this chapter. Briefly, drugs that require a viral thymidine kinase for metabolism are not effective against β-herpesviruses. Therefore, clinical treatment strategies differ by viral subfamily.

1.2 Epstein-Barr virus (EBV) - Overview

1.2.1 Discovery of EBV

In 1958, Denis Burkitt described 38 cases of a sarcoma involving the jaws of children in Uganda. (27) This malignancy bears his name – Burkitt lymphoma. Embarking on a safari to map the distribution of disease across Africa, Burkitt noticed that this lymphoma was restricted to a demarcated geographical area, and only found at low altitudes. (28) The epidemiology of cases was consistent with a transmissible agent (now known to be co-infection with malaria). Burkitt gave a lecture on these findings at the Middlesex Hospital in London in 1961. This lecture was attended by Michael Anthony Epstein, a pathologist with extensive expertise in electron microscopy. Because of the suspicion of an infectious etiology, Epstein requested samples of tumor cells to be sent to London. In 1964, Epstein and his graduate student Yvonne Barr described the existence of viral particles in cultured lymphoblasts from Burkitt lymphoma by electron microscopy. (29) These resembled the appearance of herpes simplex virus but were 20% smaller in size. Cell lines were subsequently shipped to Werner and Gertrude Henle in Philadelphia, who developed a serological test by immunofluorescence in 1966. (30) The following year, a technician in the Henle lab developed infectious mononucleosis (IM), and they were able to demonstrate seroconversion to EBV since they had previously

tested everyone in their lab. (31) The link between EBV and IM was confirmed shortly after in 1968. (32) Ensuing epidemiological studies revealed that 80%-90% of people had been infected with EBV by adulthood. (33) Unexpectedly, the search for an infectious agent responsible for the geographic distribution of Burkitt lymphoma cases led instead to the discovery of a ubiquitous worldwide pathogen.

1.2.2 Cell tropism, attachment, and entry of EBV

A schematic of EBV infection is illustrated in Figure 1.1. EBV primarily infects B-cells and epithelial cells *in vivo*. (13) Following endocytosis, entry of EBV into B-cells is mediated by two interactions - binding of the viral envelope glycoprotein gp350/220 to the CD21 or CD35 receptor, and binding of a second glycoprotein gp42 to HLA-class II. (34) This binding triggers fusion with the host cell membrane, mediated via a core complex formed by the glycoproteins gH/gL and the fusogen gB. (35) Similarly, entry of EBV into epithelial cells is facilitated by binding of gp350/220 to CD21, or BMRF2 and gH/gL to integrins on the host cell surface, (36–38) where gB subsequently triggers fusion. Importantly, EBV lacking gH cannot infect B cells or epithelial cells, making the core fusion complex a promising vaccine candidate for sterilizing immunity. (39, 40) After entry, the EBV genome is transported and released into the nucleus where it has two fates – EBV can either establish latency via EBNA-1 binding and tethering of the viral genome to host chromosomal DNA or

undergo lytic DNA replication upon expression of the major viral transcription factors BZLF1 and BRLF1. (13)

Figure 1.1

Note: this figure has been adapted with modifications from R. M. Longnecker, E. Kieff, J. I. Cohen, "Epstein-Barr Virus" in Fields Virology, 6th Ed., D. M. Knipe, P. M. Howley, Eds. (2013)

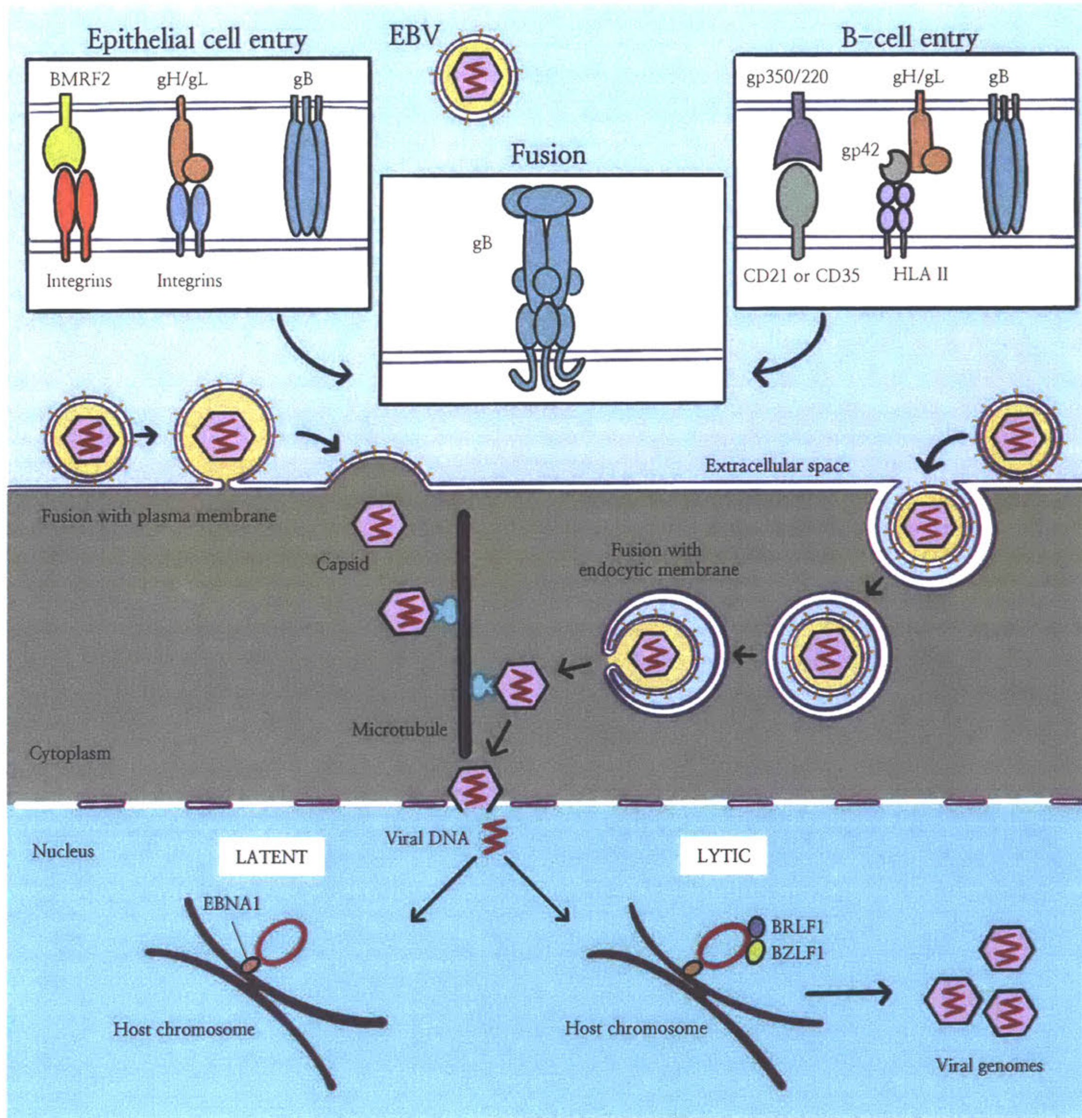


Figure 1.1 EBV infection in B-cells and epithelial cells

EBV enters B-cells via endocytosis and binding of gp350/220 to CD21 or CD35 and gH/gL/gp42 to HLA class II on endocytic membranes. For epithelial cell entry, EBV enters via BMRF2 or gH/gL binding to integrins at the plasma membrane. In both cell types, the glycoprotein gB triggers fusion. The viral capsid is released into the cytoplasm and transported to the nucleus via microtubules, where the viral DNA circularizes. The viral protein EBNA-1 tethers the EBV episome to host chromosomes, permitting viral latency. Alternatively, expression of the major viral transcription factors BZLF1 and BRLF1 can initiate the lytic cycle for production of new viral genomes.

1.2.3 EBV viral products involved in latency

The work in this thesis deals with events in the lytic cycle, so latency will only be covered briefly in this section for completeness. There are four EBV latency programs (termed latency 0-3). (13) In infected memory B-cells in the blood of healthy carriers, no viral proteins are expressed ("latency 0"). (41) This has implications for immune recognition of circulating EBV+ cells. However, in lymphoblastoid cell lines and malignancies, limited and distinct combinations of latent viral proteins can be found (termed "latency 1-3"). (13) Viral proteins involved in latency include several nuclear antigens (EBNAs), and latent membrane proteins (LMPs). EBNA-1, which is expressed in all EBV-driven malignancies, is critical for the maintenance of the viral episome during cell division. EBNA-1 binds to an 18-bp recognition sequence repeated in the viral latent origin of replication (oriP), and interacts with host chromosomes to support the association of viral DNA with host DNA during mitosis. (42–46) This interaction ensures that viral DNA is replicated by the host DNA replication machinery and maintained in progeny cells. Because of its role in episome maintenance, drugs that target EBNA-1 are an active area of investigation for potential clearance of EBV by the prevention of viral latency. (47) Additionally, EBNA-1 binding to oriP acts as an enhancer for the expression of latent genes. (48) Another viral product, EBNA-2, is required for the ability of EBV to transform primary B-cells. (49, 50) Like EBNA-1, EBNA-2 transactivates the

expression of viral genes involved in latency. (51, 52) However, it also activates the expression of cellular genes containing EBNA-2 response elements, like c-myc. (53) EBNA-LP and EBNA3A-C play essential roles in cell growth by potentiating the function of EBNA-2. (13) In addition to nuclear antigens, EBV encodes the latent membrane proteins LMP1 and LMP2A/B. These integral membrane proteins mimic CD40 and B-cell receptor signaling, thus enabling growth and survival. (54, 55) Latently infected cells also express non-coding RNAs, including EBERs and viral miRNAs. EBERs are the most abundant transcripts in latent cells. (56–58) These reside in the nucleus and form secondary structures that sequester cellular proteins (59–61) and function to increase resistance to interferon. (62, 63) Other viral products, such as miRNAs, also play essential roles in B-cell transformation and regulation of apoptosis. (13)

1.2.4 EBV viral products involved in lytic replication

Escape from latency can occur in response to various stimuli that trigger the expression of the major viral transcription factors Zta and Rta. (64–68) *In vitro*, these include epigenetic modifying agents like butyrate or 5-Aza-2′-deoxycytidine, as well as phorbol esters and calcium ionophores. These serve as artificial ways of inducing EBV in cell culture. Physiologically, the trigger for lytic reactivation is thought to be linked to the differentiation state of the cell. This is supported by evidence that EBV

DNA replication only occurs in the outermost layer of the oral epithelium, (69, 70) and that the process of plasma cell differentiation can induce the lytic cycle in EBV infected B-cells *in vivo*. (71)

Initiation of the lytic cycle by Zta and Rta leads to transcription of early genes involved in DNA replication, followed temporally by late genes involved in viral assembly. (72–74) Similar to other herpesviruses, EBV early genes are functionally differentiated from late genes by their transcription in the presence of DNA replication inhibitors. (22). Early gene products include proteins involved in nucleotide metabolism, such as the viral encoded thymidine kinase (BXLF1) and protein kinase (BGLF4). (5) Unlike the viral thymidine kinase of HSV1/2, BXLF1 cannot phosphorylate the antiviral drugs acyclovir or ganciclovir. (75–77) Correspondingly, BGLF4, but not BXLF1, is required for the sensitivity of EBV to acyclovir and ganciclovir in cell culture. (78) However, BGLF4 has not been demonstrated to phosphorylate either drug directly. These differences in viral-mediated drug metabolism will be discussed in more detail later as they are important for experiments presented later in this thesis.

DNA replicative proteins also include the viral ribonucleotide reductase large and small subunits (BORF2 and BaRF1), viral DNA polymerase and processivity subunit

(BALF5 and BMRF1), ssDNA binding protein (BALF2), primase/helicase complex (BBLF4 and BSLF1), uracil DNA glycosylase (BKRF3), and alkaline exonuclease (BGLF5). (5) Additionally, lytic DNA replication requires host enzymes, particularly the DNA topoisomerases 1 and 2. (79)

The EBV genome contains two separate lytic origins of replication positioned opposite each other in the viral episome, characterized by a duplicated 1055-bp sequence. (80) As for other herpesviruses, EBV lytic DNA replication occurs via rolling circle amplification yielding linear concatemers, which are cleaved and packaged into virions. (80) Newly synthesized viral DNA is unmethylated and only becomes methylated after infection and circularization in a recipient cell. (80) Methylation is required for Zta recognition, binding, and transcription of lytic genes, but differentially affects Rta-mediated transcription. (81–83) Thus, host-cell state at infection may play a critical role in determining the landscape of viral genome methylation, and therefore propensity for lytic reactivation, long after the establishment of latency.

Packaging of newly synthesized viral DNA requires products of late lytic genes, including a wide array of capsid, tegument, and envelope glycoproteins. The EBV genome is complex, encoding nearly 100 viral proteins that perform diverse

functions. (13) Beyond viral replication, many lytic proteins also play crucial roles in suppression of the host antiviral response, bypassing innate and adaptive immune recognition. (84–94) These proteins may be relevant to efforts to enhance the immune response to EBV *in vivo*. Importantly, since late lytic gene expression is directly tied to viral DNA replication, inhibitors of DNA replication may increase the host immune response to EBV via the downregulation of immunomodulatory viral gene products.

1.3 EBV infection and disease: short-term and long-term consequences

1.3.1 Primary EBV infection

Exposure to EBV is nearly ubiquitous worldwide, where >80-90% of people are infected with EBV by adulthood. (33, 95) In developing countries, children are infected with EBV at a younger age than in industrialized countries, with the majority infected before age 1. (96) Sharing of personal items contaminated with oropharyngeal secretions - such as toys, cups, or toothbrushes - is thought to be the predominant route of viral transmission early in life. When EBV is acquired in childhood, the initial infection either goes unnoticed or is accompanied by mild flu-like symptoms. (97, 98) In contrast, when EBV is acquired later in life, typically during adolescence, it can cause infectious mononucleosis (IM). IM is characterized by malaise, high fever, enlarged cervical lymph nodes, splenomegaly, hepatomegaly, and fatigue. Severe complications of IM can occur, including splenic rupture, encephalitis, anemia, thrombocytopenia, hepatitis, myocarditis, as well as other rare but life-threatening complications. However, most cases are self-limiting. During acute IM, up to 50% of circulating memory B cells are EBV-positive. (99) After initial infection, the incubation period preceding the development of acute IM is 4-6 weeks, after which symptoms typically resolve in 2-4 weeks. (13) IM-associated fatigue may persist for a significantly more extended period though. The reason for

this is unclear as the occurrence of disabling symptoms for six months or longer has not been reported to be correlated with either viral or immune parameters. (100) IM-associated fatigue is thought to result from the overwhelming immune response to primary EBV infection. However, fatigue is also a symptom associated with EBV-related diseases, such as multiple sclerosis, which occur years after the initial exposure. There is no antiviral therapy for IM - the current standard of care is to wait for the symptoms to pass and, if necessary, provide symptomatic relief. (101)

1.3.2 EBV and multiple sclerosis

Note: The following section is important for the work presented in this thesis, as trying to understand the role of EBV in multiple sclerosis (MS) is the reason why this work was performed. As described in later chapters, this thesis began as an effort to try to explain case reports of patients with MS who responded to antiviral drugs used clinically for the treatment of HIV by examining the effects of these drugs on EBV. While these cases will be discussed in more detail later, what follows here is a brief summary of MS, and the evidence for a causal role for EBV in MS.

MS is an inflammatory disease of the central nervous system of unknown etiology that causes demyelinating lesions in the brain and spinal cord. (102) It is diagnosed using clinical and radiological evidence of dissemination of lesions in space and

time, which may be supplemented by laboratory investigations. (102) In >90% of cases, oligoclonal bands can be found in the CSF (102), although it remains unclear if this is a cause or consequence of underlying damage to neuronal tissue. MS generally follows a relapsing-remitting course. Over time, recovery from clinical relapses is incomplete and most patients develop secondary progressive disease around age 40. (102) In a minority of patients, the disease is progressive at diagnosis. (102) The median time to death is 30 years from disease onset. (102)

MS has both a genetic and environmental component. Having a family member with MS increases the risk of developing MS, with a relative risk of 9.2 for first-degree relatives, 3.4 for second-degree relatives and 2.9 for third-degree relatives compared to a general population risk. (102) Additionally, the concordance rate is 25-30% in monozygotic twins vs. 2-5% in dizygotic twins. (103) However, most twin pairs remain discordant, suggesting the involvement of environmental risk factors. Studies looking at immigration from a country of low risk to high risk and vice versa have pinpointed the exposure to an environmental agent before the age of 15. (104–106)

Dozens of endogenous and exogenous infectious agents have been suggested to play a role in MS. However, the viral pathogen repeatedly linked to MS by

epidemiological and serological studies is Epstein-Barr virus (EBV). Patients with MS have higher EBV antibody titers than those without disease, (107–111) and the rise in antibody titers predicts the development of MS. (112, 113) Patients with MS are universally seropositive to EBV compared to 90-95% of controls. (114–116) Seropositivity to EBV is also significantly more frequent in pediatric patients with MS than in children without disease. (117–119) Among those who do not have EBV, the risk of MS is extremely low (OR 0.06), but increases sharply in the same individuals after infection. (120) MS is also associated with IM. (121, 122)

However, since the majority of the world's population has been infected with EBV, the role of this virus in the pathogenesis of MS has remained unclear. Clinical trials with the anti-herpesvirus drugs acyclovir and valacyclovir, covered later in this chapter, were unsuccessful for MS (123, 124), which is often cited as evidence for the lack of the involvement of EBV in MS. The failure of acyclovir and valacyclovir does not necessarily rule out the absence of persistent EBV infection, as discussed in detail in this thesis. To this point, patients with MS are deficient in CD8+ T-cell responses to EBV, (125, 126) and the adoptive transfer of autologous EBV-specific cytotoxic T-cells has reported promise in early clinical trials to treat advanced progressive disease refractory to standard treatment. (127, 128) While these studies establish EBV as a risk factor for the development of MS, a mechanistic role for EBV

remains ill-defined. More effective antivirals would be required to determine if EBV is a viable target in the treatment of MS.

1.3.3 EBV and cancer

EBV seropositivity is strongly correlated with neoplastic diseases in otherwise healthy non-immunocompromised persons. (129) These occur years to decades after initial infection by mechanisms that are poorly understood. The role of EBV as a causal agent in some EBV-associated cancers has been well-documented - including Burkitt's lymphoma, Hodgkin's lymphoma, nasopharyngeal carcinoma, gastric carcinoma, diffuse large B-cell lymphoma, as well as rare T-cell and NK-cell lymphomas. (130) Together, these account for ~1.5% of new cancers worldwide. (129) However, a recent study has detected EBV gene expression in a significant proportion (~10%) of tumor samples using RNA-seq data from 23 cancer types. (131) Additionally, EBV genomes have been detected by PCR in a high proportion of invasive breast tumors (51%), but only 10% of healthy tissue adjacent to the tumor ($p<0.001$). (132) Importantly, *in vitro* EBV infection of breast cancer cells has functional consequences – it confers resistance to paclitaxel and leads to overexpression of the multidrug resistance gene MDR1. (133) EBV genomes have also been detected in tissue samples from 5.2% of ovarian cancer samples, but only 0.5% of normal tissue ($p=0.011$). (134) These studies suggest that EBV may

have a broader contribution to carcinogenesis either by direct infection of target cells or indirectly as a contributor to local inflammation.

It is unclear whether or not antiviral therapy for the treatment of primary EBV infection would have an impact on the burden of EBV-associated cancers later in life. However, evidence suggests that late onset (and thus severity) of primary infection, as well as immune control of EBV, play a critical role in cancer risk. Specifically, IM is associated with Hodgkin's lymphoma, breast cancer, (135) and ovarian cancer. (136) Children with increased IgG antibody titers to the viral capsid antigen (VCA), a late lytic gene product, are at higher risk of developing Burkitt's lymphoma. (137) Furthermore, elevations in IgG or IgA to VCA and IgG to EA-D, both lytic antigens, precede the development of Hodgkin's lymphoma by several years, and elevations in these antibodies carry a 3-4 fold relative risk of disease. (138) IgA to EBV is elevated up to 10 years before the diagnosis of nasopharyngeal cancer, with a mean duration of 3 years. (139) Rising IgA to VCA confers a 21-fold increased risk of nasopharyngeal cancer. (140) Similarly, levels of IgA to VCA are higher in persons who develop EBV-positive gastric carcinoma than controls, and titers of IgG to VCA is higher in those who develop EBV-positive vs. EBV-negative cancer. (141) These studies not only suggest a lack of adequate control of EBV may play a role in the development of EBV-associated cancers but that there may be a

window of time spanning several years before the event of disease onset during which interventions with antivirals may be beneficial to prevent disease. The association of antibodies to EBV lytic antigens with risk of cancers suggests that lytic reactivation may play a role in disease.

1.3.4 EBV diseases in immunosuppressed hosts

Primary (congenital; e.g., X-linked lymphoproliferative disease) or secondary (HIV or iatrogenic) immunodeficiency can also lead to complications from EBV, underscoring the importance of immune control in preventing disease. Of particular relevance to the studies outlined in this thesis, a wide range of EBV-associated conditions present in the setting of HIV infection – including oral hairy leukoplakia, diffuse large B-cell lymphoma, CNS lymphoma, plasmablastic lymphoma, primary effusion lymphoma, and EBV-positive smooth muscle tumors. (13) These are likely related to the loss of immune control of EBV in the setting of HIV infection. Patients with HIV have higher EBV viral loads and are at higher risk of EBV-related malignancies. (142–145) These patients also have higher levels of anti-EBV antibodies, as well as poor T-cell mediated immune control of EBV. (146) However, in patients treated with highly active antiretroviral therapy (HAART), the number of lytic-specific T-cells is reduced compared to untreated patients, suggesting a decreased EBV reactivation rate. (147) Additionally, the incidence of EBV-

associated lymphomas in patients with HIV decreases with HAART treatment. (148) It is unclear if these findings are attributable to particular drugs, or are a general feature of treatment with antiretrovirals, since HAART regimens contain several concurrent medications. Outside of HIV, EBV-driven lymphoproliferative disorders can occur in the setting of drug-related immunosuppression. Post-transplant lymphoproliferative disease (PTLD) is an IM-like illness that arises in ~3% of solid organ transplant recipients. (149) High levels of EBV DNA in blood and weak CD8+ T-cell responses to EBV predict the occurrence of PTLD. (150, 151)

1.3.5 Other diseases associated with EBV

EBV has also been associated with various other diseases, although the evidence for these associations is considerably weaker than for those discussed above. The prevalence of EBV DNA in the intestinal tissue of patients with IBD (46.8%) has been found to be significantly higher than in noninflammatory disease controls (13.3%) ($p=0.001$). (152) In Alzheimer's, the clonally expanded cells in the CSF carry a signature of effector memory CD8 T-cells, and a subset are specific for EBV. (153) A study in idiopathic multicentric Castleman disease (CD) using virome capture sequencing identified EBV in 7 out of 12 unicentric CD, and 5 out of 11 idiopathic multicentric CD tissue samples. (154) EBV has also been linked to chronic fatigue syndrome. (155) Although weak evidence, these preliminary associations make a

case for further investigation of EBV in inflammatory conditions of unknown etiology.

