HISTONE DEACETYLASE INHIBITORS MODULATE HUMAN POLYOMAVIRUS JC REPLICATION

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By

Michellei Chiemi Fisher

Dissertation Committee:

Vivek R. Nerurkar, Chair

Saguna Verma

Richard Yanagihara

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ABSTRACT

The human polyomavirus JC (JCPyV) is the causative agent of progressive multifocal leukoencephalopathy (PML), a demyelinating disease of the brain. Although archetype JCPyV exists as an asymptomatic infection in the healthy population, PML occurs almost exclusively in individuals with immunodeficiencies or on immunomodulatory medication. When immune function is perturbed, the mechanisms which maintain viral latency are disrupted and can lead to the development of PML. Therefore, an important question in understanding PML pathogenesis are the molecular mechanisms which maintain JCPyV latency. The JCPyV genome incorporates host-derived histones and closely resembles host chromatin structure, therefore we hypothesize it is subject to epigenetic regulation. Histone deacetylase inhibitors (HDACi) have been demonstrated to increase rearranged JCPyV replication in a transfection model and are candidates for latency reversal agents in HIV treatment. The potential for these HDACi to reactivate HIV suggests the possibility of reactivating other viruses as well. The objective of this study is to characterize the effects of histone deacetylase inhibitors (HDACis) on archetype JCPyV infection, replication, and rearrangement *in vitro* and *in vivo*. Here, we demonstrate that HDACi, Trichostatin A, treatment of primary human brain cortical astrocytes and renal proximal tubule epithelial cells increases both early and late archetype JCPyV replication in a cell-specific manner. Further, we demonstrate in patients treated with HDACi, panobinostat, statistically higher increase in archetype JCPyV genome copy number in urine, which was abrogated after treatment. To our knowledge, this is the first study to show the effect of HDACi on archetype JCPyV replication in an *in vitro* infection model and the first to investigate JCPyV viruria during HDACi treatment. Taken together, these findings suggest that HDACi modulate archetype JCPyV replication. This study emphasizes the need to understand the effects of these global HDACi on other viruses to improve risk stratification for latency reactivation agent treatments. These findings will open new therapeutic strategies for treatment of PML aimed at preventing viral replication and maintaining JCPyV in a latent state.

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CHAPTER 1

BACKGROUND

BACKGROUND

Progressive multifocal leukoencephalopathy (PML) is a subacute demyelinating disease of the central nervous system (CNS) caused by the ubiquitous human polyomavirus JC (JCPyV). The onset of PML is insidious, typically presenting with neuropsychological deficits. The natural disease progression is usually rapid with death occurring 3 to 6 months after diagnosis. The neuropathological hallmarks of PML consist of multifocal microscopic and macroscopic demyelinating lesions typically in the subcortical white matter near the gray-white matter junction. Ultrastructural examination reveals nuclei of infected oligodendrocytes packed with electron-dense JCPyV particles, measuring approximately 40 nm in diameter (4). PML was originally recognized as a rare complication of hematological malignancies or systemic inflammatory disorders, however, a dramatic 50-fold increase in the incidence during the past 30 years occurred as a result of the acquired immunodeficiency syndrome (AIDS) epidemic (5). AIDS is the most frequent condition associated with PML (6), with approximately 6% of patients developing AIDS-related PML (7). A resurgence of PML occurred in the 2000s as a result of the use of immunomodulatory compounds, including monoclonal antibodies natalizumab, efalizumab, and rituximab, for the treatment of the autoimmune conditions, such as multiple sclerosis, Crohn's disease, severe forms of plaque psoriasis, hematologic malignancies, and rheumatoid arthritis (8). Currently, there is no treatment for PML or vaccines against JCPyV infection.