1.4 Antiviral agents for herpesviruses

Note: A list of names, type of drug, mechanism of action, and clinical features of the compounds included below is provided at the end of this section in Table 1.2.

1.4.1 Acyclovir

Acyclovir, an acyclic nucleoside analog of guanosine, was the first drug shown to have efficacy against a herpesvirus. (76, 156) Acyclovir (and the prodrug form valacyclovir with higher oral bioavailability) clinically reduces the duration of outbreaks caused by HSV1/2. Additionally, when administered intravenously at high doses, acyclovir is effective in the treatment of herpes simplex encephalitis, an otherwise lethal complication that can arise from primary infection or reactivation of HSV1. (157)

The mechanism of acyclovir against HSV1 viral replication involves two virus-mediated steps. First, a viral-encoded kinase phosphorylates acyclovir to acyclovir-monophosphate. This step is also the rate-limiting step. For HSV1, the conversion of acyclovir to acyclovir monophosphate is performed by the viral thymidine kinase. (75) Cellular kinases (or the viral thymidine kinase itself) can then convert acyclovir monophosphate to the active triphosphate form. The second virus-mediated step is the integration of acyclovir-triphosphate into viral DNA by the viral DNA

polymerase. Acyclovir-triphosphate is not integrated into cellular DNA since it is ineffectively incorporated by cellular DNA polymerases. (75) Since acyclovir lacks a 3' hydroxyl group, it inhibits viral DNA replication by acting as a chain-terminating agent. The requirement of a virus-encoded kinase for the initial phosphorylation step is thought to provide increased specificity for targeting infected cells.

In cultured cells, acyclovir not only demonstrates activity against HSV1 but also against other herpesviruses, including EBV. (158) However, the potency of acyclovir is significantly lower for EBV than HSV1. (78, 159, 160) Furthermore, the EBV thymidine kinase, encoded by the BXLF1 gene, does not phosphorylate acyclovir because it is highly restricted to thymidine analogs. (77) EBV carries a separate protein kinase encoded by the BGLF4 gene, which is required for the activity of acyclovir in cell culture. (78) However, this enzyme has not been shown to directly phosphorylate acyclovir, leaving open the question of how EBV infection leads to activation of acyclovir.

Despite its efficacy against EBV *in vitro*, acyclovir is not recommended in the treatment of IM even when IM causes serious complications. In a double-blind placebo-controlled clinical trial, acyclovir showed no benefit for any individual clinical symptom, including fever, tonsillar swelling, and patient-reported

parameters like fatigue. (161) Intriguingly, however, there was a trend towards benefit with drug treatment. Acyclovir was also shown to inhibit the shedding of EBV in saliva. (161) However, since no clinical benefit was observed with acyclovir, it is not used in the treatment of IM.

Curiously, treatment with acyclovir has shown a similar pattern of a modest trend towards benefit in other EBV-associated diseases. For prevention of PTLD, acyclovir treatment was shown to confer some protection (OR = 0.83; 95% CI = 0.59-1.16). (162) Similarly, in MS, acyclovir was shown to reduce the annualized relapse rate by 34% (p=0.083). (163, 164) If relapse rates were grouped into high vs. low MRI activity, the difference between placebo and acyclovir became significant (p=0.017). (163, 164) These results are difficult to interpret definitively, but it is important to consider even insignificant trends towards benefit, as acyclovir is a weak inhibitor of EBV. Further studies with more potent agents would be required to investigate these suggestive clinical results.

1.4.2 Ganciclovir

Ganciclovir, a guanosine analog, was developed for the treatment of HCMV because β-herpesviruses lack a viral thymidine kinase thus cannot phosphorylate acyclovir. It is instead directly phosphorylated by another HCMV-encoded viral

kinase, UL97, for which acyclovir is a poor substrate. (165) Ganciclovir is clinically licensed for the treatment of HCMV infection. (166) With respect to EBV, ganciclovir is effective *in vitro*, and also more potent than acyclovir. (78, 167) However, the oral bioavailability of ganciclovir is only 6-9%. Therefore, it is administered intravenously. (166) Although there exists an orally bioavailable form (valganciclovir), long-term or routine clinical use is limited by severe hematologic, reproductive, and renal toxicity due to off-target effects. (166) The poor safety profile of ganciclovir compared to acyclovir has restricted testing in clinical trials despite higher potency. However, in agreement with its increased potency against EBV, studies have reported a stronger trend in reducing the risk of PTLD (OR = 0.62, CI = 0.38-1.0). (162) Ganciclovir has not been tested in clinical trials for MS.

1.4.3 Other antivirals

Penciclovir, a guanosine analog licensed for the treatment of VZV, also has *in vitro* efficacy against EBV. Its mechanism of action and potency are akin to those of acyclovir. (168) Additionally, the inorganic pyrophosphate analog foscarnet, used for the treatment of HCMV and acyclovir-resistant HSV infections, demonstrates activity against EBV. (169) Foscarnet acts on the viral DNA polymerase but does not require activation by a viral kinase. However, like ganciclovir, foscarnet is limited by intravenous administration and has significant systemic toxicity. Maribavir, a

benzimidazole derivative, blocks EBV replication at least in part through its effects on the EBV protein kinase BGLF4. (170, 171) It also shows efficacy against HCMV – however, a recent clinical trial for preemptive treatment of HCMV reactivation reported a higher incidence of serious adverse events with maribavir (44%) than valganciclovir (32%) despite similar efficacy, suggesting its toxicity would impede widespread use. (172) Brivudine is a thymidine analog approved for the treatment of VZV in several European countries. It also demonstrates *in vitro* efficacy against EBV. (173) Intriguingly, after drug removal, EBV DNA replication was still inhibited more than 21 days after brivudine treatment vs. rapidly reversed after removal of acyclovir. (173) Lethal drug-drug interactions have limited its adoption as an anti-herpesviral agent on a broader scale. Finally, the HIV drug zidovudine (AZT) is reported to inhibit EBV lytic DNA replication and transformation of B-lymphocytes, although no mechanism has been defined. (174, 175) In conclusion, there are several drugs with reported efficacy against EBV. However, most are either relatively weak inhibitors of EBV replication compared to their licensed use (e.g., acyclovir, penciclovir), or have prohibitive toxicity (e.g., ganciclovir, maribavir, foscarnet). Thus, highly potent compounds with improved safety profiles that permit routine clinical use are critically needed for investigations into the treatment of EBV infection.

1.4.4 Antivirals as prophylaxis in herpesviral infections

For HIV, the use of antiviral agents to prevent viral infection before exposure ("pre-exposure prophylaxis), or shortly after exposure ("post-exposure prophylaxis"), is routine. However, the potential to prevent herpesviral infections or to limit the future consequences of primary infection is comparatively underexplored. Studies suggest that herpesviruses may also be amenable to prevention or early treatment. In mice, early intervention with acyclovir during primary HSV infection reduces latency and subsequent reactivation in the nervous system. (176) In humans, tenofovir gel reduces the incidence of HSV-2 seroconversion compared to placebo gel (incidence rate ratio, 0.45; 95% CI, 0.23 to 0.82; P=0.005). (177) Additionally, in children acyclovir prophylaxis reduces seroconversion to HSV-1 in the setting of a local outbreak (conversion rate 91% vs 27%, P< .001). (178)

Table 1.2: Anti-herpesviral drugs with *in vitro* activity against EBV

Drug	Prodrug	Type of drug	Mechanism of action	Drug activation	Clinical features
Acyclovir	Valacyclovir	deoxyguanosine analog	inhibits viral DNA pol	requires viral kinase	ineffective for EBV in clinical trials
Ganciclovir	Valgancyclovir	deoxyguanosine analog	inhibits viral DNA pol	requires viral kinase	intravenous administration; high toxicity
Penciclovir	Famciclovir	deoxyguanosine analog	inhibits viral DNA pol	requires viral kinase	similar potency to acyclovir
Foscarnet	N/A	pyrophosphate analog	inhibits viral DNA pol	does not require kinase	intravenous administration; high toxicity
Maribavir	N/A	benzimidazole derivative	unknown	unknown	high toxicity in clinical trials
Brivudine	N/A	thymidine analog	inhibits viral DNA pol	requires viral kinase	lethal drug interactions
AZT	N/A	thymidine analog	unknown	unknown	used for the treatment of HIV

1.5 Summary

Chapter 2 details a case report of a patient with relapsing-remitting multiple sclerosis treated with Combivir (AZT/3TC) who experienced resolution of fatigue and neurological symptoms, with a discussion of the possibility this effect was due to direct treatment of EBV.

Chapter 3 attempts to answer the central question raised by Chapter 2 by providing a complete characterization of all clinically licensed antiretroviral nucleoside/nucleotide analogs and their effects on EBV lytic DNA replication. This chapter also discusses alternative mechanisms for the interaction of nucleoside/nucleotide analogs with EBV, contrasting AZT with tenofovir prodrugs.

Chapter 4 provides a more detailed characterization of tenofovir prodrugs, found to be the most effective antiretroviral nucleoside/nucleotide analogs effective against EBV, with further characterization and direct comparison with standard anti-herpesviral drugs for both EBV and HSV1 at the cellular and polymerase level.

Chapter 5 summarizes the described findings detailed above and outlines future experiments and clinical studies that may be informative to elucidate the role of EBV in disease.

CHAPTER 2: COULD ANTIRETROVIRALS BE TREATING EBV IN MS? A CASE REPORT

Note: This chapter has been directly adapted from work previously accepted for publication with minimal changes to text. The organization of the figures has been modified to fit this thesis. N.D., E.E., and D.H conceptualized, wrote and edited the original manuscript.

The reference publication is:
Drosu NC, Edelman ER, Housman DE. *Could antiretrovirals be treating EBV in MS? A case report.* Mult Scler Relat Disord. 2018;22:19-21.

2.1 Abstract

We present the case of an HIV-negative patient clinically diagnosed with relapsing-remitting multiple sclerosis who achieved significant disease improvement on Combivir (zidovudine/lamivudine). Within months of treatment, the patient reported complete resolution of previously unremitting fatigue and paresthesiae, with simultaneous improvements in lesion burden detected by MRI. All improvements have been sustained for more than 3 years. This response may be related to the action of AZT as a known inhibitor of EBV lytic DNA replication, suggesting future directions for clinical investigation.

2.2 Case Report

The epidemiological link between multiple sclerosis (MS) and the Epstein-Barr virus (EBV) is well-established, (179) but the role of EBV in MS pathology and clinical progression remains controversial. Classic anti-herpesviral drugs, such as acyclovir and valacyclovir, have no significant clinical benefit in MS, (180) and are similarly ineffective for infectious mononucleosis, known to be caused by primary EBV infection. (161) Here we report a case of a patient with severe MS who experienced a dramatic improvement after treatment with Combivir (zidovudine/lamivudine). Zidovudine is known to effectively inhibit EBV (and no other herpesviruses) *in vitro,* (174) but has never been tested in a randomized clinical trial of MS.

In September 2014, a 25-year old female student presented to her university's health services clinic complaining of progressive inability to feel her right leg for two months. She also felt severe fatigue and worsening bilateral leg pain aggravated by walking. On examination, she had bilateral lower extremity numbness and extensor plantar response on the right side.

MRI revealed multiple small brain lesions. Additional lesions were detected in the right peripheral cord at the C4 and C6 vertebral levels, with gadolinium

enhancement at C4. The thoracic cord contained extensive lesions spanning T7-T11 as follows: T7-T8 measuring 8 mm, T9-T10 measuring 7 mm, T11 superiorly measuring 1.3 cm, and T11 inferiorly measuring 1.2 cm.

The patient recalled a long history of relevant clinical symptoms including: sudden change in vision in the right eye at age 13, an episode of unilateral lower extremity weakness at 17, bilateral leg numbness at 20, and upper extremity weakness at 24 causing her to drop a pot of boiling water leading to second degree burns. All symptoms had lasted at least 24 hours. While the patient had sought medical care throughout this time, she was instructed each time to wait for improvement and return to the clinic if symptoms did not go away on their own. Since each symptom resolved, no follow-up occurred. Medical attention was only sought at presentation because of decline without improvement in right leg numbness, which was atypical compared to prior numbness, which had disappeared on its own.

Serological testing performed at presentation was negative for HIV, HBV, HCV, HSV1/2, syphilis, lyme, and anti-AQP4 antibody. Subsequently, the patient also tested negative for HTLV1/2 and HCMV. No EBV testing was performed at presentation. EBV serology was later performed on July 31, 2017 and showed the following: EBNA1 IgG of 191, VCA IgM negative and IgG >600, EA-D IgG negative.

There was no history of infectious mononucleosis. After MRI imaging, her primary care provider referred her to the MS clinic at Brigham and Women's Hospital, where she was diagnosed with clinically definite relapsing-remitting MS. CSF was not examined because the patient met the diagnostic criteria for RRMS based on the McDonald criteria (2010).

In October 2014, she was started on glatiramer acetate injections. While on glatiramer acetate, she was sleeping more than 16 hours a day without feeling rested, developed increasingly disabling pain in her arms and legs, and could not walk more than 100 feet without needing to sit down. On December 2, 2014, a 3-day course of corticosteroids was initiated for suspicion of a relapse, but decline continued.

Because of this clinical decline and local skin reactions, she stopped treatment with glatiramer acetate in December. Repeat MRI performed on January 9, 2015 (Fig 2.1) showed a new lesion in the cervical spine at C3 (Fig S2.1) as well as more pronounced lesions at C4 and C6. The patient, a medical student, after reading a case report of MS with sustained resolution of symptoms on HAART, (181) independently started therapy with Combivir (zidovudine 300mg/lamivudine 150mg) twice daily.

A few days after starting Combivir, she noticed dramatic improvement in fatigue. After 2 months, she had gradual improvement in numbness and pain in her arms and legs. Her neurologic exam on March 24, 2015 (11 weeks after starting Combivir) was normal. After 9 months, she had minimal numbness in her feet, only noticeable after walking for long stretches. She could go jogging for the first time in years.

Repeat MRI was performed on September 22, 2015 (after 8.5 months of Combivir). The previously described cervical and thoracic cord abnormalities were significantly less distinct compared to prior study (Fig 2.1). The brain MRI abnormalities remained stable with no change on subsequent imaging. The patient has now been on Combivir for more than 3 years. During this time, she has experienced no new symptoms, and all described improvements, including complete cessation of MS-related fatigue, have been sustained to date. Follow-up MRIs performed on December 7, 2016 and December 6, 2017 (Fig 2.1) revealed no new focal lesions.

Fig. 2.1

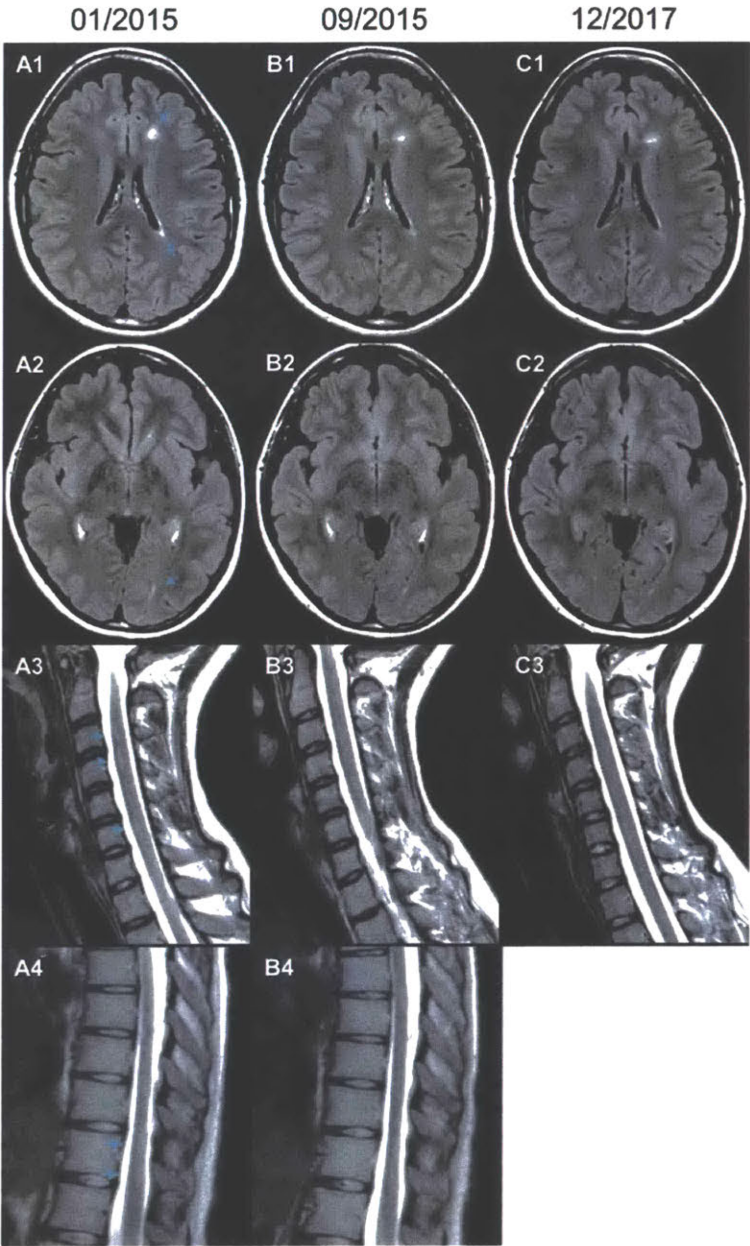


Fig. 2.1

Axial T2-FLAIR MRI images of the brain (A1-C1, A2-C2) immediately prior to, 8.5 months after, and 35 months after starting Combivir. Sagittal T2-weighted MRI images of the cervical (A3-C3) and thoracic cord (A4, B4) before and after starting Combivir. MRI of the thoracic cord at 35 months was not performed. All imaging was done without gadolinium contrast on 12/2017 due to lack of clinical symptoms.

Fig. S2.1

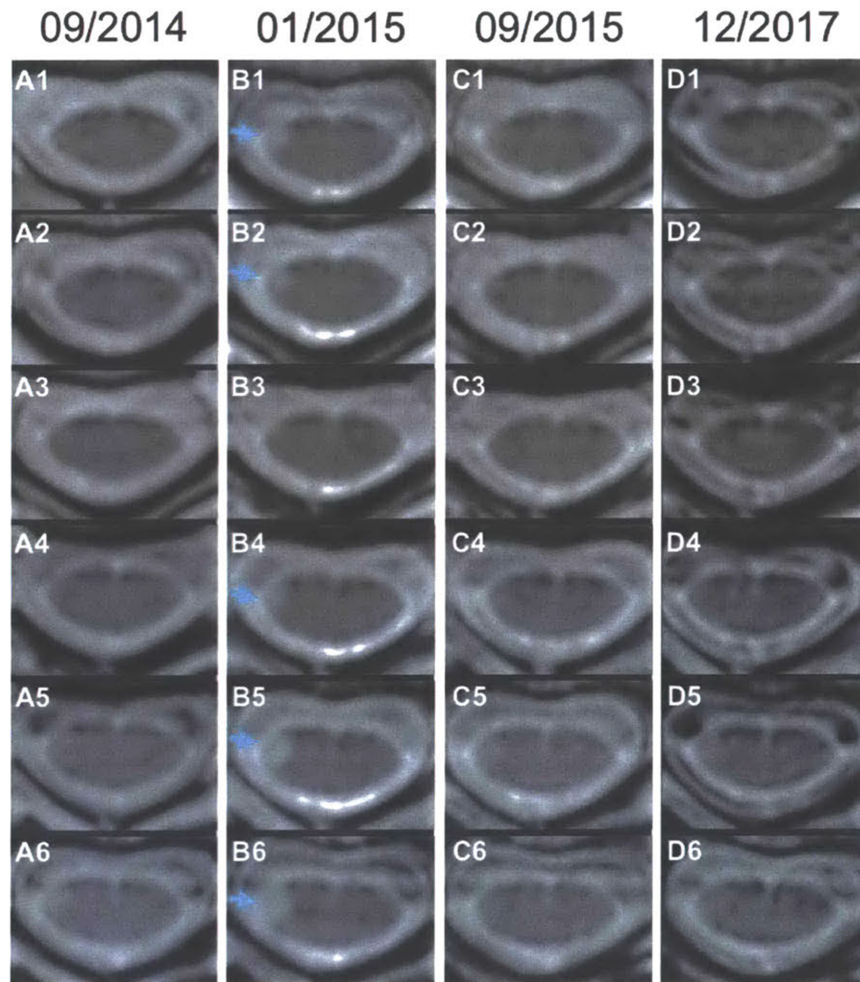


Fig. S2.1

Serial (3mm offset) axial T2-weighted MRI images of the cervical cord between cord levels C3 (top) and C4 (bottom) at diagnosis, before and after starting Combivir showing presence of a new lesion at C3 on 01/2015. All imaging was done without gadolinium contrast on 12/2017 due to lack of clinical symptoms.

2.3 Discussion

Multiple cases of patients with HIV and MS who experienced indefinite remission or resolution of MS symptoms on HAART regimens have been reported in the literature. (181–183) These have raised an important question of whether or not it is HIV infection that modulates MS or if treatment with HAART could directly impact MS. We report this case of an HIV-negative patient on antiretroviral therapy to support the possibility that antiretroviral drugs may directly affect MS. In particular, this case supports careful examination of drugs in the class of nucleoside/nucleotide analogues.

Addressing the question of mechanism is critical to guide future clinical studies in MS and most importantly, to inform drug selection for these studies. Several mechanisms have been proposed for the impact of HAART on MS. These mechanisms include treatment of a human endogenous retrovirus by inhibition of an endogenous reverse transcriptase, (181) and chemical similarity of the fumaric acid component of tenofovir disoproxil fumarate with dimethyl fumarate. (182, 183)

We propose a different mechanism. Given the link between EBV and MS, it is possible that nucleoside analogues could have a direct effect on EBV, a dsDNA

virus, by inhibiting lytic EBV DNA replication. Zidovudine, a component of Combivir, is known to inhibit EBV DNA replication. However, any mechanism must account for the lack of clinical efficacy of acyclovir-class drugs in MS and infectious mononucleosis. Acyclovir drug metabolism is different from drug metabolism of antiretroviral nucleoside analogues because acyclovir requires a viral kinase for phosphorylation. Bypassing this requirement facilitates the accumulation of active drug intracellularly. This unique feature of antiretroviral nucleosides may be important during low-level viral replication or for pre-treatment during a period of viral latency prior to reactivation in the CNS.

To account for the other reported cases of HIV and MS with improvement on HAART, this theory would require the activity of other nucleoside analogues on lytic EBV DNA replication. We suggest this should be tested *in vitro*, and that the results be used to inform drug selection for a future clinical trial. Meanwhile, it should be emphasized that neither Combivir nor other antiretroviral drugs have been as of yet proven to be effective treatments for MS.

CHAPTER 3: INVESTIGATING THE POTENTIAL OF ANTIRETROVIRAL NUCLEOSIDE/NUCLEOTIDE ANALOGS AS INHIBITORS OF EBV LYTIC DNA REPLICATION

Note: This chapter is not intended to stand alone, but to serve as a bridge between Chapters 2 and 4. In Chapter 2, we presented a case report of an HIV-negative patient with multiple sclerosis who responded to Combivir (AZT/3TC) and briefly discussed similar case reports in HIV-positive patients. Since these patients were each on different medication regimens, we raised the question of whether antiretrovirals could be treating EBV in MS. Here we attempt to answer that question.

3.1 Background and Introduction

EBV is one of the most common human pathogens worldwide, infecting >90% of people by adulthood. (33) Despite being nearly ubiquitous, EBV is associated with an increased risk for a wide range of diseases, including cancers, (129) autoimmune diseases, (184) and diseases of immunodeficiency. (145, 185, 186) While a substantial amount is known about EBV as an oncogenic virus due to its potential to drive cellular transformation, its association with diseases classified as 'autoimmune' is comparatively less clear. Epidemiological studies have linked EBV to multiple sclerosis (MS), (187) rheumatoid arthritis, Sjögren's syndrome, and lupus. (188) Of these, the association of EBV with MS is most robust, while associations with the other diseases are significantly weaker. However, the search

for autoantibodies has not yielded any conclusive evidence of autoimmunity in MS to date. A re-evaluation of whether MS is actually a true 'autoimmune' disease is warranted. Other classifications, including perhaps 'infectious disease', should be considered.

The link between EBV with MS is covered in Chapter 1. Briefly, the odds ratio of MS in EBV-seronegative individuals is <0.001-0.06 but increases sharply after infection. (115, 120, 187, 189) Patients with MS have higher EBV antibody titers than those without the disease, (107, 111, 114, 190) and the rise in antibody titers predicts the development of MS. (112, 113) However, a mechanistic role for EBV in the pathogenesis of MS remains controversial.

Antiviral trials for MS using standard herpesviral drugs have had modest, but unconvincing results. Acyclovir (ACV), a guanosine analog repurposed from the treatment of α-herpesviruses that inhibits the viral DNA polymerase, (76) was shown to reduce the annualized relapse rate in patients with MS by 34% (p=0.083). (163, 164) When relapse rates were grouped into high vs. low MRI activity (based on number of lesions), the difference between placebo and ACV became significant (p=0.017). (163, 164) While these results are inconclusive in light of the epidemiology, the trends are suggestive of the possibility that anti-EBV

interventions may have an impact on disease pathogenesis. Given that the potency of ACV is significantly lower for EBV than HSV, (78, 159, 160) more effective drugs are needed to answer the question of whether EBV is a viable target in established MS.

Intriguingly, several case reports of patients with MS with concurrent HIV infection, as reviewed in (191), and one without HIV (Chapter 2 of this thesis), have described resolution of symptoms and extended remissions that coincided with the initiation of antiretroviral therapy. Importantly, in the HIV-positive cases, benefit was noted regardless of HIV acquisition before, at the same time as, or after the onset of MS. Additionally, an epidemiological study found HIV infection to be the most protective environmental factor ever described for subsequent diagnosis of MS. (192) The rate ratio of developing MS in people with HIV relative to those without HIV was 0.38 (95% CI 0.15 to 0.79). (192) The rate ratio also decreased with time since HIV infection – 0.25 at 1+ year and 0.15 at 5+ years. (192) However, information about drug treatment for HIV was unavailable.

No compelling explanation has been put forth to explain these case reports of patients with MS responding to antiretroviral treatment. Because of the overwhelming epidemiological evidence for EBV in MS, we chose to investigate the

possibility that some antiretroviral drugs may be directly treating EBV. Additionally, in the case report presented in Chapter 2, the patient tested positive for EBV but negative for known human retroviruses (both HIV and HTLV), arguing against a retroviral etiology.

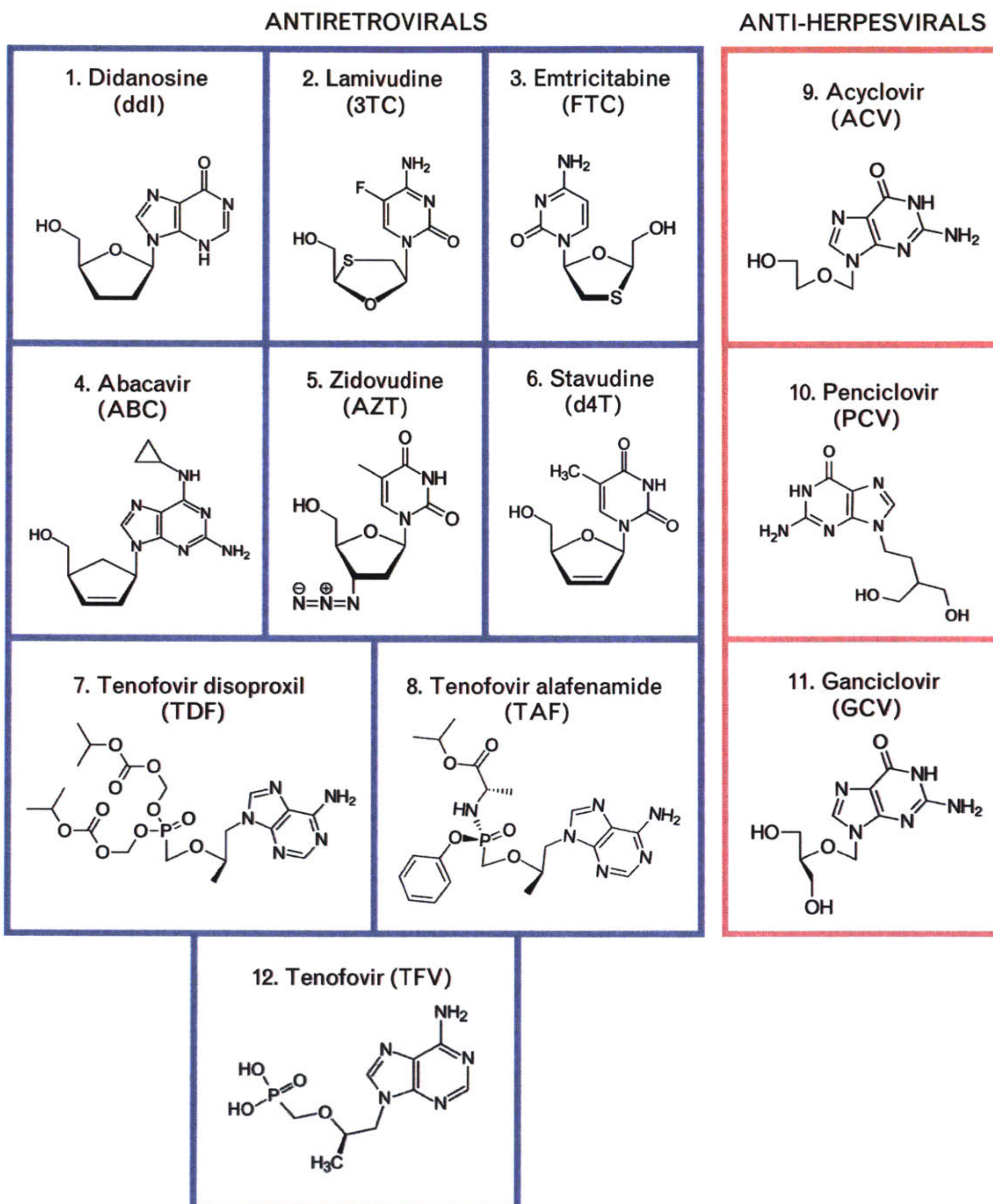
This case also directed us to focus on nucleoside/nucleotide analogs since Combivir is composed of two drugs that are part of this class of HIV medications – AZT and 3TC. Preliminary evidence emerged from previously published reports that the AZT inhibits EBV lytic DNA replication. (174) Also, tenofovir (a component of the antiretroviral nucleotide analogs TDF and TAF) has anti-herpesviral activity. This observation was pertinent since EBV is a herpesvirus. (193) Therefore, we asked whether anti-EBV activity may extend to other nucleoside/nucleotide analogs used in the treatment of HIV infection.

Note: A list of analogs, abbreviations, analog base, licensed use, and chemical structure for the drugs used in this chapter is provided in Table 3.1.

Table 3.1: List of antiviral nucleoside/nucleotide analogs, abbreviations, analog base, and licensed use.

Abbv.	Drug name	Base	Triphosphate form	Clinical use (licensed)	Drug target
ddI	Didanosine	dI (dA)	ddA-TP	HIV	Reverse transcriptase
3TC	Lamivudine	dC	3TC-TP	HIV	Reverse transcriptase
FTC	Emtricitabine	dC	FTC-TP	HIV	Reverse transcriptase
ABC	Abacavir	dG	(-)-CBV-TP	HIV	Reverse transcriptase
AZT	Zidovudine	dT	AZT-TP	HIV	Reverse transcriptase
d4T	Stavudine	dT	d4T-TP	HIV	Reverse transcriptase
TDF	Tenofovir disoproxil fumarate	dA	TFV-DP	HIV	Reverse transcriptase
TAF	Tenofovir alafenamide	dA	TFV-DP	HIV	Reverse transcriptase
ACV	Acyclovir	dG	ACV-TP	HSV1/2	Viral DNA polymerase
PCV	Penciclovir	dG	PCV-TP	VZV	Viral DNA polymerase
GCV	Ganciclovir	dG	GCV-TP	HCMV	Viral DNA polymerase
TFV	Tenofovir	dA	TFV-DP	N/A (metabolite of TDF and TAF)	Reverse transcriptase

Structures of antiviral nucleoside/nucleotide analogs listed in Table 3.1



3.2 Results

3.2.1 Establishing an in vitro model of EBV lytic DNA replication for testing candidate nucleoside/nucleotide analogs

In vitro models of lytic DNA replication for EBV involve the addition of chemical stimuli to cell lines harboring latent EBV to trigger the expression of the major viral transcription factors Zta and Rta. (64–68) Different established cell lines require different stimuli, presumably because of cell-specific requirements for Zta/Rta expression. A small proportion of cells enter the lytic cycle spontaneously (5-10%) during *in vitro* culture in the absence of chemical stimuli. (66) To increase the number of cells that enter the lytic cycle, several protocols have been described in the literature. Chemicals used for induction in cultured cells are broadly acting and include epigenetic modifying agents like butyrate or 5-Aza-2′-deoxycytidine (5-AzadC), as well as phorbol esters and calcium ionophores. The B95-8 marmoset cell line and the P3HR-1 Burkitt lymphoma cell line can be induced by phorbol 12-myristate 13-acetate (PMA). Similarly, the HH514-16 cell line, a subclone of P3HR-1 selected for low spontaneous reactivation, (194) responds to both the histone deacetylase inhibitor butyrate or the DNA methyltransferase inhibitor 5-AzadC. (195)

We attempted lytic DNA replication and drug treatment in several models. However, we noticed that B95-8 cells or P3HR-1 cells induced with PMA, or HH514-16 cells induced with 5-AzadC (Fig 3.1A) continued to divide after the initiation of lytic EBV DNA replication. In contrast, HH514-16 cells induced with butyrate ceased to divide following lytic induction (Fig 3.1A). Both models produced similar viral copy numbers after 96 hours of induction (Fig 3.1B).

Dividing cells are known to contain higher levels of nucleotides than non-dividing cells. (196) In line with this, we observed that the anti-herpesviral drug ACV was significantly less effective as an inhibitor of EBV lytic DNA replication in dividing HH514-16 cells induced with 5-AzadC than in non-dividing HH514-16 cells induced with butyrate (Fig 3.1B). At a 50μM dose, ACV reduced the number of viral copies by 95.5% when cells were induced with butyrate. However, ACV used at the same dose only led to a decrease of 76.2% when cells were induced with 5-AzadC. Based on these results, we chose to use butyrate instead of 5-AzadC for subsequent experiments for two reasons: (1) EBV lytic DNA replication is known to occur physiologically in coordination with terminal cellular differentiation in both epithelial and plasma cells, (69–71) which are non-dividing cells and (2) effects of drugs would be easier to detect.

Figure 3.1

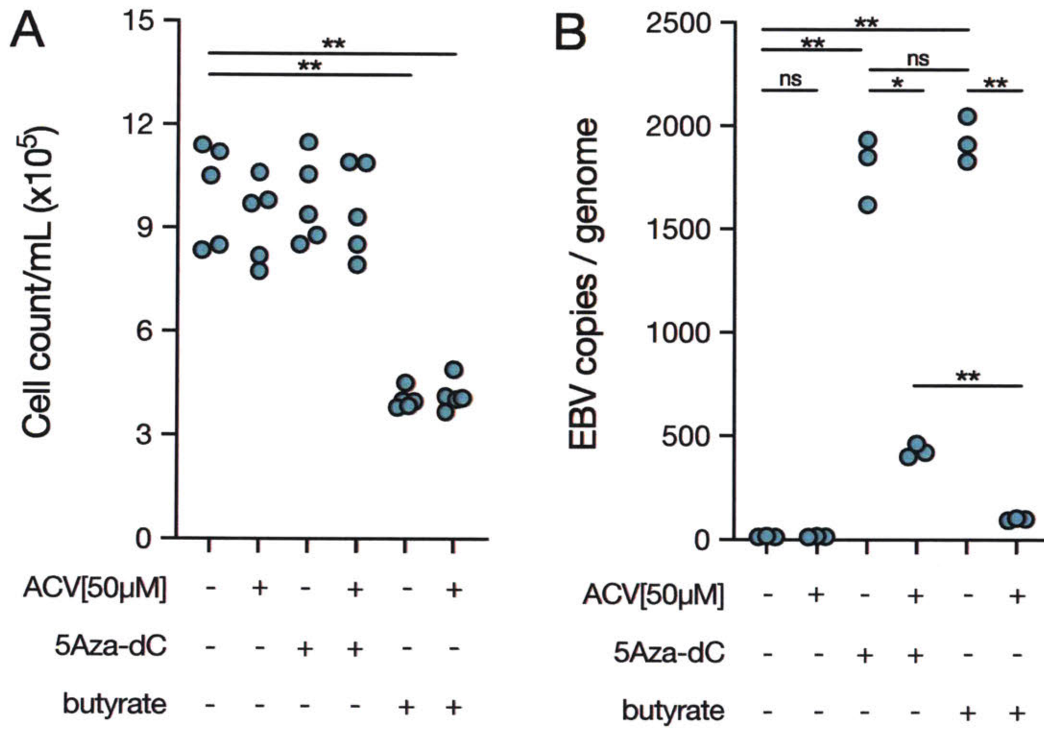


Figure 3.1: Establishing an In vitro model of EBV lytic DNA replication for testing candidate nucleoside/nucleotide analogs

(A) HH514-16 cells were induced with 5μM 5-AzadC or 3mM butyrate and treated with the established anti-herpesviral drug ACV at a 50μM dose. Cell count per mL was measured after 96 hours of treatment. Results were analyzed from 5 independent experiments, each value shown. One-way ANOVA ($p<0.05$) was followed by multiple hypothesis testing between control and each condition. Statistical significance is highlighted by P values as follows: *$P<0.05$; **$P < 0.01$ (B) EBV copies per genome were measured by qPCR after 96 hours of treatment. Results were analyzed from 3 independent experiments, each value shown. One-way ANOVA ($p<0.05$) was followed by multiple hypothesis for pairs shown with statistical significance calculations, highlighted as follows: ns = not significant: *$P<0.05$; **$P < 0.01$

3.2.2 Some, but not all, antiretroviral nucleoside/nucleotide analogs inhibit lytic EBV DNA replication

Using our model of lytic induction with butyrate in HH514-16 cells, we next decided to test all nucleoside/nucleotide analogs licensed for the treatment of HIV. A list of analogs, abbreviations, analog base, licensed use, and chemical structure is provided earlier in this chapter in Table 3.1. Briefly, these include the thymidine analogs zidovudine (AZT) and stavudine (d4T), the deoxyguanosine analog abacavir (ABC), the deoxycytidine analogs lamivudine (3TC) and emtricitabine (FTC), and the deoxyadenosine analogs dideoxyinosine (ddI), tenofovir disoproxil fumarate (TDF) and tenofovir alafenamide (TAF).

First, we wanted to determine the potential cytotoxicity of these compounds at a 50µM dose. Therefore, we measured the viability of drug-treated cells. Lytic induction with butyrate slightly reduced viability in comparison to latency (Fig. S3.1). However, we did not observe any reduction of viability at a 50µM dose of any drug compared to butyrate alone, except for TDF (Fig. S3.1). Thus, any potential observed effects against EBV, except in the case of TDF, were not likely to be mediated through cytotoxicity.

Next we tested the ability of these drugs to inhibit EBV. After 96 hours of butyrate treatment, the average number of viral copies per genome increased 128-fold (Fig 3.2). At 50μM concentrations, the effect of antiretroviral nucleotide analogs fell into two categories – drugs either inhibited EBV lytic DNA replication by (1) <50% or (2) >90% (Fig 3.2). Based on these results, we classified the latter as 'effective' and the former as 'ineffective'. Our assay validated AZT as an inhibitor of EBV lytic DNA replication, as previously reported. (174) We also found that ABC, d4T, TDF, and TAF were effective at inhibiting EBV in our model. In contrast, ddI, 3TC, and FTC were ineffective. Of the effective drugs, the inhibitory capacity at a 50μM dose was ordered as follows: ABC<AZT<d4T<TDF<TAF (Fig 3.2). Except in the case of ddI, these were grouped by base (Fig 3.2), where dC analogs were ineffective, and for the others the order of effectiveness was: dG<dT<dA.

Figure S3.1

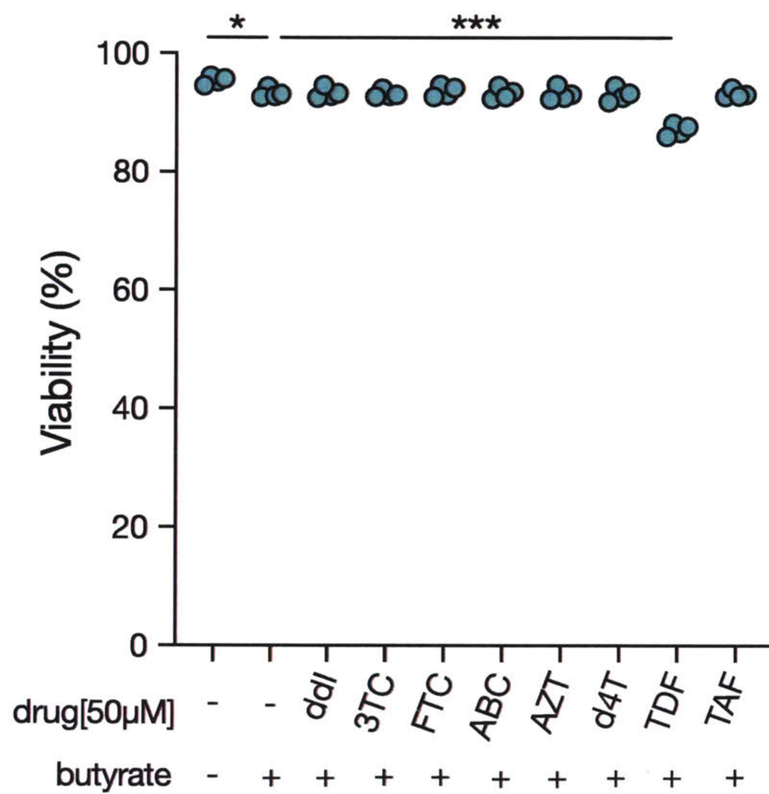


Figure S3.1 Effects of antiretroviral drugs on cellular viability in HH514-16 cells induced with butyrate

HH514-16 cells were induced with 3mM butyrate for 96 hours and treated with the antiretroviral drugs ddI, 3TC, FTC, ABC, AZT, d4T, TDF, and TAF. Viability was measured by trypan blue exclusion after 96 hours of treatment at a 50μM dose. Results were analyzed from 4 independent experiments, each value shown. One-way ANOVA ($p<0.05$) was followed by multiple hypothesis testing between butyrate and each condition. Statistical significance is highlighted by P values as follows: *$P<0.05$; ***$P < 0.001$.