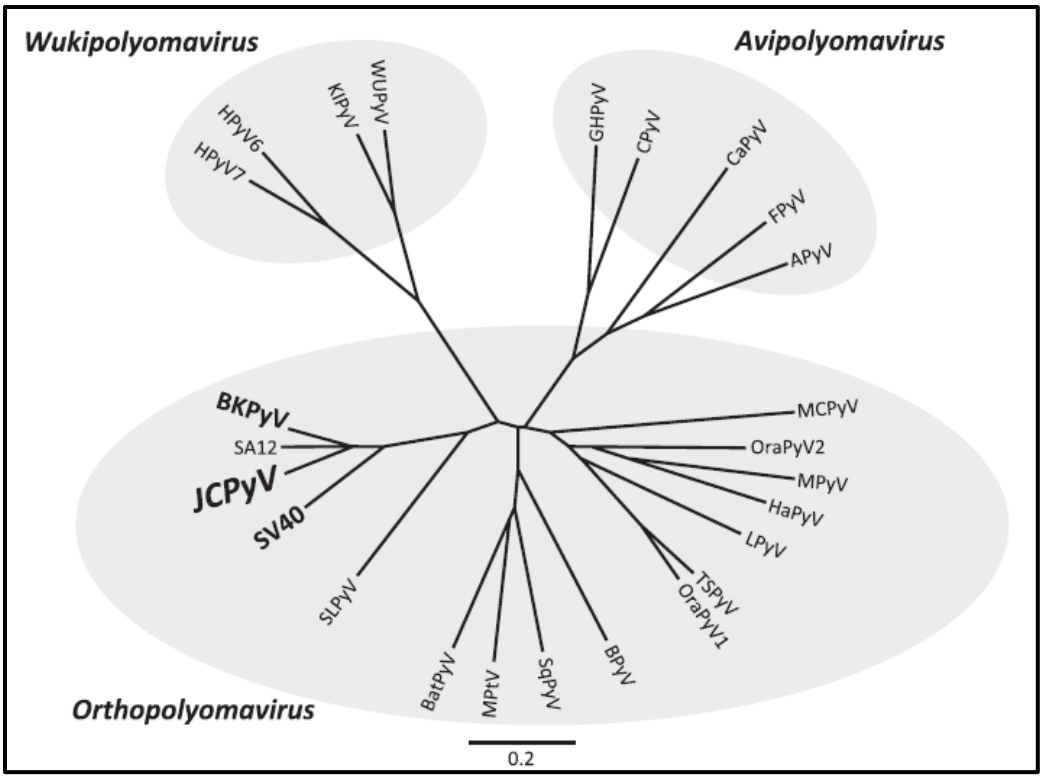

Figure 1. Phylogenetic tree of *Polyomaviridae*. Based on whole genome sequencing, the members of the *Polyomaviridae* family have been classified into four distinct genera: *Alphapolyomavirus* with members Merkel cell polyomavirus (MCPyV) and Trichodysplasia spinulosa-associated polyomavirus (TSPyV); *Betapolyomavirus* with notable human viruses, BK polyomavirus (BKPyV), JC polyomavirus (JCPyV), *Deltapolyomavirus* containing orphan human viruses, including human polyomavirus 6 (HPyV6) and 7 (HPyV7); and *Gammapolyomavirus* which includes canary polyomavirus (CaPyV) and goose hemorrhagic polyomavirus (GHPyV) (9).

JCPyV

JCPyV is a neurotropic human polyomavirus belonging to the genus *Betapolyomavirus* in the family *Polyomaviridae*. It was first isolated in 1971 from the brain of a patient, with the initials JC, suffering from PML, for whom the virus is named (10, 11). Polyomaviruses have been found in humans, monkeys, rodents, and birds . According to the International Committee on Taxonomy of Viruses, the family *Polyomaviridae* consists of four genera: *Alphapolyomavirus* with members Merkel cell polyomavirus (MCPyV) and Trichodysplasia spinulosa-associated polyomavirus (TSPyV), *Betapolyomavirus* with the species simian vacuolating virus 40 (SV40), BK polyomavirus (BKPyV), and JCPyV; *Deltapolyomavirus* including orphan human viruses human polyomavirus 6 (HPyV6) and 7 (HPyV7); and *Gammapolyomavirus* with respective polyomaviruses infecting birds (13, 14) . In addition to JCPyV, the polyomaviruses that have the ability to infect humans include BKPyV, KIPyV, WUPyV, MCPyV, TSPyV, HPyV6, HPyV7, HPyV9, and Malawi polyomavirus (MWPyV) .