Figure 3.2

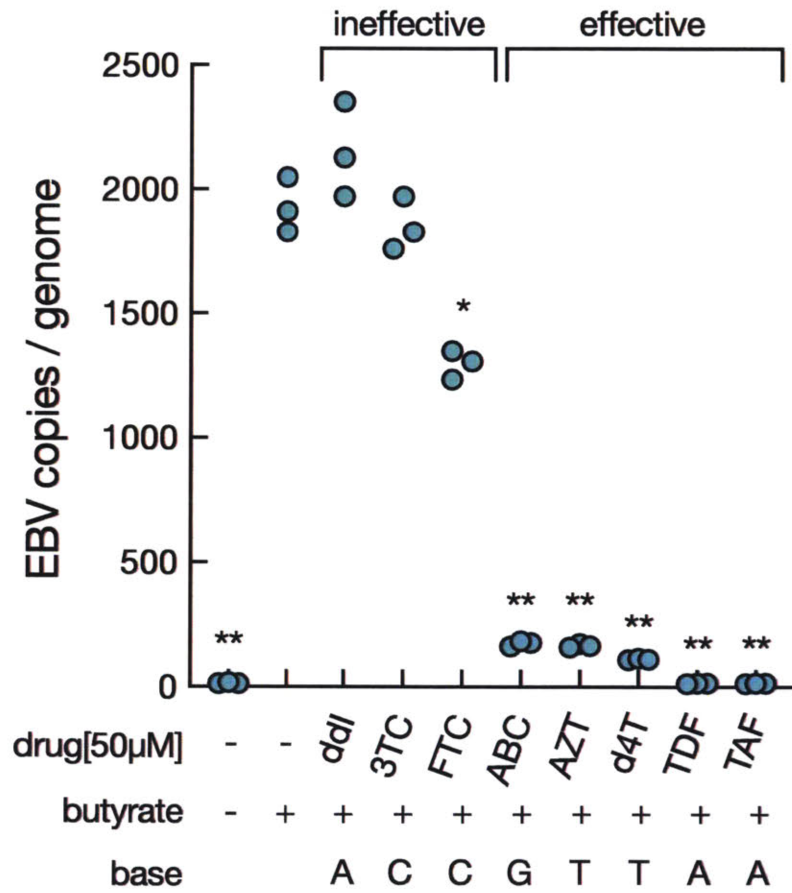


Figure 3.2: Antiretroviral drugs inhibit EBV lytic DNA replication in HH514-16 cells induced with butyrate.

HH514-16 cells were induced with 3mM butyrate for 96 hours and treated with the antiretroviral drugs ddI, 3TC, FTC, ABC, AZT, d4T, TDF, and TAF. EBV copies per genome were measured by qPCR after 96 hours of treatment at a 50μM dose. Results were analyzed from 3 independent experiments, each value shown. One-way ANOVA ($p<0.05$) was followed by multiple hypothesis testing between butyrate and each condition. Statistical significance is highlighted by P values as follows: *$P<0.05$; **$P < 0.01$.

3.2.3 The inhibition of EBV DNA replication by antiretroviral nucleoside/nucleotide analogs is specific to the lytic cycle

EBV DNA replication during the lytic cycle is mechanistically distinct from replication during latency. (13) Since viral episomes replicated during latency serve as templates for lytic DNA replication via rolling circle replication, we wanted to determine whether antiretroviral nucleoside/nucleotide analogs were inhibiting a process specific to the lytic cycle. Therefore, we treated cells with each drug in the absence of butyrate induction. There was no effect on viral copy number at a 50μM dose with any drug during latency (Fig 3.3), suggesting that these drugs act only on lytic EBV DNA replication.

EBV encodes several enzymes involved in lytic DNA replication – including a viral DNA polymerase and processivity factor, as well as a viral DNA primase, primase accessory protein, helicase, ssDNA binding protein, and ribonucleotide reductase (RNR). (13) We considered both the viral DNA polymerase and viral RNR as potential targets since these classes of enzymes are regulated and may be inhibited by nucleoside/nucleotide analogs. (197, 198) Furthermore, both of these targets would be specific to the lytic cycle.

Figure 3.3

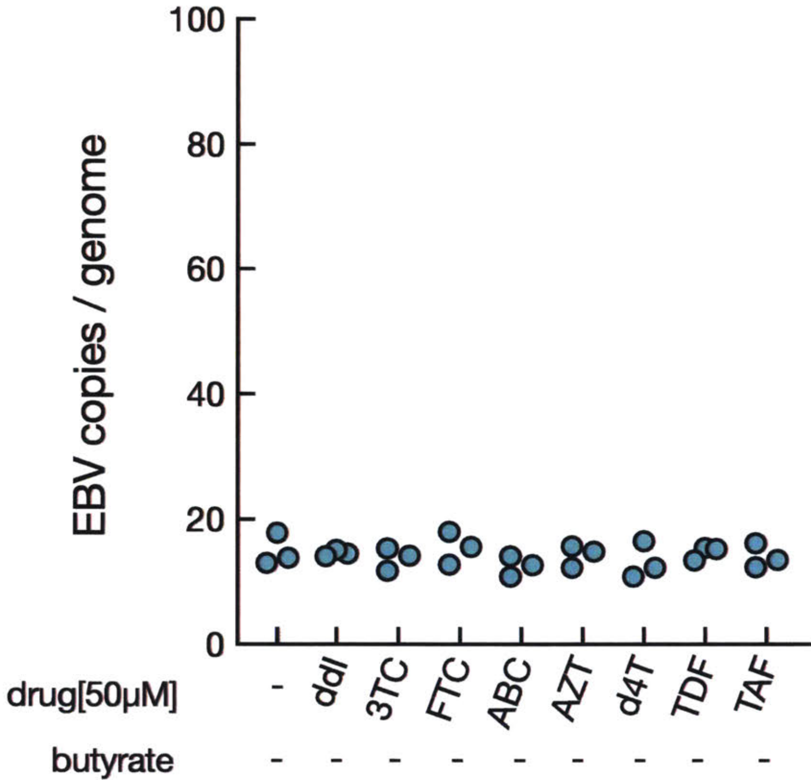


Figure 3.3 The inhibition of EBV DNA replication by antiretroviral nucleoside/ nucleotide analogs is specific to the lytic cycle

HH514-16 cells were grown in the absence of butyrate for 96 hours and treated with the antiretroviral drugs ddI, 3TC, FTC, ABC, AZT, d4T, TDF, and TAF. EBV copies per genome were measured by qPCR after 96 hours of treatment at a 50μM dose. Results were analyzed from 3 independent experiments, each value shown. One-way ANOVA was performed ($p>0.05$).

3.2.4 The target of antiretroviral nucleoside/nucleotide analogs is not the EBV ribonucleotide reductase

First, we hypothesized a plausible role for drug action on the viral RNR. In our assays, we observed that the inhibitory capacity of antiretroviral nucleoside/nucleotide analogs was structurally restricted to the thymidine, deoxyguanosine, and deoxyadenosine analog classes. (Note: only one deoxyguanosine analog exists for the treatment of HIV – ABC). In contrast, the deoxycytidine analogs 3TC and FTC had little or no effect on EBV lytic DNA replication. Of note, the deoxyadenosine analogs TDF and TAF were most potent. This pattern is similar to that observed for the regulation of class I eukaryotic RNRs, which are allosterically regulated by dTTP, dGTP, and dATP (with dATP acting as a pan-inhibitor of dNTP synthesis), but unresponsive to dCTP. (199) Furthermore, acyclic nucleoside phosphonates – a class of drugs that contains tenofovir as a member – have been shown to function as inhibitors of the HSV RNR. (200, 201)

The HSV RNR is known to be dispensable for viral replication in cultured cells due to the presence of compensatory human RNR activity. (15) We hypothesized that if the target of antiretroviral nucleoside analogs was the viral RNR, we would expect drug resistance in a model where replication occurs independently of the viral RNR.

Thus, we sought to establish a similar *in vitro* replication system where the EBV RNR was not required for replication and test the ability of antiretroviral drugs to inhibit viral lytic DNA replication. Since the EBV RNR is resistant to hydroxyurea (HU) while the human RNR is sensitive, (202) we treated cells with HU and measured viral copies after lytic induction (Fig 3.4A). When using butyrate induction, we observed that lytic viral DNA replication plateaued at 50% even at high doses of HU, suggesting that the viral RNR was required, at least for 50% of nucleotide biosynthesis in this model system (Fig 3.4A). However, using 5-AzadC, viral replication was completely inhibited by HU (Fig 3.4A), indicating that in this system viral replication occurs independently of the viral RNR. These differences likely reflect the coordination of viral RNR activity in dividing vs. non-dividing cells. Using 5-AzadC induction as a model for viral RNR-independent replication, we still observed complete inhibition by the antiretroviral drug TAF (Fig 3.4B) and similarly with the EBV polymerase inhibitor ganciclovir (GCV) (Fig 3.4C). Dose-response curves were shifted by approximately 10-fold for both TAF and GCV. However, this may be accounted for by higher dNTP levels in cells treated with 5-AzadC as discussed earlier in this chapter. Similar results were obtained with AZT and ABC. Thus, the viral ribonucleotide reductase was unlikely to be the primary target of nucleoside/nucleotide analogs, leaving the viral DNA polymerase as an alternative hypothesis.

Figure 3.4

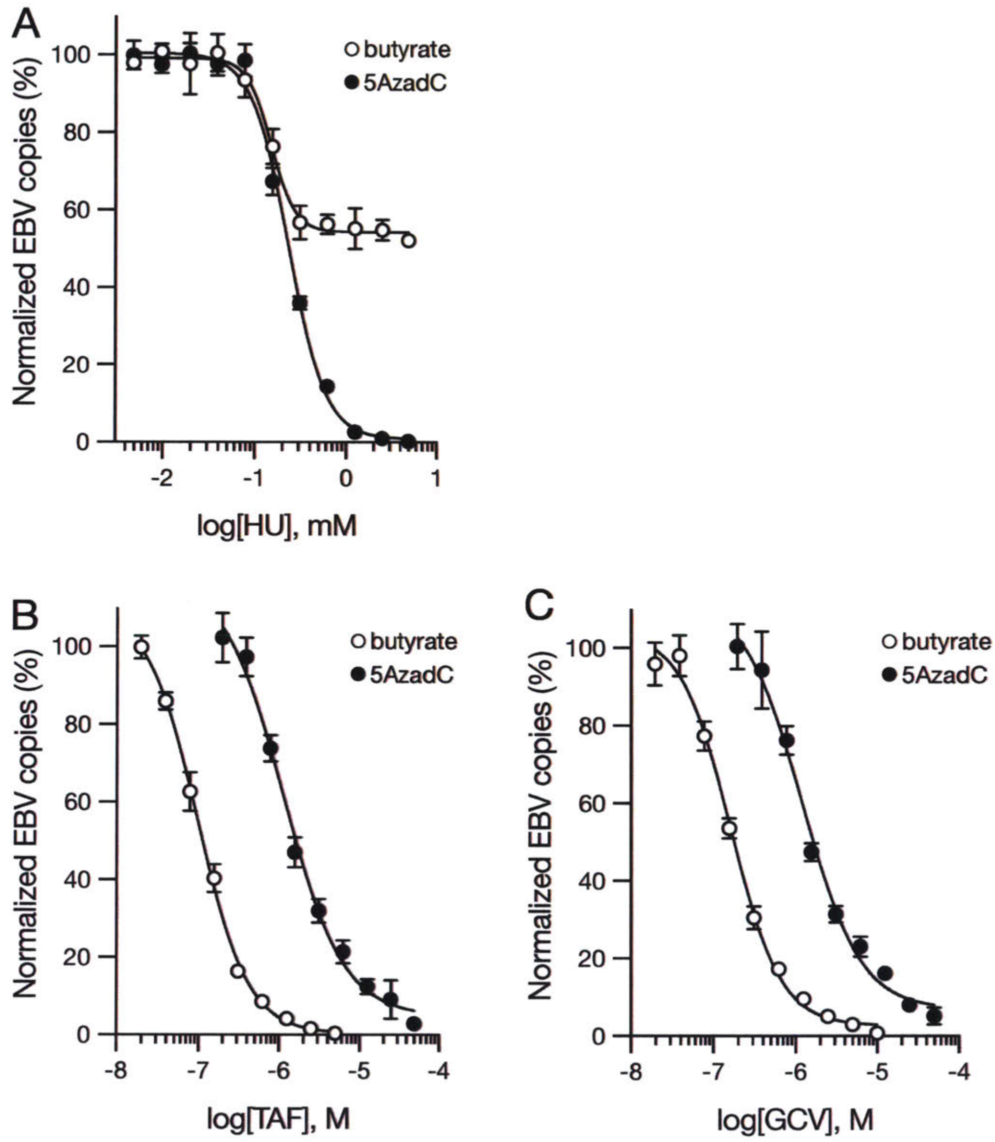


Figure 3.4 The target of antiretroviral nucleoside/nucleotide analogs is not the EBV ribonucleotide reductase

HH514-16 cells were induced with either 5μM 5-AzadC or 3mM butyrate and treated with (A) hydroxyurea, (B) TAF, or (C) the established anti-herpesviral drug GCV over a range of concentrations. Each point represents the mean and SD obtained from 3 independent experiments. EBV copies were normalized to results obtained with no drug, which were not significantly different between butyrate and 5-AzadC.

3.2.5 Drug-triphosphates of antiretroviral nucleoside/nucleotide analogs inhibit the EBV DNA polymerase except for AZT-triphosphate

We hypothesized that the triphosphate forms of antiretrovirals may inhibit the viral DNA polymerase by directly acting as chain terminating agents, similar to their mechanism against the HIV reverse transcriptase. To test the ability of nucleoside/nucleotide analogs to inhibit the EBV DNA polymerase directly, we carried out *in vitro* polymerase assays. The EBV polymerase catalytic subunit (BALF5) and processivity factor (BMRF1) were produced by transcription/translation in Rabbit reticulocyte lysate. Polymerase assays were performed by measuring the incorporation of [3H]dNTPs into activated calf thymus DNA upon addition of drug-triphosphates (TPs). All assays were carried out in the presence of 1µM of the competing nucleotide for each drug to approximate physiological levels of dNTPs. Drug-TP concentrations were varied from 0-50µM.

To validate our assay, we first tested two positive controls, ACV-TP and GCV-TP, which are known inhibitors of the EBV DNA polymerase (Fig 3.5A). We then tested five drug-TPs representing the active metabolites of six antiretroviral nucleoside analogs. A list of drug-TPs is provided at the start of this chapter in Table 3.1. Three drug-TPs represent drugs that were found to be effective in cell-based assays:

carbovir-triphosphate ((-)-CBV-TP) – the active metabolite of ABC; TFV-DP – the active metabolite of TDF and TAF; and AZT-TP – the active metabolite of AZT (Fig 3.5B). We also included two drugs that did not affect EBV lytic DNA replication in cell-based assays: ddA-TP – the active metabolite of ddI; and 3TC-TP – the active metabolite of 3TC (Fig 3.5C).

We observed that drug-TPs could be divided into two categories by IC_{50} in the in vitro polymerase assay: either <50μM or >50μM. We used this threshold to define the former as 'effective' and latter as 'Ineffective'. Our results indicate that drugs previously classified as ineffective in cell-based assays (ddI and 3TC) were also ineffective for inhibiting the EBV DNA polymerase (IC_{50} > 50μM). However, drugs that were classified as effective in cell-based-assays (AZT, ABC, and TDF/TAF) were not all effective against the polymerase. Specifically, AZT could not be distinguished from cell-based assay ineffective drugs based on the action of drug-TPs on the EBV polymerase. AZT-TP was as ineffective (IC_{50} > 50μM) as 3TC-TP and ddA-TP. As a positive control, we tested AZT-TP on the HIV reverse transcriptase (RT). In contrast to its effects the EBV polymerase, AZT-TP was a more potent inhibitor of the HIV RT than either TFV-DP or (-)-CBV-TP (Fig S3.2).

We further compared the cell-based assay IC_{50} vs. polymerase IC_{50} for drugs that were effective in both assays (Fig 4.5D). A general trend was observed, where drugs that were more effective on the polymerase as triphosphates were also more effective in cell-based assays. However, the relationship between the two was not linear, suggesting that drug metabolism in cells also plays a role in determining potency.

These findings raise questions about whether AZT-TP could plausibly mediate effects observed *in vivo*. Based on these results, other AZT metabolites should be examined for their capacity to inhibit EBV by other mechanisms. The effects of TDF and TAF are discussed in more detail in Chapter 4 of this thesis.

Figure 3.5

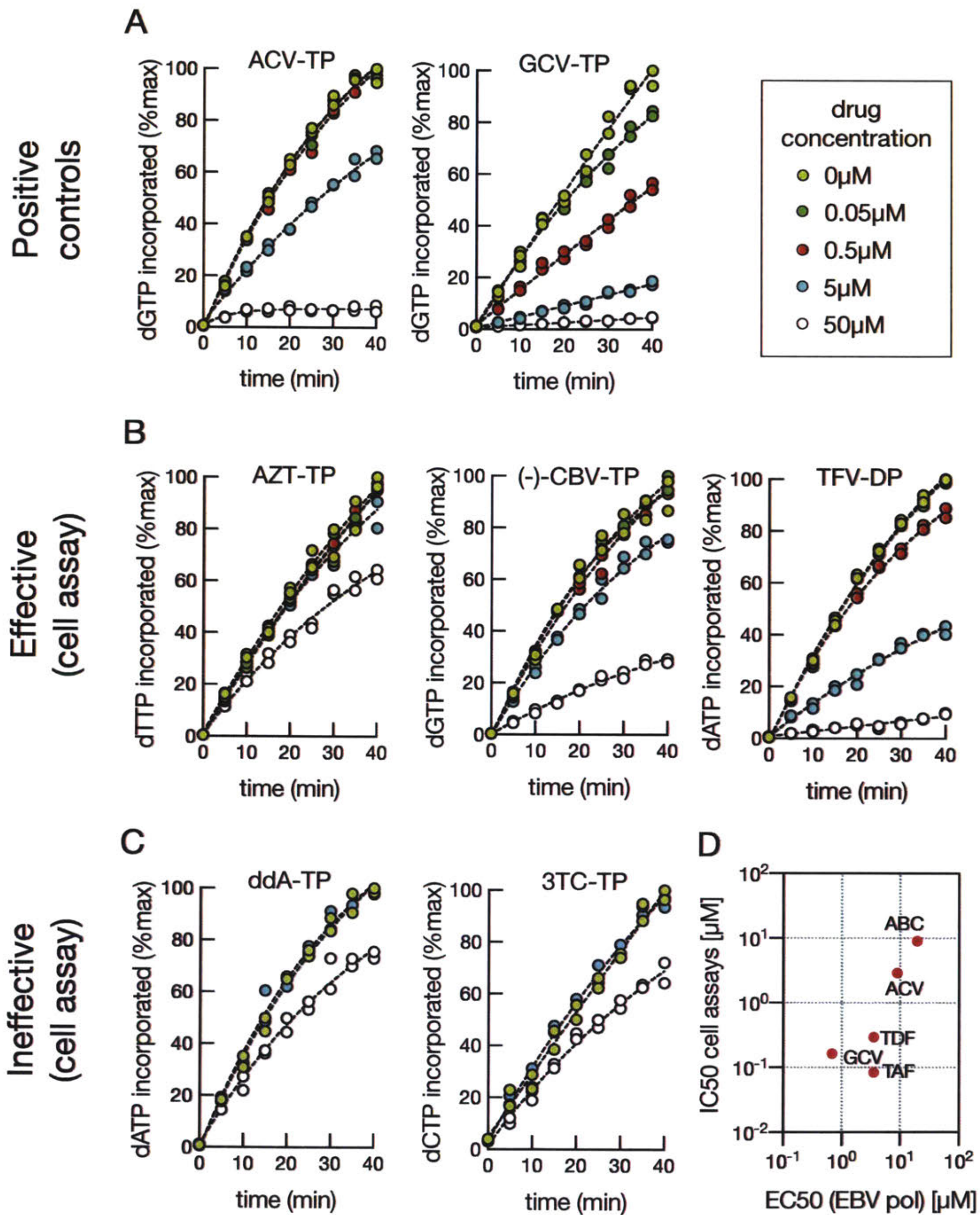


Figure 3.5 Drug-triphosphates of antiretroviral nucleoside/nucleotide analogs effectively inhibit the EBV DNA polymerase except for AZT-TP

In vitro polymerase assays were performed by measuring the incorporation of 1 μM competing [^{3}H]dNTPs into activated calf thymus DNA during a 40 min reaction followed by DEAE filter-binding and scintillation counting. The positive controls (A) ACV-TP and GCV-TP; the cell-based assay effective drugs (B) AZT-TP, (-)-CBV-TP, and TFV-DP; and the ineffective drugs (C) ddA-TP and 3TC-TP were added to the reaction from 0-50μM. Every 5 minutes, aliquots were removed and quenched with EDTA. Counts per minute were normalized to maximum counts obtained at 40 min. Each time point represents 2 independent experiments, each value shown. (D) The IC_{50} for each drug in cell-based assays was compared to the EC_{50} for the corresponding drug-triphosphate.