EPIDEMIOLOGY

JCPyV infection is defined by serological or virological evidence of virus exposure including both replicative and non-replicative states (15). Serological surveys from widely separated geographic regions have shown that between 30-91% of healthy individuals have anti-JCPyV-specific antibodies (Table 1) (16). Although JCPyV is prevalent in urban populations, isolated and rural Pacific island populations demonstrate antibody acquisition in early childhood that remained stable throughout adult life (15, 17, 18). Infection usually occurs during childhood and is typically subclinical. The route of JCPyV transmission and the primary sites of replication are unknown. Viruria is common (20-23) in which JCPyV is detected in urine. Therefore, transmission via urine to oral or respiratory route and primary replication in tonsillar tissue has been proposed . Virus-infected lymphocytes or cell-free virus presumably spread by the hematogenous route from the primary site to secondary sites, such as kidneys, lymphoid tissues and brain, to establish focal areas of infection or persistence . PCR analyses have suggested that JCPyV may persist in brain, tonsils and lymphocytes of individuals with or without PML , and that PML arises from reactivation of JCPyV (28).

Table 1. JCPyV global seroprevalence rates

Country	N	Age (years)	Population	Seropositive	Reference
Finland	590 50	0-13 >25	Normal	33% (VLPEIA) 72% (VLPEIA)	(29)
UK	2435 356	1-69 60-69	Normal	35% (HI) 50% (HI)	(30)
USA	1501	>21	Normal	39% (VLPEIA)	(31)
USA	415 90	19-78 40-64	Normal Cancer	44% (VLPEIA) 52% (VLPEIA)	(32)
Belgium	106 225	20-80 11-70	Normal Crohn's	52% (VLPEIA) 76% (VLPEIA)	(33)
Switzerland	400	20-59	Normal	58% (VLPEIA)	(34)
USA	622 724	20-74 20-74	Normal Cancer	59%(VLPEIA) 49% (VLPEIA)	(15)
USA	277	>20	Normal	69% (HI)	(35)
Japan	480	0-80+	Normal	71% (HI)	(16)
USA Malaysian aborigines	70 20	All age groups All age groups	Neurological Normal	72% (HI) 75% (HI)	(36)
Germany	49 36	4-81 17-55	Normal MS	86% (VP1 EIA) 76% (VP1 EIA)	(37)
Portugal	171 63	3-75 25-75	Normal HIV	91% (VLPEIA) 91% (VLPEIA)	(38, 39)

*****VLPEIA, Virus-like particle enzyme immunoassay; HI, Hemagglutination inhibition; VP1 EIA, VP1 enzyme immunoassay

STRUCTURE AND GENOME ORGANIZATION

JCPyV has a naked icosahedral capsid and a circular double-stranded DNA genome of approximately 5.1 kb . The viral genome is functionally divided into an early region (2.4 kb) encoding large and small T antigen proteins along with the recently described T' proteins generated by alternative splicing of the early mRNA; a late region (2.3 kb) encoding viral capsid proteins VP1, VP2 and VP3, and the accessory agnoprotein; and a non-coding regulatory region (0.4 kb) referred to as the noncoding control region (NCCR) (Fig. 2). Based upon the structure of the NCCR, two types of JCPyV have been identified: the archetypal form, which is predominantly detected in kidney and urine of people with and without PML (23, 41); and the rearranged form which is predominantly detected in brain, tonsils, and lymphocytes of individuals with PML (Figure 2) . The archetype JCPyV NCCR, which consists of six regions, designated A to F, displays minimal sequence variation, whereas the NCCR of JCPyV isolated from PML patients are highly variable and contain rearrangements, such as deletions, duplications, tandem repeats, and insertions . It is thought that all other rearranged forms of JCPyV arise from the archetype form, and most likely arise during immunosuppression or immunomodulation (44).

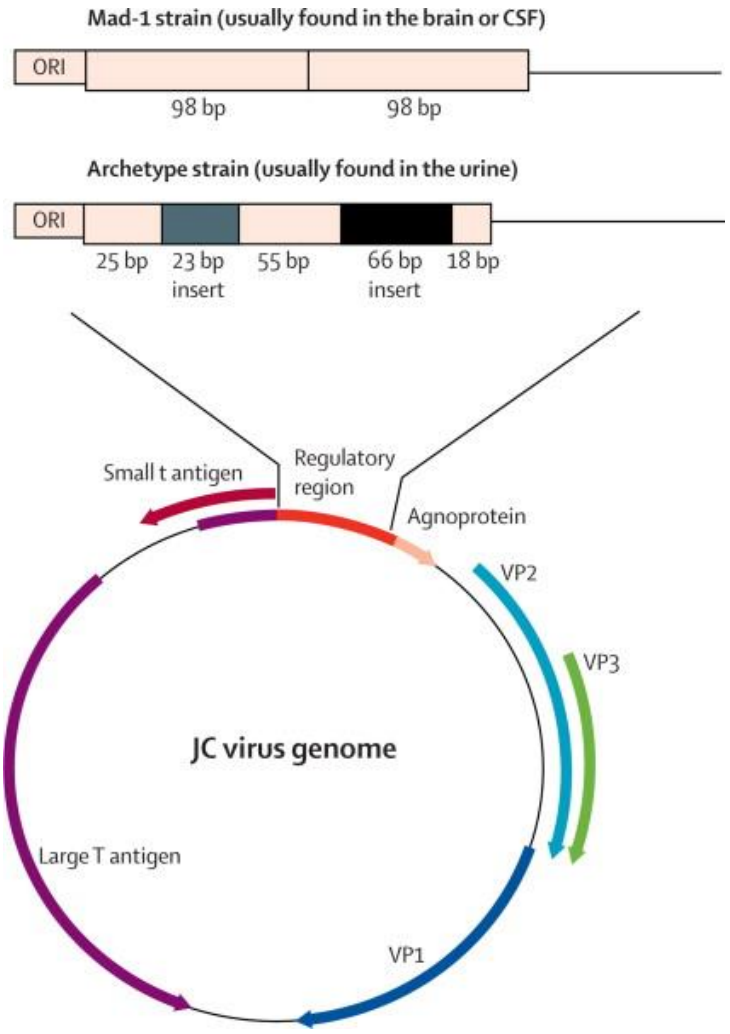

Figure 2. JCPyV genome. JCPyV has a circular double stranded DNA genome about 5.13 Kb in length consisting of three regions: the early region, coding for the small and large T antigen; the late region, coding for the structural proteins VP1, VP2, VP3 and the agnoprotein; and the non-coding control region (NCCR) or regulatory region (RR). The archetype JCPyV NCCR shows little variation, whereas rearranged strains are characterized by deletions, duplications, and/or tandem repeats in their NCCR's (45).

REPLICATION CYCLE

JCPyV infects cells by first binding to a receptor on the outer membranes of susceptible cells (Figure 3). JCPyV possesses intrinsic hemagglutination activity which allows it to engage alpha 2-6-linked sialic acid residues, suggesting binding to the oligosaccharide is an important step in JCPyV infection (46). It has also been shown that JCPyV can interact with the serotonin receptor 2A (5HT2AR) (47), which leads to virus internalization into glial cells. Virus is taken up by clatherin-dependent endocytosis (13) followed by its transportation to the nucleus where the removal of the viral capsid proteins occurs. Early transcription results in a primary transcript that is alternatively spliced into two mRNAs which code for the large T-antigen (TAg), a nuclear phosphoprotein that is essential for viral DNA replication, and the small t-antigen (48). Once TAg initiates DNA replication it stimulates transcription from the late promoter. JCPyV relies on host cell enzymes and cofactors for DNA replication. Since expression of these proteins are confined to the S-phase of the cell cycle, TAg stimulates the cell cycle by modulating cellular signaling pathways by binding key cellular control proteins including p53**,** retinoblastoma protein (pRB)**,** and insulin receptor substrate 1 (IRS-1) (13, 49). Ultimately, the capsid proteins, VP1, VP2 and VP3, are expressed from the late region and assemble with the replicated viral DNA to form intranuclear virions, which are released upon cell lysis .