Figure S3.2

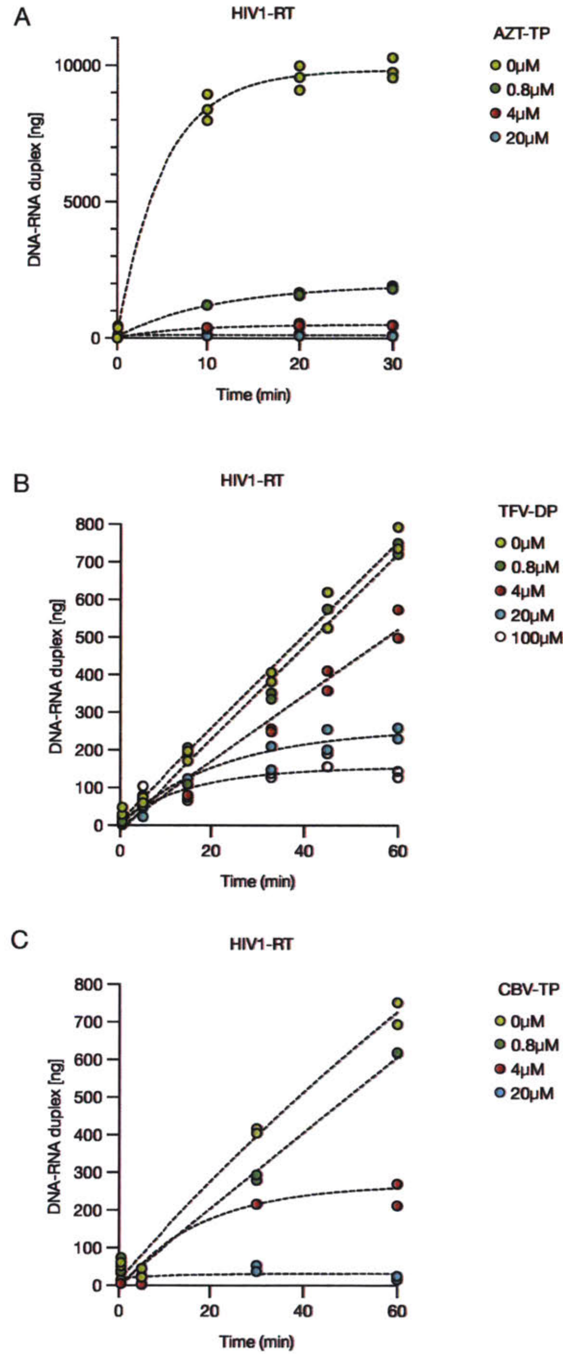


Figure S3.2 Drug-triphosphates of antiretroviral nucleoside/nucleotide analogs, including AZT-TP, inhibit the HIV reverse transcriptase

In vitro reverse transcriptase assays were performed using a poly(rN) oligo (dN) annealed primer-template, with extension by recombinant HIV-RT. Reactions were quantified by measuring the incorporation of PicoGreen reagent into the RNA-DNA heteroduplex. Fluorescence was normalized using a lambda DNA standard curve. (A) AZT-TP, (B), TFV-DP, and (C) (-)-CBV-TP were added to the reaction from 0-20μM. At indicated time points, aliquots were removed and quenched with EDTA. Each time point represents independent experiments, each value shown.

3.3 Discussion

In this chapter, we classify antiretroviral drugs into two distinct categories with respect to their ability to inhibit EBV lytic DNA replication: those that are effective and those that are ineffective. These distinctions can be made both at the cellular level and the polymerase level. Drugs that are effective in cell-based assays include ABC, AZT, d4T, TDF, and TAF. Further testing of available drug-triphosphates (all except d4T) revealed that (-)-CBV-TP and TFV-DP are also effective inhibitors of the EBV polymerase. However, AZT-TP is not effective and could not be distinguished from cell-based assay ineffective drugs based on this property. Two potential explanations for this observation exist – either AZT is metabolized to AZT-TP at very high concentrations in cell cultures, or other AZT metabolites direct the anti-EBV effects of AZT.

While it is possible that AZT is converted to AZT-TP at high concentrations in cells, the metabolism of AZT to AZT-TP is known to be hindered by the poor utilization of AZT-monophosphate (MP) as a substrate for human thymidylate kinase (TMPK). (203, 204) This enzyme is rate-limiting for AZT phosphorylation, as AZT is an efficient substrate for the human thymidine kinase (TK). (204) EBV also encodes a viral TK, which is capable of phosphorylating both thymidine and AZT. (77) However, unlike

the HSV TK, where the primary product of thymidine phosphorylation is thymidine diphosphate, the EBV TK has very little thymidylate kinase activity, and the primary product formed is thymidine monophosphate. (77) Thus, the metabolism of AZT either by the human or viral TK, would likely lead to the accumulation of AZT as the monophosphate form. In line with this, the maximal intracellular AZT-MP concentration achieved in PBMCs *in vivo* is 22-fold higher than the level of AZT-TP. (205) Interestingly, the concentration of AZT-MP at standard clinical dosing reached in humans is well above the IC_{50} for inhibition of protein glycosylation and glycolipid biosynthesis by direct competition with pyrimidine sugars for transport into Golgi membranes. (206, 207) The potential contribution of protein glycosylation to the anti-EBV effects of AZT merits further investigation. A specific inhibitor of protein glycosylation, tunicamycin, has been shown to reduce virus yield from EBV-producing cells. (208) While it is possible that the effect of AZT on EBV DNA replication in cultured cells is due to AZT-TP direct effects on the EBV polymerase if the concentrations reached are supra-physiological, any potential clinical effects of AZT *in vivo* are more likely to be mediated through alternative effects, perhaps by AZT-MP on protein glycosylation.

These experiments were performed to attempt to explain the reported cases of patients with MS who responded to antiretroviral therapy (191). The treatment

regimens for some cases included AZT-based combination therapy. However, others contained tenofovir-based regimens (191). In contrast to AZT, our results showed that the tenofovir prodrugs TDF and TAF were effective in both cell-based and polymerase assays. (These drugs are examined in more detail in Chapter 4).

Some hypotheses have been proposed for the effects of HIV drugs in MS, including the reconstitution of cellular immunity to EBV, the treatment of an endogenous retrovirus, and immunomodulation by fumarate as a component of TDF. However, HIV acquisition following MS (182) argues against immune reconstitution to EBV as a mechanistic explanation, since MS was present before HIV. Furthermore, there is no compelling mechanism by how antiretroviral drugs could affect an endogenous retrovirus because these drugs act upstream of integration to prevent infection of naïve cells. By definition, an endogenous retrovirus would already be integrated into all cells. Evidence against a retroviral hypothesis is also provided by the failure of the integrase inhibitor raltegravir in a clinical trial for MS. (209) The premise for the fumarate explanation is equally weak as the fumarate component of TDF has been equated to the MS drug dimethyl fumarate (DMF) due to similarity of name. However, fumarate has a different chemical structure with no reported immunosuppressive properties. Therefore, alternative explanations for the effect of antiretroviral drugs in MS are needed.

The evidence provided in this chapter raises the possibility that the observed effects on MS in several case reports of patients taking antiretroviral drugs may be explained by the anti-EBV effects of antiretroviral drugs. The results of our investigations also suggest that tenofovir-class drugs are likely to be mechanistically distinct from AZT with respect to EBV. Further exploration of the mechanism of action of AZT would be informative. TDF and TAF are covered in more detail in chapter 4.

3.4 Methods

3.4.1 Lytic induction and drug treatment

The EBV+ HH514-16 cell line was a kind gift from Prof. George Miller's lab at Yale School of Medicine. Cells were seeded at a concentration of $4x10^5$ cells/mL in RPMI-10% FBS supplemented with penicillin (100 units/mL), streptomycin (100 μg/mL), and Amphotericin-B (1μg/mL). Sodium butyrate (Sigma) was used to induce EBV lytic DNA replication at a concentration of 3mM. 5-AzadC (Sigma) was used to induce EBV at 5μM. Didanosine (Sigma), lamivudine (Sigma), emtricitabine (SelleckChem), abacavir sulfate (Sigma), zidovudine (Sigma), stavudine (Sigma), tenofovir disoproxil fumarate (Sigma), tenofovir alafenamide (SelleckChem), acyclovir (Sigma), and ganciclovir (Sigma) were added at concentrations and time points indicated in the text. Working solutions of all drugs were dissolved in dimethyl sulfoxide (DMSO) except abacavir sulfate, which was dissolved in sterile water. For all experiments, the final concentration of DMSO was below 0.1% (v/v).

3.4.2 Quantification of EBV copy number by qPCR

Cells were centrifuged at 500xg for 3 min and washed once with PBS. Genomic DNA was extracted using the Qiagen DNeasy Blood & Tissue Kit (Qiagen) and subjected to qPCR. A 20μL reaction was set up with 250ng genomic DNA. The PCR

mix contained 1x PrimeTime qPCR Master Mix (Integrated DNA Technologies), 500 nM each primer, and 250 nM probe.

Primers and fluorescent probes (5'FAM/ZEN/3'IBFQ) for the BALF5 gene of EBV and GAPDH (Integrated DNA Technologies) are listed below:

BALF5 primers: 5'-CGGAAGCCCTCTGGACTTC-3',

5'-CCCTGTTTATCCGATGGAATG-3',

BALF5 probe: 5'-TGTACACGCACGAGAAATGCGCC-3',

GAPDH primers: 5'-ACATCACCCCTCTACCTCC-3',

5'-CTCCCACCTTTCTCATCCAAG-3',

GAPDH probe: 5'-AAAGCCAGTCCCCAGAACCCC-3'.

The following cycling conditions were used: 95°C for 10 min followed by 40 cycles with 95°C for 15s and 60°C for 45s carried out on a LightCycler 480 system (Roche Diagnostics). GAPDH was used as an internal standard. The calculation of the relative amounts of BALF5 DNA compared to GAPDH DNA was performed using the $2^{-\Delta\Delta Cp}$ method using the Cp (second derivative maximum) calculated by the LightCycler software. Each experiment was performed with three biological replicates. To calculate the absolute number of EBV copies, the EBV BALF5 gene was cloned by PCR from HH514-16 genomic DNA into pcDNA3.1+ vector. Absolute

EBV DNA copy number was calculated from a standard curve generated by serial dilutions of pcDNA3.1-BALF5 added to human genomic DNA extracted from the EBV-negative A549 cancer cell line (ATCC).

3.4.3 Recombinant EBV DNA polymerase assays

The EBV DNA polymerase subunits BMRF1 and BALF5 were separately cloned by PCR from HH514-16 genomic DNA into pcDNA3.1+ vector. Recombinant proteins were expressed using the Promega TnT T7 Coupled Reticulocyte Lysate System according to the manufacturer's instructions. Protein expression in reticulocyte lysates was validated by using the FluoroTect GreenLys *in vitro* Translation Labeling System (Promega), gel electrophoresis, and fluorescence imaging using a Typhoon 9400 scanner (Amersham/GE). The recombinant polymerase proteins in reticulocyte lysates were then desalted using PD-10 Sephadex-G25 columns to remove any potential interfering salts or nucleotides present in reticulocyte lysates. Polymerase assays were set up as follows in a total volume of 100µL on ice: 50 mM Tris-HCl (pH 7.5), 100 mM ammonium sulfate (Sigma), 50 µg/ml BSA (Sigma), 1 mM DTT (Sigma), 3 mM $MgCl_2$ (Sigma), 10 µg/ml activated calf thymus DNA (GE Healthcare Life Sciences), 100 µM non-limiting dNTPs (New England Biolabs), 1 µM limiting ^{3}H-dNTP (dGTP for ACV-TP/GCV-TP/(-)-CBV-TP, dATP for ddA-TP/TFV-DP, dTTP for AZT-TP) (Moravek), or 1 µM ^{32}P-dCTP final concentration diluted with

cold dCTP for 3TC-TP (Perkin Elmer), 0-50 μM drug-triphosphate (ACV-TP, TFV-DP, and AZT-TP were from Moravek; GCV-TP from TriLink Biotechnologies; (-)-CBV-TP from Toronto Research Chemicals; 3TC-TP from Abcam; ddA from Sigma), and 10μL reticulocyte lysate (desalted) containing both recombinant EBV polymerase proteins mixed at a 1:1 ratio. Reactions were incubated at 37°C for 40min in a heating block. Aliquots (10 μL) were removed every 5 min, mixed with 5 μL EDTA (200mM), and incubated on ice to stop the reaction. Reactions (7 μL) were spotted on DEAE anion exchange filter paper (Perkin Elmer) and dried for 10 min. Filters were washed twice with 5% (w/v) dibasic sodium phosphate for 5 min, water for 5 minutes, and rinsed with ethanol. Filters were then allowed to dry for 10 min, and added to 10mL Ultima Gold Scintillation Cocktail (Perkin Elmer) and counted on a Beckman LS6000 Scintillation Counter. All values were normalized to maximum counts observed with no drug at 40 min. Dose-response curves for inhibition of DNA replication by the EBV polymerase were fit by a five-parameter logistic regression model using the Prism 8 software (GraphPad), and EC_{50} measurements were calculated by the software using best-fit values.

3.4.4 Measurement of cell viability by trypan blue

Cells (seeded at an initial concentration of 4×10^5 cells/mL), were examined at time points and drug concentrations indicated in the text. Viability was measured by trypan blue exclusion using a 1:1 mixture of a cell suspension with 0.4% trypan blue in PBS, and quantified using an Auto T4 Cellometer Cell Counter (Nexcelom Biosciences).

3.4.5 Recombinant HIV reverse transcriptase (RT) assays

In vitro RT reactions were performed using the EnzChek™ Reverse Transcriptase Assay Kit (Thermo Fisher) and recombinant HIV-RT (Sigma). To test AZT-TP, the kit was used according to manufacturer's instructions with extension of a poly(rA) template using oligo(dT_{16}) primers. To test TFV-DP, a poly(rU) template (Sigma) was used with oligo(dA_{16}) (IDT). Similarly, to test (-)-CBV-TP, a poly(rC) template (IDT) was used with oligo(dG_{16}) (IDT). Briefly, RNA templates and DNA primers were mixed and incubated for 1 hour at room temperature. These were diluted into polymerization buffer for a final reaction volume of 25µL containing 60mM Tris-HCl (pH 8.1), 60mM KCl, 8mM $MgCl_2$, 13mM DTT, and 100µM each dNTP, and 0.5U HIV-RT. Reactions were incubated at room temperature for times indicated in text and stopped by the addition of 2µL 200mM EDTA. RNA-DNA duplexes were quantified by PicoGreen reagent according to the manufacturer's instructions.

3.4.5 Statistical Analysis

As indicated in text, a Brown-Forsythe and Welch ANOVA for unequal variances was initially used. Multiple hypothesis testing was then conducted, with correction for multiple testing, where appropriate, using the Prism 8 software (GraphPad).

CHAPTER 4: TENOFOVIR PRODRUGS POTENTLY INHIBIT EPSTEIN-BARR VIRUS LYTIC DNA REPLICATION BY TARGETING THE VIRAL DNA POLYMERASE

Note: This chapter has been adapted from work in preparation for publication as of January 2020

4.1 Abstract

Epstein-Barr virus (EBV) is a ubiquitous human **γ**-herpesvirus that establishes life-long infection. Antivirals repurposed from the treatment of α-herpesviruses have had limited clinical translation to EBV, despite *in vitro* effectiveness. However, the potency of antiviral drugs is known to differ substantially between α- and **γ**-herpesviruses, raising issues for clinical efficacy. In the present study, we aimed to characterize compounds that bypass virus-dependent drug activation in order to increase potency. We show that the nucleotide analogs tenofovir disoproxil fumarate (TDF) and tenofovir alafenamide (TAF) inhibit EBV lytic DNA replication, with an IC_{50} of 0.297μM and 0.084μM, respectively. In a cell-based assay, TDF and TAF are more potent than α-herpesviral drugs that are clinically ineffectual against EBV. TAF has a 35- and 24-fold, and TDF has a 10- and 7-fold lower IC_{50} than acyclovir and penciclovir, respectively. TAF is also twice as potent as the β-

herpesviral drug ganciclovir. The active metabolite of tenofovir prodrugs, tenofovir-diphosphate (TFV-DP), inhibits incorporation of dATP into a primed DNA template by the EBV DNA polymerase *in vitro*, and is more potent than acyclovir-triphosphate. Finally, we show that a functional consequence of bypassing viral-dependent drug metabolism is the ability to initiate treatment prior to the viral lytic cycle. Treatment of cells in latency for 24 hours by TAF, but not ACV, still inhibited EBV lytic DNA replication 72 hours after drug was removed. In summary, low potency and dependence on viral enzymes may potentially contribute to the limited clinical translation of α-herpesviral drugs to EBV. These issues are addressed by tenofovir prodrugs.

4.2 Background and Introduction

Epstein-Barr virus (EBV) is a B-cell tropic dsDNA γ-herpesvirus that infects >90% of humans by adulthood. (33) While primary EBV infection is typically self-limiting in children, it can become pathogenic later in life. EBV causes infectious mononucleosis (IM), which is a significant cause of morbidity in adolescents and young adults. (13) Additionally, EBV is associated with an increased risk for a wide range of diseases - molecular and epidemiological studies have linked EBV to malignancies, (129) autoimmune diseases, (184) and diseases of immunodeficiency. (145, 185, 186) Cancers linked directly to EBV are estimated to account for at least 1.5 % of global cancer burden. (129) In addition, breast cancer (135) and ovarian cancer (136) are associated with IM. In the context of autoimmunity, the odds of multiple sclerosis in EBV-negative individuals is extremely low, but increases dramatically after infection. (120) MS is also strongly associated with IM. (122) These studies speak to the risk of disease following EBV infection, particularly late-onset EBV, and beg the question of whether or not treatment of primary infection would have an impact on the subsequent development of pathology.

Antiviral agents repurposed from α-herpesviruses have had minimal clinical efficacy against EBV despite *in vitro* effectiveness. Acyclovir (ACV), an acyclic guanosine

analog clinically effective against herpes simplex virus-1 and -2 (HSV-1/2), has shown no significant benefit in the treatment of IM. (161) However, there was a trend toward shorter duration of symptoms with drug treatment. (161) Similarly, clinical results in EBV-associated diseases have been suggestive but ambiguous. (162, 163) More potent agents like ganciclovir (GCV) have not been widely tested for EBV-related indications due to severe hematological toxicity and need for intravenous administration. (166) The lack of efficacy of ACV has been interpreted to indicate an absence of viral replication in disease pathogenesis. (210) Nonetheless, there are substantial differences in ACV metabolism and drug action between α and γ-herpesviruses that make ACV a comparatively weaker drug for EBV. The half-maximal inhibitory concentration (IC_{50}) of ACV for EBV is significantly higher than for HSV, (78, 159, 160) and ACV has poor selectivity for the EBV DNA polymerase compared to the HSV-1 DNA polymerase. (198)

Like ACV, tenofovir (TFV) is also an acyclic nucleoside/nucleotide analog. (201) TFV is the primary metabolite of the prodrugs tenofovir disoproxil fumarate (TDF) and tenofovir alafenamide (TAF). (211) These drugs are licensed for the treatment of human immunodeficiency virus (HIV) infection, HIV prophylaxis, and Hepatitis B. Both TDF and TAF are orally bioavailable drugs with highly favorable safety profiles. (212) A significant difference between ACV and TFV lies in the initial phosphorylation

step - ACV requires a viral kinase while TFV does not because it is itself a monophosphorylated nucleotide. (201) TDF and TAF contain chemical modifications which mask the negatively charged phosphate group of TFV, allowing the drugs to reach higher intracellular concentrations. In contrast to ACV, TFV prodrugs are metabolized by host enzymes to their active triphosphate form, tenofovir-diphosphate (TFV-DP). (211) Between the two prodrugs, TAF has better distribution into lymphoid tissues, including lymphocytes, lymph nodes, and the spleen. (213)

A recent study established that in addition to targeting the HIV reverse transcriptase, TFV targets the HSV-1/2 DNA polymerase. (193) Here we show that prodrugs of TFV - TDF and TAF - are also effective at inhibiting EBV lytic DNA replication. Furthermore, TDF and TAF resolve key issues that may potentially have limited the clinical translation of ACV to EBV.

4.3 Results

4.3.1 TDF and TAF inhibit EBV lytic DNA replication

Since compounds that have efficacy against α-herpesviruses (76, 156) can inhibit EBV lytic DNA replication *in vitro,* (78) we asked if TFV prodrugs can have antiviral activity against EBV. In the EBV+ HH514-16 cell line, a subclone of P3HR-1 cells selected for low spontaneous EBV reactivation, (194) we induced lytic DNA replication with sodium butyrate. After 96 hours of butyrate treatment, the average number of viral copies per genome increased 128-fold (Fig 4.1A). We used the nucleoside analogs ACV and GCV, two known inhibitors of EBV lytic DNA replication, as positive controls. (78) At 50μM concentrations, both ACV and GCV effectively suppressed lytic DNA replication, reducing the number of new viral copies produced by >95.5% and >99.9%, respectively (Fig 4.1A). In line with previous studies, GCV was more inhibitory than ACV. (78) The TFV prodrugs TDF and TAF also reduced the number of viral copies produced after lytic induction, each by >99.9% (Fig 4.1A).

EBV DNA replication occurs both during the lytic cycle and during latency but is mediated by distinct mechanisms during each state. In latency, the EBV episome is

replicated by the host DNA replication machinery. (13) However, lytic viral DNA replication is mediated by the viral replication machinery independent of the host. EBV encodes its own DNA polymerase and processivity factor, as well as a viral DNA primase, primase accessory protein, helicase, ssDNA binding protein, and ribonucleotide reductase. (13) To determine if the reduction in EBV copy number mediated by TDF and TAF was specific to the lytic cycle, we also treated cells with each drug in the absence of butyrate induction. We saw no significant reduction in viral copy number at a 50μM dose with either drug during latency (Fig S4.1A), supporting a role for TDF and TAF specifically during lytic DNA replication.

Next, we asked whether the reduction in viral copies could be due to general cytotoxicity of TDF and TAF. While lytic induction with butyrate slightly reduced viability compared to latency, we saw no significant additional reduction in viability during ACV or GCV treatment at a 50μM dose compared to butyrate alone (Fig. 1B). The lack of toxicity of these drugs may be due, in part, to the absence of cellular proliferation after butyrate treatment, as we saw no increase in cell number following butyrate induction (Fig S4.2). Similarly, there was no reduction in viability with TAF treatment (Fig. 1B). However, we observed a decrease in viability with TDF. Unlike TAF, TDF is known to be highly unstable *in vitro* in the presence of serum. (214) To check if the toxicity of TDF was specific to cells in the lytic phase, we treated cells

in the absence of butyrate (Fig. S1B). While ACV, GCV, and TAF showed no reduction in viability, TDF-treated cells had reduced viability, suggesting the toxicity of TDF is independent of lytic induction. Our results suggested that toxicity alone could not account for the ability of tenofovir prodrugs to inhibit EBV lytic DNA replication.

Figure 4.1

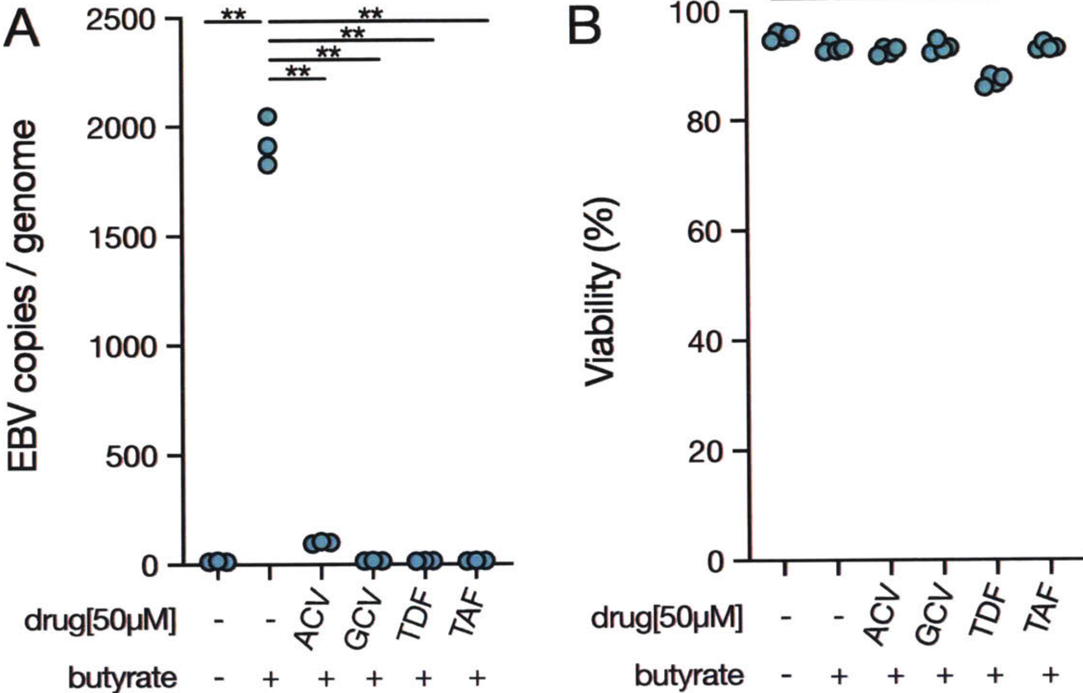


Figure 4.1: TDF and TAF inhibit EBV lytic DNA replication.

(A) HH514-16 cells were induced with 3mM butyrate for 96 hours and treated with the tenofovir prodrugs TDF and TAF, or the established anti-herpesviral drugs ACV and GCV. EBV copies per genome were measured by qPCR after 96 hours of treatment at a 50µM dose. Results were analyzed from 3 independent experiments, each value shown. One-way ANOVA ($p<0.05$) was followed by multiple hypothesis testing between butyrate and each condition. Statistical significance is highlighted by P values as follows: **$P < 0.01$ (B) Viability was measured by trypan blue exclusion after 96 hours. Results were analyzed from 4 independent experiments, each value shown. One-way ANOVA ($p<0.05$) was followed by multiple hypothesis testing between butyrate and each condition. Statistical significance is highlighted by P values as follows: *$P < 0.05$; ***$P < 0.001$.

Figure S4.1

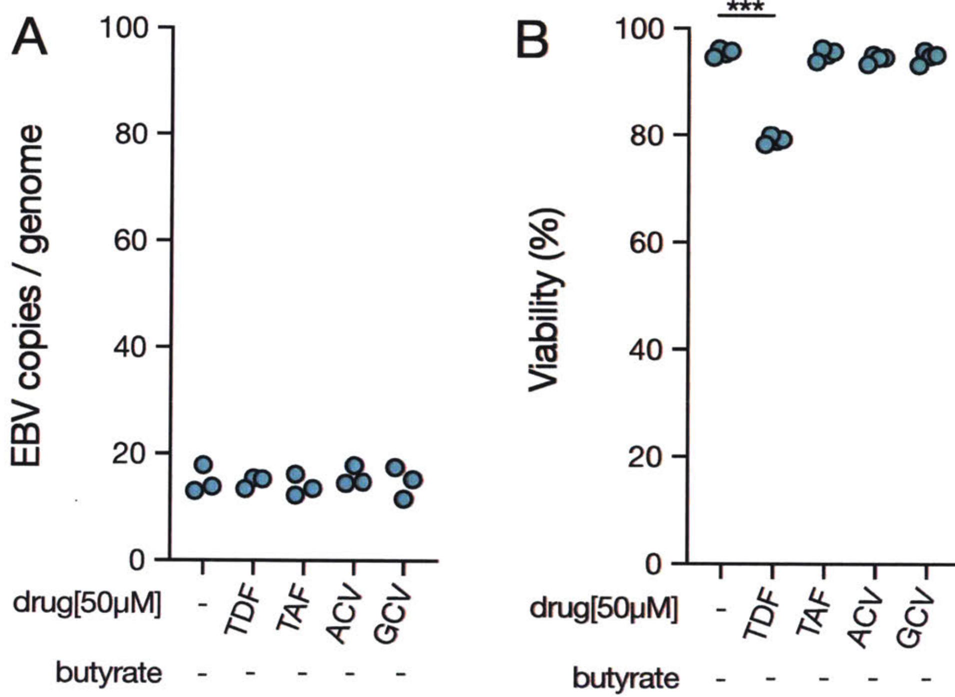


Figure S4.1: Effects of TDF and TAF on EBV DNA copies during latency.

(A) HH514-16 cells were grown in the absence of butyrate for 96 hours and treated with the tenofovir prodrugs TDF and TAF, or the established anti-herpesviral drugs ACV and GCV. EBV copies per genome were measured by qPCR after 96 hours of treatment at a 50μM dose. Results were analyzed from 3 independent experiments, each value shown. One-way ANOVA was performed ($p>0.05$). (B) Viability was measured by trypan blue exclusion after 96 hours. Results were analyzed from 4 independent experiments, each value shown. One-way ANOVA ($p<0.05$) was followed by multiple hypothesis testing between no drug and each condition. Statistical significance is highlighted by P values as follows: ***$P < 0.001$.

Figure S4.2

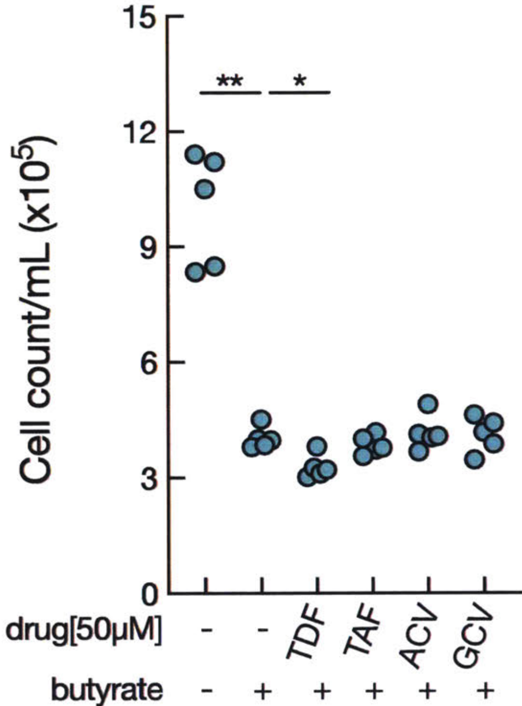


Figure S4.2: Effects of butyrate and drug treatment on number of cells after 96 hours.

HH514-16 cells were treated with the tenofovir prodrugs TDF and TAF, or the established anti-herpesviral drugs ACV and GCV. Cell count per mL was measured after 96 hours of treatment at a 50µM dose. Results were analyzed from 5 independent experiments, each value shown. (B) One-way ANOVA ($p<0.05$) was followed by multiple hypothesis testing between butyrate and each condition. Statistical significance is highlighted by P values as follows: *$P<0.05$; **$P < 0.01$

4.3.2 TAF is a highly potent inhibitor of EBV lytic DNA replication in cell-based assays

TFV is classified as ineffective against EBV, (215) but TDF and TAF have not been previously tested. TFV is also known to have poor intracellular uptake in comparison to TDF and TAF, and is less potent against HIV *in vitro*, with a 100- and 1000-fold higher IC_{50} than the two prodrugs. (213) We sought to determine if HH514-16 cells exhibited differential sensitivity to TFV vs. its prodrugs. In line with the literature, (215) we found TFV to have little effect against EBV DNA replication, with an IC_{50} of ~100μM (Fig 4.2A), while the prodrugs were potent. TDF was 337-fold (IC_{50} = 0.297μM) and TAF 1190-fold (IC_{50} = 0.084μM) (Fig 4.2A) more potent than TFV.

To rank drug potency in the context of established inhibitors, we compared TFV prodrugs to standard anti-herpesviral drugs. ACV, GCV, and penciclovir (PCV) are clinically licensed for the treatment of α- and β-herpesviruses. These compounds also have efficacy against EBV *in vitro.* (78, 168) In direct comparison, TAF had a 2-, 24-, and 35-fold lower IC_{50} than GCV (0.163μM), PCV (2.03μM), and ACV (2.94μM), respectively (Fig. 2B). TDF had a 7- and 10-fold lower IC_{50} than PCV and ACV. Thus, both TDF and TAF were more potent than standard α-herpesviral drugs.

Since TAF is known to be metabolized by cellular enzymes to its active form, we asked if TAF may be selective for viral DNA replication. The cytotoxic concentration required to inhibit total cell viability during latency by 50% (CC_{50}) was 178µM (Fig S4.3), a 2119-fold difference compared to the IC_{50} for inhibition of EBV lytic DNA replication. Therefore, TAF was selective for EBV.

Figure 4.2

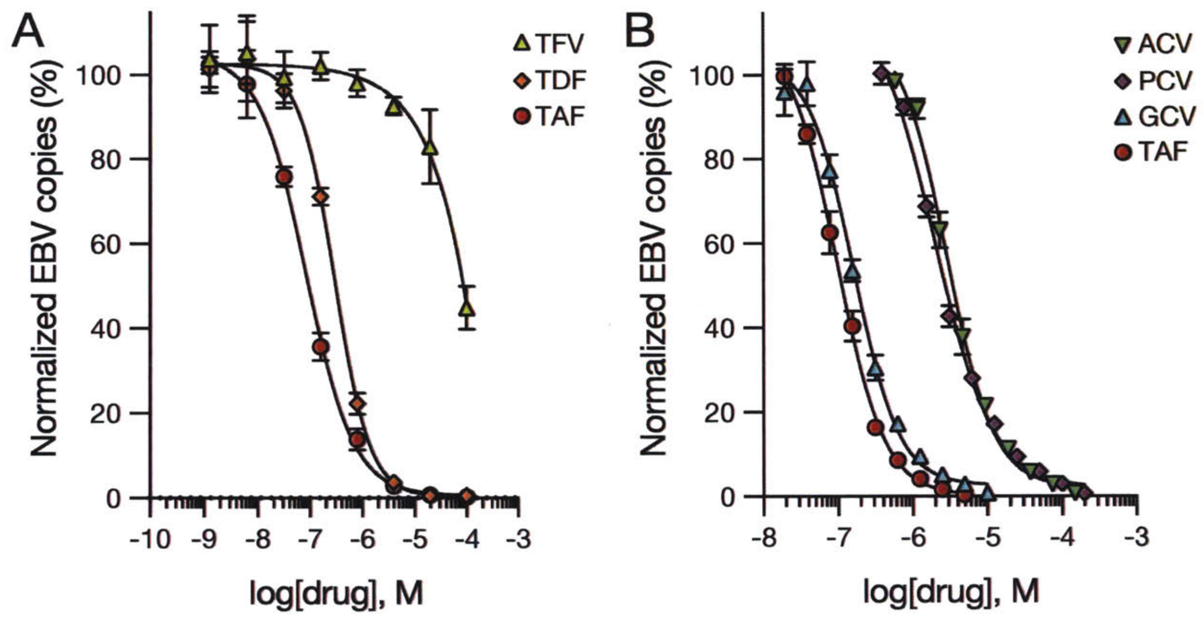


Figure 4.2: TAF is a highly potent inhibitor of EBV lytic DNA replication in cell-based assays.

(A) HH514-16 cells were induced with 3mM butyrate for 96 hours and treated with drugs over a range of concentrations. TFV was compared with the TFV prodrugs TDF and TAF, and (B) TAF was directly compared with the known anti-herpesviral drugs ACV, PCV, and GCV. Each point represents the mean and SD obtained from 3 independent experiments. Dose-response curves (black lines) were generated by four-parameter logistic regression analysis.

Figure S4.3

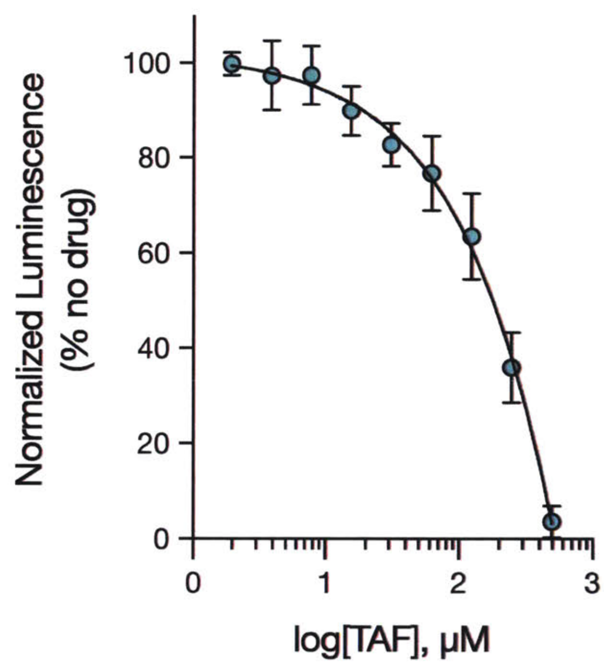


Figure S4.3: Effect of TAF dose of number of viable cells in latency after 96 hours.

HH514-16 cells were treated with TAF at indicated doses in the absence of butyrate. Total cell viability was measured by an ATP-dependent luminescence assay after 96 hours of treatment, which is dependent on both cell proliferation and percent viability. Luminescence was normalized using the signal from untreated samples (no cytotoxicity) and the signal from samples treated with 10µM podophyllotoxin (max. cytotoxicity). Results were analyzed from 3 independent experiments, data are plotted as mean ± SD.

4.3.3 TAF acts downstream of induction to block EBV lytic DNA replication

We first asked if TAF could be blocking lytic induction. To make direct comparisons between TAF and other anti-herpesviral drugs, we standardized the concentration of each drug to the dose required to inhibit 95% of EBV lytic DNA replication (IC_{95}) at 96 hours – 2.5µM for TAF, 62.5µM for ACV, and 5µM for GCV, respectively. We examined protein expression of the viral early antigen Ea-D, by western blot after 72 hours of drug treatment. Ea-D expression was not affected by TAF treatment, suggesting that TAF was not blocking induction. (Fig 4.3A). In line with this, when adding drug late (24 hours) after lytic induction, we saw a similar decrease in lytic DNA replication as when adding drug early (0 hours), further suggesting TAF acts downstream of induction (Fig 4.3B).

Figure 4.3

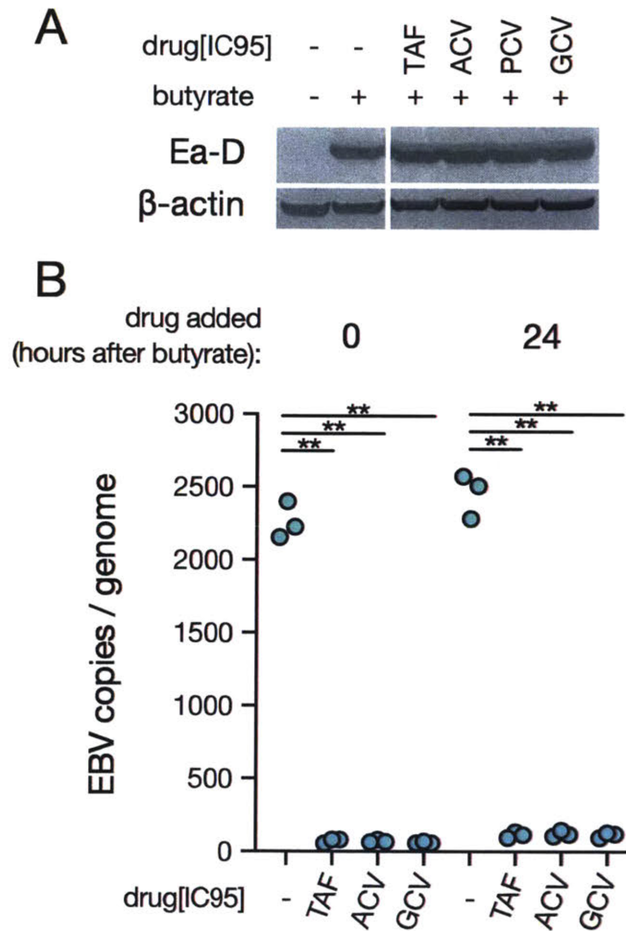


Figure 4.3: TAF acts downstream of viral lytic induction.

(A) HH514-16 cells were induced with 3mM butyrate in the presence of drugs standardized to IC_{95}. Total protein was collected at 72 hours and stained for the early viral antigen Ea-D or β-actin by western blot. Results are shown from one experiment representative of 3 independent experiments. (B) HH514-16 cells were induced with 3mM butyrate. Drug concentrations were standardized to IC_{95}. Drugs were added either 0 hours or 24 hours following butyrate addition, and the number of EBV copies per genome were quantified by qPCR. Results were analyzed from 3 independent experiments, each value shown. One-way ANOVA ($p<0.05$) was followed by multiple hypothesis testing between butyrate and each condition. Statistical significance is highlighted by P values as follows: **$P < 0.01$.

4.3.4 TAF inhibits a viral transcriptional process dependent on continuous lytic DNA replication

We hypothesized that TAF might be directly blocking a step in DNA synthesis by the viral DNA replication machinery. During γ-herpesviral lytic replication, continuous DNA synthesis is required for the transcription of late lytic viral genes, but not for early lytic genes. (22) We performed qPCR for six late lytic viral transcripts: BVRF1, BVRF2, BILF2, BDLF1, BDLF2 and BcLF1, and six early lytic viral transcripts: BMRF1, BaRF1, BALF2, BGLF4, BALF5, and BBLF2/3 (5) after 72 hours of drug treatment. Expression of all six late lytic viral genes decreased with TAF treatment at IC_{95} by >10-fold (Fig 4.4B). However, five of the six early viral transcripts were only minimally altered by TAF treatment (<2-fold change) (Fig 4.4A). We obtained the same patterns of gene expression during ACV and GCV treatment (Fig S4.4), suggesting TAF may act by a similar mechanism.

Figure 4.4

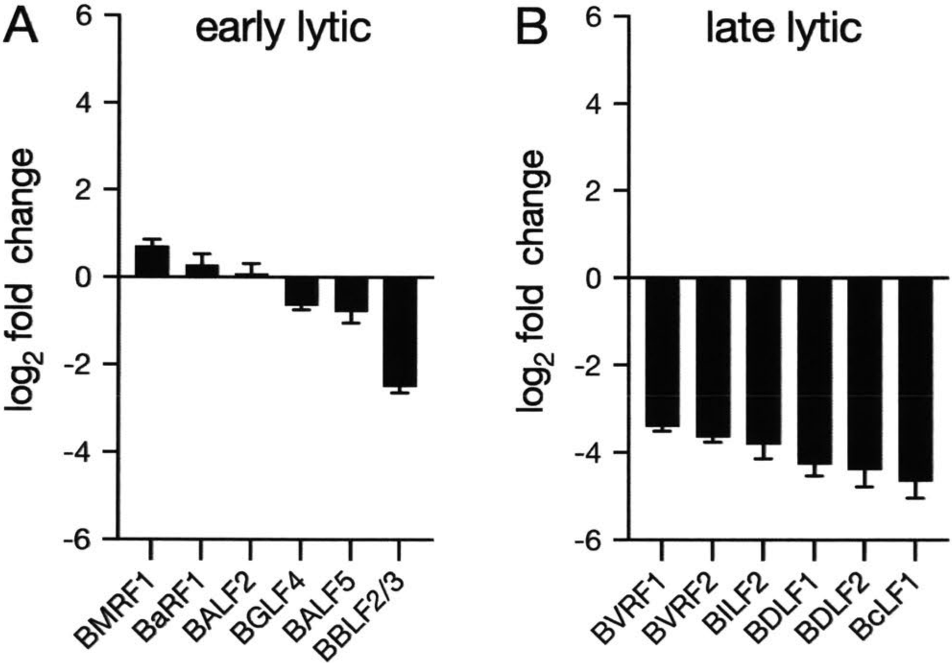


Figure 4.4: TAF inhibits a viral transcriptional process dependent on continuous lytic DNA replication.

(A) HH514-16 cells were induced with 3mM butyrate and treated with TAF[2.5μM] or no drug. Total RNA was collected at 72 hours and used to measure the expression of six early and (B) six late lytic genes by RT-qPCR. Gene expression was normalized to the housekeeping gene HPRT1. Each column represents the mean and SD obtained from 3 independent experiments.

Figure S4.4

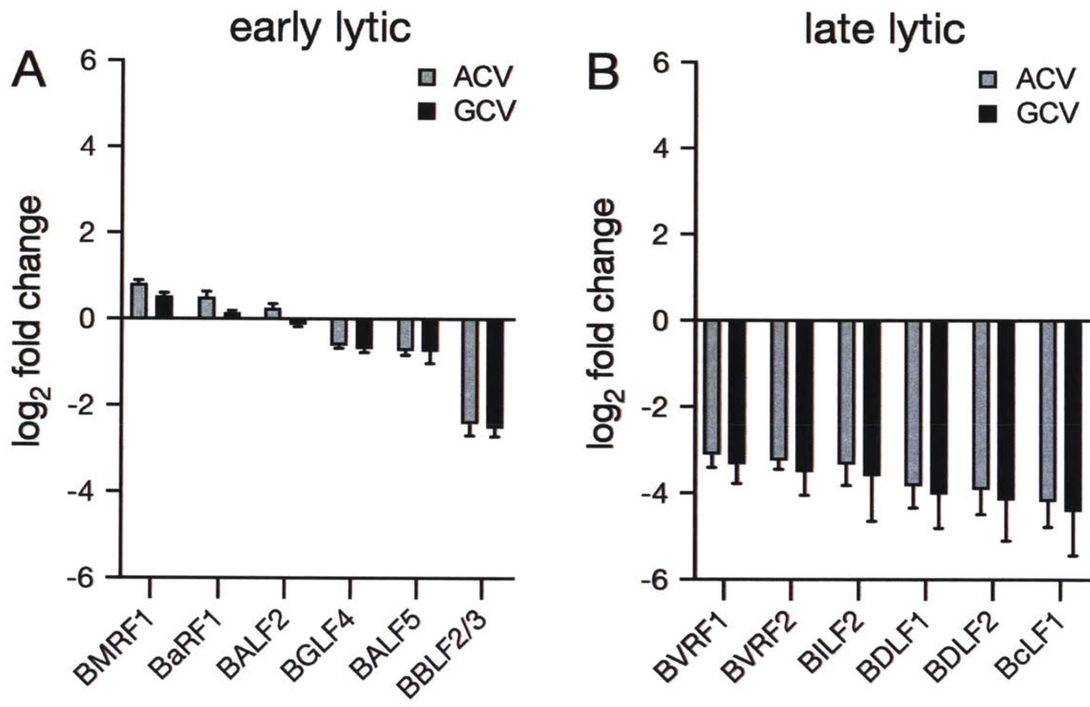


Figure S4.4: Effects of ACV and GCV on expression of early and late lytic viral genes.

(A) HH514-16 cells were induced with 3mM butyrate and treated with ACV[62.5μM], GCV[5μM], or no drug. Total RNA was collected at 72 hours and used to measure the expression of six early and (B) six late lytic genes by RT-qPCR. Gene expression was normalized to the housekeeping gene HPRT1. Each column represents the mean and SD obtained from 3 independent experiments.

4.3.5 TFV-DP inhibits the EBV DNA polymerase by competing with dATP

Since herpesviral DNA polymerases contain motifs conserved across subfamilies, (216) we investigated whether or not TAF can inhibit the EBV DNA polymerase. To do so, we carried out *in vitro* polymerase assays. The EBV polymerase catalytic subunit (BALF5) and processivity factor (BMRF1) were produced using a transcription/translation system in Rabbit reticulocyte lysates. Protein expression was confirmed by gel electrophoresis (Fig 4.5A). Polymerase assays were performed by measuring the incorporation of [3H]dNTPs into activated calf thymus DNA. Both BALF5 and BMRF1 were required for viral polymerase activity (Fig 4.5B), as previously reported. (216)

Measurements of dNTP levels in human primary B cells have not been reported. However, concentrations of dNTP contents of resting human primary T-cells range from 0.28-0.35 pmol/10^6 cells, while in other non-dividing cell types (e.g., macrophages), dNTP contents are significantly lower. (217) Using the mean volume of lymphocytes as 206 fL, (218) we calculated the range of average concentrations of dNTPs in T-cells as 1.36-1.70μM. From these values, we estimated near-physiologic levels of dNTPs in lymphocytes as 1μM. In our polymerase assays, we used either 1μM [3H]dATP or 1μM [3H]dGTP as the competing nucleotide for TFV-

DP or ACV-TP/GCV-TP, respectively. Non-competing unlabeled dNTPs were added to each reaction in excess. Drug-TPs were added at a range of concentrations up to 50μM (Fig 4.5C-E). Aliquots were removed every 5 min for filter binding assays. Dose-response curves for each drug were generated from the mean inhibition of DNA replication after 40min. We observed that GCV-TP was the most potent inhibitor of the EBV DNA polymerase (Fig 4.5F). In comparison to GCV-TP (IC_{50} = 0.65μM), TFV-DP was 5.4-times less potent (IC_{50} = 3.4μM), and ACV-TP was 13.2-times less potent (IC_{50} = 8.6μM) (Fig 4.5F).

We also asked whether TDF and TAF at standard clinical doses can reach intracellular concentrations of TFV-DP that inhibit EBV. We used the reported values of TFV-DP concentrations in PBMCs in human subjects following directly observed daily treatment with TDF (median 71; IQR: 53, 97 fmol/10^6 cells) and TAF (median 685; IQR: 566, 751 fmol/10^6 cells), (219) and the reported mean corpuscular volume of PMBCs (282.9 fL). (220) From these values, we calculated that the concentration of intracellular TFV-DP in PBMCs following TAF treatment would reach a concentration needing to block approximately 40% of DNA replication (EC40) mediated by the EBV polymerase after 40 min in our *in vitro* assay (Fig 4.5G). Intracellular concentrations of TFV-DP on daily TDF treatment fell below the EC10 (Fig 4.5G). Thus, TAF may be more effective against EBV.

Finally, to compare the anti-EBV activity of ACV-TP and TFV-DP with the anti-HSV activity of ACV-TP, we performed similar assays using the HSV-1 polymerase catalytic subunit, UL30 (Fig. S5). The IC_{50} of TFV-DP for EBV was only marginally higher (1.3-fold) than the IC_{50} of ACV-TP for HSV-1, suggesting that TFV-DP for EBV is comparable to ACV-TP for HSV-1.

Figure 4.5

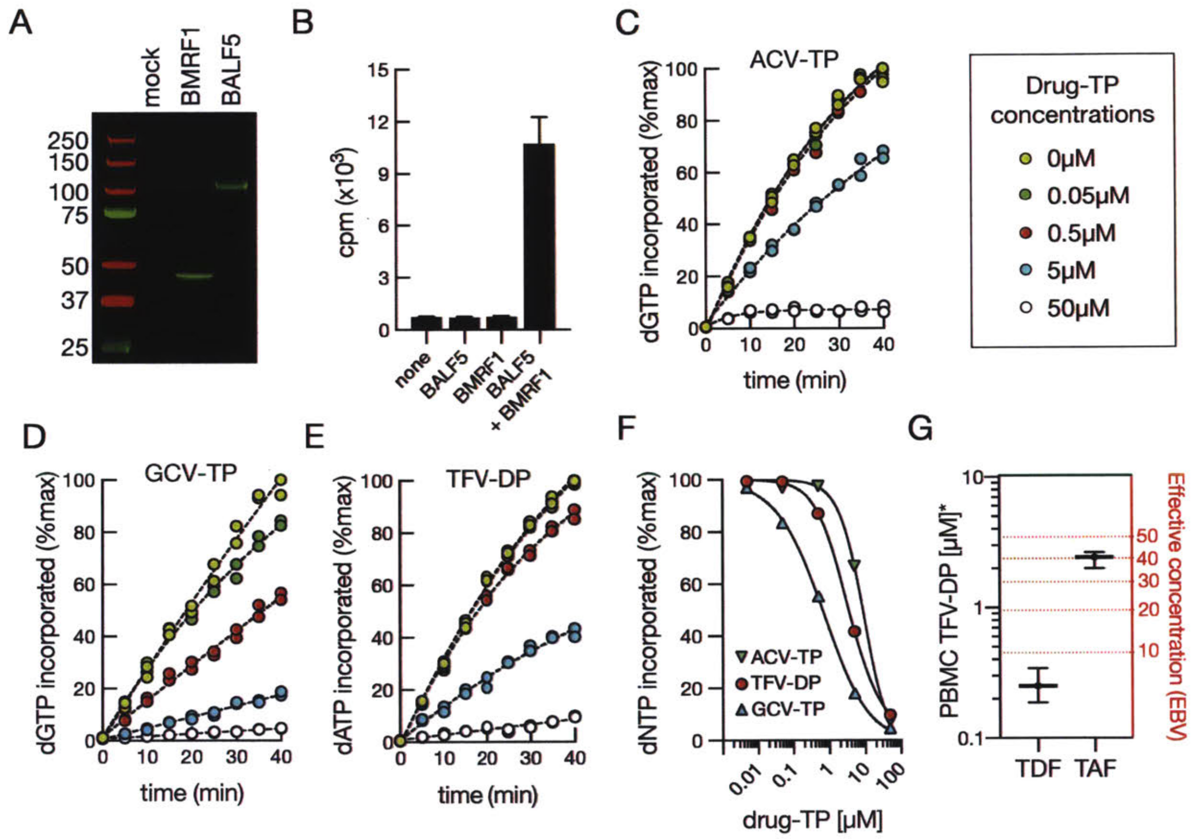


Figure 4.5: TFV-DP inhibits the EBV DNA polymerase by competing with dATP. (A) The EBV polymerase catalytic subunit (BALF5) and processivity factor (BMRF1) were cloned into pcDNA3.1+ vector and produced using a transcription/translation system in Rabbit reticulocyte lysate. Protein expression was confirmed using a fluorophore-labeled lysine-charged tRNA followed by gel electrophoresis and imaging. (B) *In vitro* polymerase assays were performed by measuring the incorporation of 1μM [^{3}H]dGTP into activated calf thymus DNA during a 40 min reaction followed by DEAE filter-binding and scintillation counting. Each column represents the mean and SD of counts per minute (cpm) obtained from 3 independent experiments. (C) ACV-TP and (D) GCV-TP were added to reactions containing 1μM [^{3}H]dGTP from 0-50μM and (E) TFV-DP was added to reactions containing 1μM [^{3}H]dATP from 0-50μM. Every 5 minutes, aliquots were removed and quenched with EDTA. Counts per minute were normalized to maximum counts obtained at 40 min. Results were analyzed from 2 independent experiments, each value shown. (F) Dose-response curves were generated from mean inhibition of dATP or dGTP incorporation at 40 min by five-parameter logistic regression analysis. (G) Effective doses were calculated from reported concentrations of TFV-DP in PBMCs during directly observed daily therapy with TDF or TAF *ref. (219) Black lines represent the median and interquartile range (IQR). Effective inhibitory

concentrations (red lines) were calculated using the EC_{50} and Hill slope from the best-fit line generated for TFV-DP in (F).

Figure S4.5

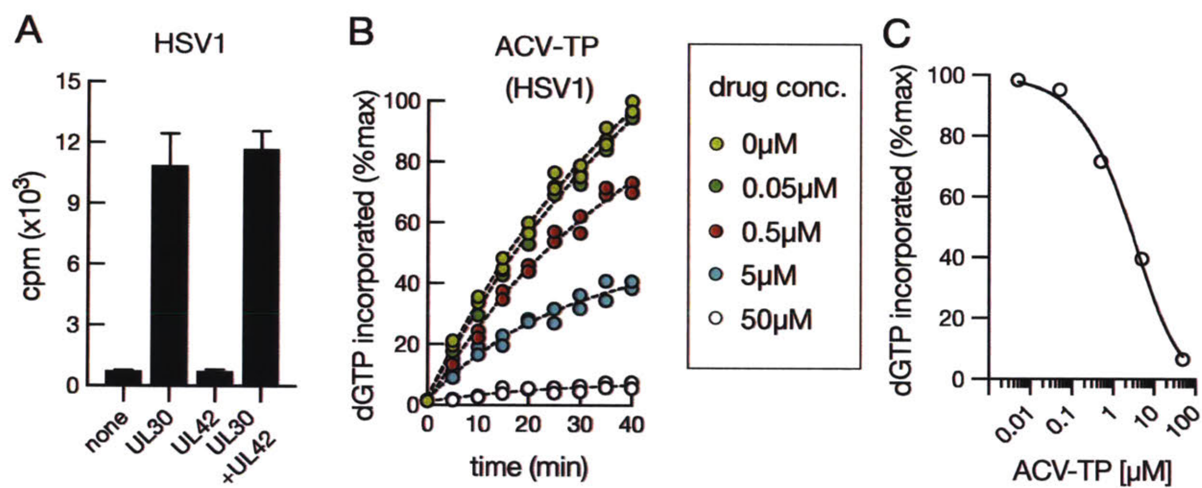


Figure S4.5: ACV-TP effects on the HSV DNA polymerase

The HSV-1 polymerase subunits (UL30 and UL42) were cloned into pcDNA3.1+ vector and produced using a transcription/translation system in Rabbit reticulocyte lysate. (A) *In vitro* polymerase assays were performed by measuring the incorporation of [^{3}H]dGTP into activated calf thymus DNA during a 40 min reaction followed by DEAE filter-binding and scintillation counting. Each column represents the mean and SD of counts per minute (cpm) obtained from 3 independent experiments. (B) ACV-TP was added to the reaction containing 1µM [^{3}H]dGTP from 0-50µM. Every 5 minutes, aliquots were removed and quenched with EDTA. Counts per minute were normalized to maximum counts obtained at 40 min. Results were analyzed from 2 independent experiments, each value shown. (C) Dose-response curves were generated from mean inhibition of dGTP incorporation at 40 min by five-parameter logistic regression analysis.

4.3.6 TAF, but not ACV, is effective even when treatment precedes lytic induction

The intracellular metabolism of TAF is well-established. In lymphocytes, TAF is first cleaved to TFV by the lysosomal enzyme cathepsin A (CTSA) and subsequently phosphorylated by AMP kinase and nucleotide diphosphate kinases to TFV-DP. (221) Since neither HIV nor Hepatitis B encodes a viral kinase, the metabolism of TAF is mediated by the host. Conversely, ACV requires a viral enzyme, the EBV protein kinase BGLF4, for antiviral activity. (78) The requirement of a virus-encoded kinase is thought to provide more specificity and thus decreased toxicity. (75) However, tenofovir-class drugs bypass the requirement for a viral kinase and yet still show a highly favorable safety profile. (212)

Since TAF is metabolized by host enzymes, we hypothesized that it may be possible to pre-treat cells prior to lytic induction. We added either TAF or ACV at IC_{95} for 24 hours. Before butyrate induction, drug was washed away until the final supernatant did not inhibit lytic DNA replication in cells unexposed to drug (Fig 4.6B). After washing away extracellular drug, TAF-treated cells were minimally permissive to lytic viral DNA replication, even after 72 hours (Fig 4.6A). However, pre-treatment of cells with ACV showed no effect (Fig 4.6A). This suggested to us that host metabolism of antiviral drugs confers the advantage of being able to initiate treatment prior to lytic induction.

Figure 4.6

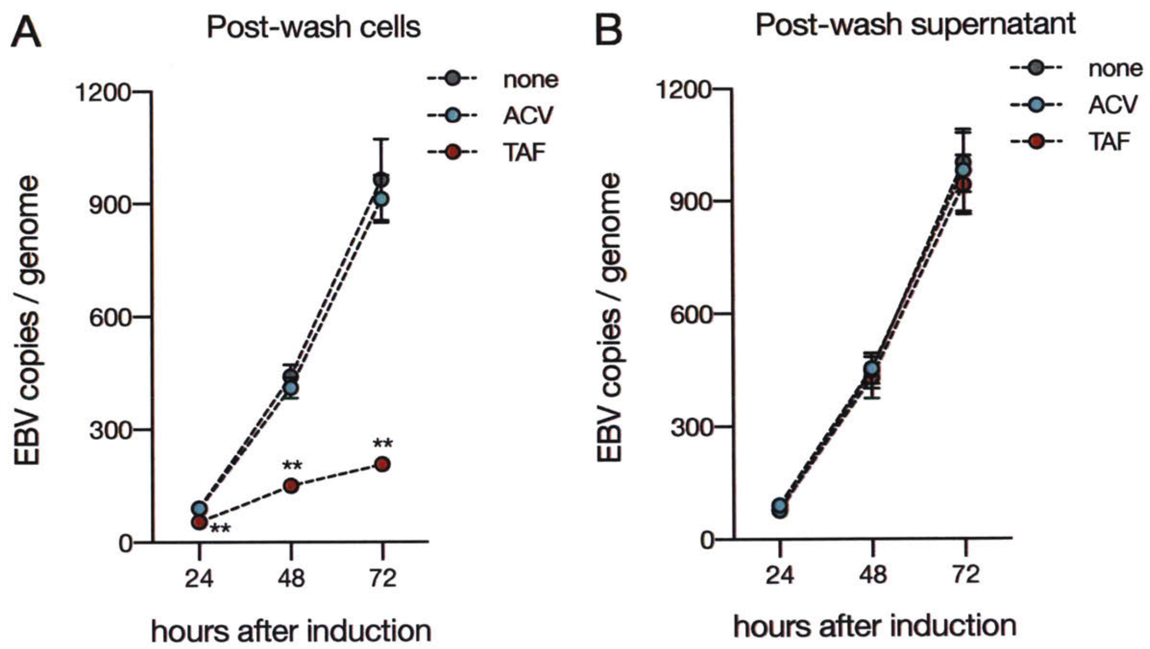


Figure 4.6: TAF, but not ACV, is effective even when treatment precedes lytic induction.

(A) HH514-16 cells were treated with ACV[62.5μM], TAF[2.5μM], or no drug for 24 hours in the absence of butyrate, after which cells were washed five times with fresh media without drug. After the final wash, cells were resuspended at a concentration of $4x10^5$ cells/mL in media containing 3mM butyrate. Genomic DNA was removed for measurements of EBV copy number every 24 hours. Each point represents the mean and SD obtained from 3 independent experiments. Two-way ANOVA ($p<0.05$) was followed by multiple hypothesis testing between butyrate and each drug. Statistical significance is highlighted by P values as follows: **$P < 0.01$. (B) After the final wash, a sample of the butyrate-containing supernatant was removed, filtered, and tested for inhibitory activity in cells previously unexposed to drug. Each point represents the mean and SD obtained from 3 independent experiments. Two-way ANOVA was performed ($p>0.05$).

4.4 Discussion

Anti-herpesviral drugs like ACV have demonstrated limited clinical benefit against EBV despite effectiveness in cell culture models. It has been suggested that EBV lytic replication does not contribute to pathogenesis in primary infection or EBV-associated diseases. (210) A second possibility is that the drugs that have been tested clinically are ineffective inhibitors of EBV. To answer this question, more potent inhibitors of EBV are needed. In this study we show that the TFV prodrugs TDF and TAF inhibit EBV lytic DNA replication. In cell-based assays, we demonstrate that TFV prodrugs are more potent than standard α-herpesviral drugs. TAF is also more potent than the β-herpesviral drug GCV. Furthermore, we provide strong evidence to suggest that, like standard herpesviral drugs, these compounds target the EBV DNA polymerase.

Although ACV has shown limited clinical benefit in the treatment of EBV, it is highly effective for the treatment of HSV-1/2. Therefore, differences between EBV and HSV may account for the failure of clinical translation. With respect to ACV, EBV and HSV can be distinguished by three characteristics: (1) viral enzymes involved in ACV metabolism, (2) potency of ACV both at the cellular and polymerase levels, and (4)

dNTP contents of infected cell types. Each of these issues may have limited clinical translation of ACV to EBV and should be addressed.

First, the rate-limiting step in ACV metabolism is performed by distinct, non-homologous enzymes in HSV and EBV. In contrast to EBV, the HSV thymidine kinase (TK) directly phosphorylates ACV. (75, 77) A separate EBV enzyme, the viral protein kinase (PK), is required for the susceptibility of EBV to ACV *in vitro.* (78) The EBV TK cannot phosphorylate ACV, (77) and the PK has not been shown to directly phosphorylate ACV. (78, 222) This leaves open the question of how ACV is activated by EBV and highlights the mechanistically distinct rate-limiting steps in drug metabolism between HSV and EBV.

In addition, ACV is markedly weaker against EBV than HSV. In cell-based assays, the reported IC_{50} of ACV for wild-type EBV ranges from 4.1-10μM. (78, 159) This is significantly higher than for clinical isolates of HSV (0.084-0.34μM). (160) Strains of HSV with an IC_{50} above 8.8 -13.2μM have been classified as clinically resistant to ACV. (223, 224) Furthermore, previous studies have shown that ACV-triphosphate (-TP) is a weaker inhibitor of the EBV DNA polymerase compared to the HSV-1 DNA polymerase. (198) This could indicate that ACV is clinically ineffective against EBV due to differences in potency.

The final characteristic considered was the intracellular concentration of dNTPs. In virus-producing cells, ACV-TP competes with dGTP for incorporation into newly synthesized viral DNA. (198) Therefore, ACV may have cell-type specificity depending on dNTP levels. *In vivo*, neurons and epithelial cells compose the cell types supporting HSV replication. (225) However, EBV is present in both epithelial cells and B-cells. (13) While ACV has been shown to reduce shedding of EBV in saliva, (226) and is effective in EBV-driven oral hairy leukoplakia, (13) both of these responses reflect processes occurring in epithelial cells. Lymphocytes contain higher dNTP concentrations than other non-dividing cells, (217) thus EBV-infected B-cells may be intrinsically more resistant to ACV than EBV-infected epithelial cells or HSV-infected cells.

TFV prodrugs have unique features that address the three issues described above. First, TDF and TAF bypass viral-dependent drug metabolism, (211) since activation of these drugs is mediated by host enzymes. Host drug metabolism permits treatment during latency, as we demonstrate in this study, which may be particularly relevant for lymphocyte trafficking to a compartment that contains lower drug concentrations (e.g., blood to cerebrospinal fluid). (227) Also, host-mediated metabolism allows global measurements of intracellular concentrations of TFV-DP

in vivo at steady-state (228) which can be used for correlation with clinical effects. Second, we show that TDF and TAF each have higher potency against EBV than ACV in cell-based assays, with an IC_{50} in the range reported for ACV against HSV. Increased potency was also observed at the polymerase level for TFV-DP against EBV comparable to ACV-TP for HSV-1. Finally, TAF specifically addresses the issue of lymphocyte tropism since it is metabolized by CTSA, an enzyme preferentially expressed in lymphoid tissues and PBMCs. (213)

In conclusion, directing drugs to clinical translation requires an approach that takes into account the specific characteristics of drug metabolism, potency, and target cell type. When considered, these issues may help explain relative clinical efficacy and can serve to guide drug selection. In the specific case of EBV, TFV prodrugs address issues that could have limited the clinical translation of ACV. Effective antiviral agents for EBV are critically needed. Data from molecular and epidemiological studies demonstrating increased risk of cancers and autoimmune diseases after EBV infection remain largely unexplained. These studies raise the question of whether treatment of primary EBV infection could reduce the burden of disease linked to EBV. Further clinical studies are needed to determine if TDF and TAF could aid in efforts for disease prevention.

4.5 Methods

4.5.1 Lytic induction and drug treatment

The EBV+ HH514-16 cell line was a kind gift from Prof. George Miller's lab at Yale School of Medicine. Cells were seeded at a concentration of $4x10^5$ cells/mL in RPMI-10% FBS supplemented with penicillin (100 units/mL), streptomycin (100 µg/mL), and Amphotericin-B (1µg/mL). Sodium butyrate (Sigma) was used to induce EBV lytic DNA replication at a concentration of 3mM. Tenofovir disoproxil fumarate (Sigma), tenofovir alafenamide (SelleckChem), acyclovir (Sigma), ganciclovir (Sigma), and penciclovir (SelleckChem), were added at concentrations and time points indicated in the text. Working solutions of drugs were dissolved in dimethyl sulfoxide (DMSO). For all experiments, the final concentration of DMSO was below 0.1% (v/v).

4.5.2 Quantification of EBV copy number by qPCR

Cells were centrifuged at 500xg for 3 min and washed once with PBS. Genomic DNA was extracted using the Qiagen DNeasy Blood & Tissue Kit (Qiagen) and subjected to qPCR. A 20µL reaction was set up with 250ng genomic DNA. The PCR mix contained 1x PrimeTime qPCR Master Mix (Integrated DNA Technologies), 500 nM each primer, and 250 nM probe.

Primers and fluorescent probes (5'FAM/ZEN/3'IBFQ) for the BALF5 gene of EBV and GAPDH (Integrated DNA Technologies) are listed below:

BALF5 primers: 5'-CGGAAGCCCTCTGGACTTC-3',

5'-CCCTGTTTATCCGATGGAATG-3',

BALF5 probe: 5'-TGTACACGCACGAGAAATGCGCC-3',

GAPDH primers: 5'-ACATCACCCCTCTACCTCC-3',

5'-CTCCCACCTTTCTCATCCAAG-3',

GAPDH probe: 5'-AAAGCCAGTCCCCAGAACCCC-3'.

The following cycling conditions were used: 95°C for 10 min followed by 40 cycles with 95°C for 15s and 60°C for 45s carried out on a LightCycler 480 system (Roche Diagnostics). GAPDH was used as an internal standard. The calculation of the relative amounts of BALF5 DNA compared to GAPDH DNA was performed using the $2^{-\Delta\Delta Cp}$ method using the Cp (second derivative maximum) calculated by the LightCycler software. Each experiment was performed with three biological replicates. To calculate the absolute number of EBV copies, the EBV BALF5 gene was cloned by PCR from HH514-16 genomic DNA into pcDNA3.1+ vector. Absolute EBV DNA copy number was calculated from a standard curve generated by serial

dilutions of pcDNA3.1-BALF5 added to human genomic DNA extracted from the EBV-negative A549 cancer cell line (ATCC).

4.5.3 Recombinant EBV DNA polymerase assays

The EBV DNA polymerase subunits BMRF1 and BALF5 were separately cloned by PCR from HH514-16 genomic DNA into pcDNA3.1+ vector. Recombinant proteins were expressed using the Promega TnT T7 Coupled Reticulocyte Lysate System according to the manufacturer's instructions. Protein expression in reticulocyte lysates was validated by using the FluoroTect GreenLys *in vitro* Translation Labeling System (Promega), gel electrophoresis, and fluorescence imaging using a Typhoon 9400 scanner (Amersham/GE). The recombinant polymerase proteins in reticulocyte lysates were then desalted using PD-10 Sephadex-G25 columns to remove any potential interfering salts or nucleotides present in reticulocyte lysates. Polymerase assays were set up as follows in a total volume of 100µL on ice: 50 mM Tris-HCl (pH 7.5), 100 mM ammonium sulfate (Sigma), 50 µg/ml BSA (Sigma), 1 mM DTT (Sigma), 3 mM $MgCl_2$ (Sigma), 10 µg/ml activated calf thymus DNA (GE Healthcare Life Sciences), 100 µM non-limiting dNTPs (New England Biolabs), 1 µM limiting ^{3}H-dNTP (dGTP for ACV-TP/GCV-TP or dATP for TFV-DP) (Moravek), 0-50 µM drug-triphosphate (ACV-TP and TFV-DP were from Moravek, and GCV-TP from TriLink Biotechnologies), and 10µL reticulocyte lysate (desalted) containing both

recombinant EBV polymerase proteins mixed at a 1:1 ratio. Reactions were incubated at 37°C for 40min in a heating block. Aliquots (10 µL) were removed every 5 min, mixed with 5 µL EDTA (200mM), and incubated on ice to stop the reaction. Reactions (7 µL) were spotted on DEAE anion exchange filter paper (Perkin Elmer) and dried for 10 min. Filters were washed twice with 5% (w/v) dibasic sodium phosphate for 5 min, water for 5 minutes, and rinsed with ethanol. Filters were then allowed to dry for 10 min, and added to 10mL Ultima Gold Scintillation Cocktail (Perkin Elmer) and counted on a Beckman LS6000 Scintillation Counter. All values were normalized to maximum counts observed with no drug at 40 min. Dose-response curves for inhibition of DNA replication by the EBV polymerase were fit by a five-parameter logistic regression model using the Prism 8 software (GraphPad), and EC_{50} measurements were calculated by the software using best-fit values.

4.5.4 Measurement of cell viability by trypan blue

Cells (seeded at an initial concentration of $4x10^5$ cells/mL), were examined at timepoints and drug concentrations indicated in the text. Viability was measured by trypan blue exclusion using a 1:1 mixture of a cell suspension with 0.4% trypan blue in PBS, and quantified using an Auto T4 Cellometer Cell Counter (Nexcelom Biosciences).

4.5.5 Luminescent total cell viability assay

Cells were seeded at a density of $4x10^5$ cells/mL in a volume of 200µL in 96 well plates. After 96 hours, the cells were mixed with CellTiterGlo reagent (Promega) according to manufacturer's instructions, and luminescence was quantified using a Varioskan Flash plate reader (Thermo Fisher). Luminescence was normalized using the signal from untreated samples (no cytotoxicity) and the signal from samples treated with 10µM podophyllotoxin (Sigma) (max. cytotoxicity).

4.5.6 Dose-response curves for inhibition of EBV replication and calculation of IC_{50}

Dose-response curves for inhibition of EBV lytic DNA replication were fit by four-parameter logistic regression analysis using the Prism 8 software (GraphPad), and IC_{50} measurements were calculated by the software using best-fit values.

4.5.7 Calculation of effective inhibitory concentration of TFV-DP in PBMCs from in vitro polymerase assays

Dose-response curves for inhibition of EBV lytic DNA replication were fit by five-parameter logistic regression analysis using the Prism 8 software (GraphPad) with top and bottom plateaus specified as 100 and 0, respectively. EC_{50} measurements and Hill slope were calculated by the software using best-fit values. The following equation derived from the Hill equation was used to calculate EC_F, the concentration

required to produce F, the percent response. Here, EC_{50} is the concentration that leads to 50% maximal response, and H is the Hill slope:

$$EC_F = \left(\frac{F}{100 - F}\right)^{\sqrt{H}} \times EC_{50}$$

4.5.8 Western blotting for early antigen expression

Cells were centrifuged at 500xg for 3 min and washed once with ice-cold PBS. Cells were lysed in RIPA buffer supplemented with protease inhibitor tablets (Sigma) and phosphatase inhibitor cocktail (Thermo Fisher), on ice for 30 minutes. Cell lysates were centrifuged at 14,000xg for 30 min at 4°C to remove debris, and supernatants were assayed for protein concentration by BCA assay to normalize for total protein. Samples were boiled in 1X NuPAGE LDS sample buffer (Thermo Fisher) with 2-mercaptoethanol at a final concentration of 2.5% for 10 minutes and loaded in a NuPAGE 10% Bis-Tris Protein Gel (Thermo Fisher). The gel was transferred to a nitrocellulose membrane using the iBlot gel transfer device (Thermo Fisher). Membranes were blocked for 30 min at room temperature in PBS + 0.1% Tween-20 (PBS-T) + 5% nonfat dry milk (Bio-Rad) and incubated with primary antibodies in with PBS-T with 10% StartingBlock blocking buffer (Thermo Fisher) overnight. Membranes were then washed with PBS-T for 15 min, incubated with HRP-linked secondary antibodies (Cell Signaling) for 2 hours at room temperature, washed in PBS-T for 30 minutes, and developed using 1 Step Ultra TMB Blotting Solution

(Thermo Fisher). Blots were imaged with the Bio-Rad ChemiDoc XRS imaging system. Antibodies were obtained and used at the following dilutions: mouse anti-EBV EA-D (Thermo Fisher) at 1:100, rabbit anti-β-actin (Cell Signaling) at 1:1000, anti-rabbit IgG HRP (Cell Signaling) at 1:1000, and anti-mouse IgG HRP (Cell Signaling) at 1:1000.

4.5.9 Quantitative RT-PCR for measurement of gene expression

Total RNA was isolated from HH514-16 cells using the RNeasy Mini Kit (Qiagen). RNA (2.5μg) for synthesis of cDNA using the SuperScript IV VILO Master Mix with ezDNase kit (Thermo Fisher), and cDNA was then used at a 1:10 dilution for qPCR. Each reaction contained 1x PrimeTime qPCR Master Mix (Integrated DNA Technologies), 500 nM each primer, and 250nM fluorescent (5'FAM/ZEN/3'IBFQ) probe. The following cycling conditions were used: 95°C for 10 min followed by 45 cycles with 95°C for 15s and 60°C for 45s carried out on a LightCycler 480 system (Roche Diagnostics). HPRT1 was used as a housekeeping gene for normalization of gene expression. The calculation of relative gene expression was done by the $2^{-\Delta\Delta Ct}$ method using the Cp (second derivative maximum) calculated by the LightCycler software. Primers and fluorescent probes for gene expression (IDT) are listed in supplemental Table 4.1.

4.5.10 Pre-treatment of cells in latency prior to induction of viral lytic DNA replication

Cells were seeded at an initial concentration of $4x10^5$ cells/mL and incubated for 48 hours in media without butyrate. After this time, drugs were added at the indicated concentrations, and cells were incubated for an additional 24 hours. Cells were then centrifuged at 500xg for 3 min and washed five times with fresh media without drug. After the final wash, cells were resuspended at a concentration of $4x10^5$ cells/mL in media containing 3mM butyrate. To remove supernatant for drug testing, half of the cell suspension from this step was re-centrifuged, and the butyrate-containing supernatant was filtered using a 0.2μM cellulose acetate syringe filter. Cells previously unexposed to drug were resuspended in this post-wash supernatant at a concentration of $4x10^5$ cells/mL. Genomic DNA was removed for measurements of EBV copy number every 24 hours.

4.5.11 Recombinant HSV-1 DNA polymerase assays

Assays were performed as described for EBV DNA polymerase assays except that the UL30 and UL42 subunits were cloned by PCR from HSV-1 genomic DNA, MacIntyre strain (ATCC) into pcDNA3.1+ . For drug-triphosphate testing, only the UL30 protein was used for polymerase assays since the UL42 protein was dispensable for activity. After desalting, samples were diluted to normalize for polymerase activity to that obtained with the EBV polymerase.

4.5.12 Statistical Analysis

A Brown-Forsythe and Welch ANOVA for unequal variances was initially used. Multiple hypothesis testing was then conducted, with correction for multiple testing, where appropriate using the Prism 8 software (GraphPad).

Table S4.1: Primers and probes for qPCR

Gene	Primer 1 (5'-3')	Primer 2 (5'-3')	Probe (FAM/ZEN/Iowa Black FQ)
BALF2	CTACCAGGAGGGAGAACTACAT	GTACAGGCTTGAGAGCTTAGTG	TCCTCAACACCTACCACAAGACCCTA
BALF5	CGGAAGCCCTCTGGACTTC	CCCTGTTTATCCGATGGAATG	TGTACACGCACGAGAAATGCGCC
BGLF4	GGCTGGTCCTGACTGATTATG	TCTGGCAATAGAGGCGATAGA	ATGCTGGATGTGCGGCTAAAGTCT
BaRF1	GATTCTTGTGTTCCTGCTGATTG	GAGCAGCTCATCCCTACTTATG	TGGCATCTGCCTGGCCAATAACTA
BBLF2/3	CAGTCCTCCTCAGATCTCAAAC	CTGCGGTCTGGGTAACATTAT	AATGATGGAAACACCCGCGGAGA
BMRF1	TTTACAGGTCTGGCATCATAGC	CTTCGGAGGCGTGGTTAAATA	AACACTAAGATCCAACGGCAGGTCC
BILF2	GGGAAGAAGACGACCAATACC	TTGTGGTGTGGGAGACTAATG	ACCCTCACTCAGCGTATGCATCTT
BcLF1	GTGGAGGCATGGATCGTAAA	TTGAGGCTGTTTAGGGTATGG	AATGCTCTCAAGGTTCTCTGTCACCC
BDLF1	AAATGGTGCCGGATGAGATAG	GATGCCAAAGACTGTAGGTAGG	TGACCTGTCAGTGGCGGATGATTT
BDLF2	GTGTCCGACCAATCCATTCT	ATGGCTGTCCGTGTTGTT	TATCCCGGCCGTGGTCATTAACAA
BVRF1	CCCAGTATGCAGTCTCAATCTC	GGTACCCACTGGTTGATGTT	ACGCTGCTTGGTAATCTGTACGGG
BVRF2	CTTCTTTGACCACGTGTCTATCT	GACGGTTCCAGGTCACTAAAG	ACGGCAGTCTACGGTACAGACCTT
HPRT1	TTGTTGTAGGATATGCCCTTGA	GCGATGTCAATAGGACTCCAG	AGCCTAAGATGAGAGTTCAAGTTGAGTTTGG

CHAPTER 5: CONCLUSIONS & FUTURE RESEARCH DIRECTIONS

5.1 Premise

This work investigates antiviral drug candidates for the treatment of EBV. While EBV infection is regarded as benign and self-limiting, it is associated with significant risks for the development of diseases later in life. Both cancers (129) and autoimmune diseases (184) are strongly linked to EBV infection, and in particular, to late-onset EBV. (122, 135, 136) In the absence of an EBV vaccine, practical interventions for clinical practice could involve antivirals. Whether treatment of primary infection may impact the large burden of disease attributable to EBV remains an open question, but one that is critically important to address. However, current anti-herpesviral drugs are either poor inhibitors of EBV or have prohibitive toxicity for routine clinical use, as outlined in Chapter 1. Thus, better antivirals with activity against EBV are urgently needed.

Our focus on HIV drugs emerged from the clinical case presented in Chapter 2 (ref. (229)), as well as case reports of patients with MS who experienced extended remissions after the initiation of HAART regimens. (reviewed in (191)) We sought to not only provide an explanation for these cases but also to define a systematic

approach to determine which of these drugs would be most promising and informative for clinical testing. A fundamental understanding of the mechanism of action of different drugs is critically required to not only select antiviral agents, but to accurately interpret clinical results, and to define parameters for optimal drug design in the future.

5.2 Summary of research goals

The studies presented in this thesis aimed to achieve three key goals:

(1) To describe the case report of a patient with MS, an EBV-associated disease, who responded to the antiretroviral drug Combivir (AZT/3TC). (Chapter 2)

(2) To define the ability of all antiretroviral nucleoside/nucleotide analogs to inhibit EBV lytic DNA replication in cell-based and polymerase assays *in vitro* in an attempt to answer the questions raised by Chapter 2 . (Chapter 3)

(3) To characterize TDF and TAF as inhibitors of the EBV DNA polymerase by using quantitative metrics for direct comparison with a clinically effective anti-herpesviral drug for a known pathogen – acyclovir for HSV. (Chapter 4)

5.3 Findings and Implications

We first established a cell-based assay for EBV lytic DNA replication in non-dividing cells in order to test candidate antiretroviral nucleoside/nucleotide analogs. Using this assay, several drugs were found to be effective against EBV – including ABC, AZT, d4T, TDF, and TAF (>90% inhibition at 50μM). Others were found to be ineffective (<50% inhibition at 50μM) – specifically ddI, 3TC, and FTC. Of the effective drugs, a subset was found to inhibit the EBV DNA polymerase in the form of drug-triphosphates, with TFV-DP being the most potent. In contrast, AZT could not be distinguished from cell-based ineffective drugs by its effects on the EBV polymerase as AZT-TP. This finding has important implications because it suggests that the mechanism of AZT against EBV may differ from standard herpesviral drugs, and should be the subject of further exploration. This also suggests the effects observed in the case report presented in Chapter 2 are unlikely to be mediated by the inhibition of AZT-TP on the EBV DNA polymerase. Mechanistic understanding of the effects of AZT on EBV would provide clearer insights into the potential mode of action of AZT.

We further explored the effects of TDF and TAF to show that these compounds are not only effective but highly potent inhibitors of lytic EBV DNA replication. TAF has

a 35- and 24-fold, and TDF has a 10- and 7-fold lower IC_{50} than acyclovir and penciclovir, respectively. TAF is also twice as potent as the β-herpesviral drug ganciclovir. *In vitro*, the active metabolite of TDF and TAF, TFV-DP, is more potent than acyclovir-triphosphate at inhibiting dNTP incorporation into a DNA template by the EBV DNA polymerase. Importantly, these compounds bypass viral-dependent drug metabolism, which enables initiation of treatment prior to the viral lytic cycle. These findings imply that tenofovir-class drugs may serve as improved antiviral drugs for EBV by solving several problems that may be limiting the clinical effectiveness of existing herpesviral drugs. TDF and TAF have increased potency, highly favorable safety profiles, and can be used for pre-treatment during latency. The latter feature may be especially relevant for lymphocyte trafficking from systemic circulation to areas that reach lower extracellular drug concentrations, particularly the central nervous system.

5.4 Future research directions

5.4.1 Expanded mechanistic understanding of AZT on EBV

The mechanism of inhibition of EBV by AZT remains unclear. Questions remain about which AZT metabolite(s) are responsible for the observed effects on EBV DNA replication in cell culture. Further studies are needed to provide a clearer understanding of AZT phosphorylation in EBV-infected cells. Hypotheses may be addressed by quantitative measurements of drug monophosphate, diphosphate, and triphosphate species *in vitro* using HPLC-MS. Comparative metabolic studies would be informative not only for AZT, but also for drugs that are ineffective in cell-based assays yet have matched potency with AZT at the polymerase level – such as 3TC and ddI. Perhaps the inhibition of EBV DNA replication is attributable simply to artificially high levels of AZT-TP concentrations during the lytic cycle in cultured cells. Next steps may seek to characterize AZT metabolism, as well as explore alternative mechanisms.

5.4.2 Characterizing the effects of antivirals in vivo

Similar cell-based and polymerase assays, as performed in this thesis, could be complemented by *in vivo* studies. Specifically, murine gamma-herpesvirus 68 (MHV-68), a rodent pathogen with homology to EBV, could be used as a mouse

model system of primary γ-herpesvirus infection. Viral load in peripheral blood and tissues, as well as the establishment of latency, could be examined for response to antiviral treatment. However, it is also possible that antivirals are differentially effective for EBV vs. other γ-herpesviruses. Therefore, detailed molecular studies *in vitro* using MHV-68 infected cells and/or the MHV-68 polymerase with direct comparison to EBV would first be required to determine if drug parameters (cell-level and/or polymerase-level potency) are matched.

5.4.3 Potential use of TAF for prophylaxis in EBV-negative individuals

TAF, the most effective anti-EBV drug we found in our studies, is approved for pre-exposure prophylaxis for HIV. Given its high potency as an antiviral for EBV *in vitro*, TAF may also be useful as prophylaxis for EBV. Studies using antivirals to prevent infection with HSV1/2 have reported benefit. (177, 178) Even if TAF does not establish sterilizing protection, treatment of primary EBV infection may still be helpful. In particular, those at higher risk of long-term complications from infectious mononucleosis (e.g., EBV-negative young adults with a significant family history of EBV-associated lymphoma, or children with genetic immunodeficiency syndromes such as XLP) may have improved outcomes with EBV prophylaxis. Since infectious mononucleosis presents in the clinic weeks after initial exposure, the use of TAF as a continuous treatment would solve the problem of "catching" primary exposure

before clinical presentation. However, the treatment of infectious mononucleosis at presentation may still provide benefit. It remains an open question whether treatment of IM may reduce the risk of EBV-associated cancers and autoimmune diseases later in life.

5.4.4 Clinical testing of antiretrovirals with activity against EBV in EBV-associated diseases

This thesis work began as an attempt to explain the effects of antiretroviral drugs in several case reports of patients with MS. However, clinical translation of this finding has been impeded by a lack of mechanistic insight. Previous selection of antiviral drugs with no or low efficacy against EBV (e.g., acyclovir, raltegravir) (163, 209) has led to negative trial results. The consequences of this have been unwarranted interpretations that viruses are either not involved in the pathogenesis of MS, or that antivirals would not provide benefit. Thus, we sought to develop a systematic way to define which antiretroviral drugs would be most promising candidates to trial for the treatment of MS based on the hypothesis that these drugs may be treating EBV. Based on our studies, TAF would be a good candidate to evaluate the effects of treatment of EBV in MS. Other EBV-associated diseases as outlined in Chapter 1 may also benefit from antiviral treatment.

In addition to TAF, AZT would be a candidate for clinical testing as a follow-up to the case report presented in Chapter 2. Intriguingly, a small open-label trial in 7 patients with primary Sjogren's syndrome also reported significant benefit during treatment with AZT. (230) A more extensive exploration of the clinical effects of AZT in EBV-associated diseases would be instructive. Furthermore, mechanistic insights into the mode of action of AZT against EBV may better inform the interpretation of these and future clinical results.

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