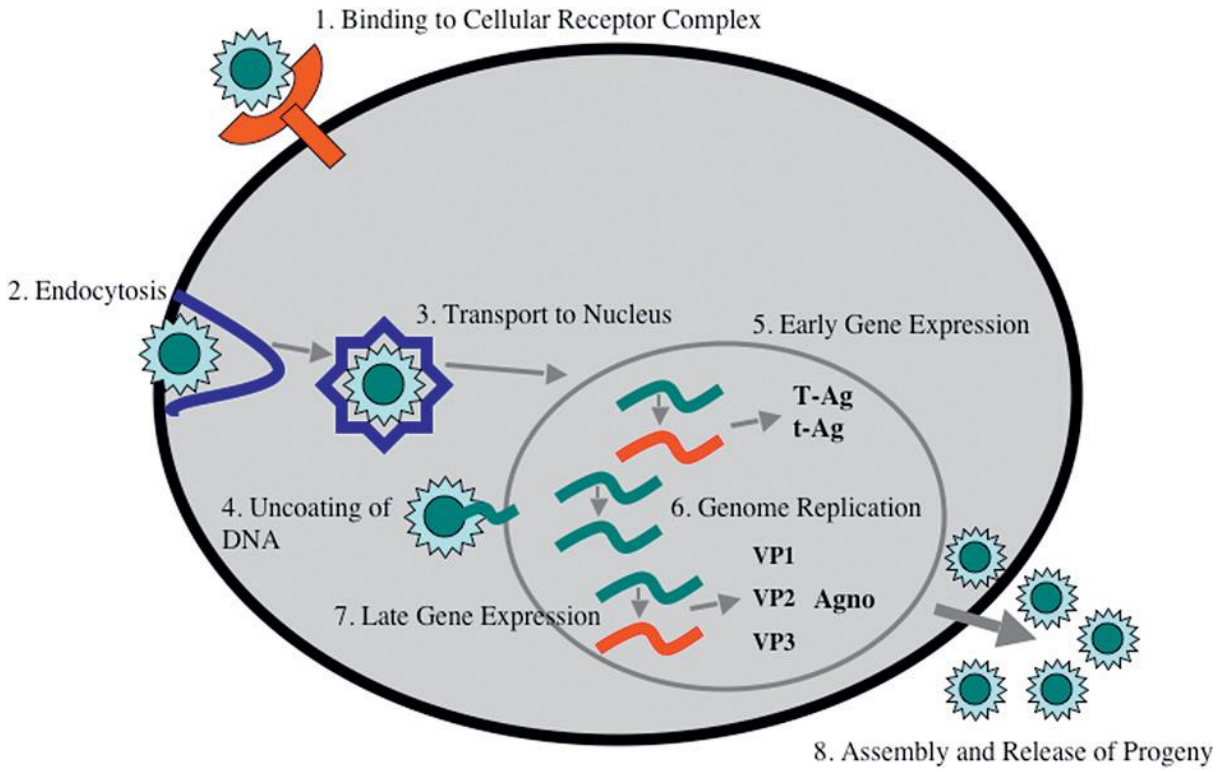

Figure 3. JCPyV replication cycle. 1) JCPyV first binds to a cellular receptor complex followed by **2)** virus internalization into the cytosol via clathrin-dependent endocytosis **3)** The virus is then transported to the nucleus where **4)** uncoating of viral DNA occurs exposing viral DNA for **5)** early gene expression **6)** Viral DNA synthesis occurs followed by **7)** late gene expression **8)** New virions are assembled and released, establishing productive infection (50).

PERSISTENCE AND LATENCY

Infection with JCPyV produces a self-limiting infection in immunocompetent individuals with intermittent viruria. Primary JCPyV infection is cleared by the immune system to where viremia is undetectable, however archetype JCPyV can persist in the kidney with occasional viruria during low level replication within kidney cells. Prevalence of viruria varies and can be sporadic, but it is estimated that between 10 and 50% of healthy asymptomatic adults excrete JCPyV in their urine depending on the population (50-53). It is thought that JCPyV enters the bloodstream from its portal of entry, with the fecal-oral hypothesis implicating the nasopharynx or gut. JCPyV DNA has been detected in peripheral blood mononuclear leukocytes from immunocompetent individuals, however productive infection has not been shown (17). It is hypothesized that JCPyV is spread hematogenously and establishes latent infections in the kidneys, bone marrow, and lymphoid organs (55). Interestingly, both archetype and rearranged sequences are detected in peripheral blood mononuclear cells, suggesting blood may be the compartment in which neurotropic virus emerges and traffics to the brain (56).

REACTIVATION AND REARRANGEMENT

JCPyV reactivation and rearrangement are hypothesized to take place after immunological alterations of the host, though the exact mechanism of rearrangement remains unclear. NCCR structure changes in relation to anatomical sites within the same patient (42). Detailed analysis of JCPyV DNA populations demonstrated that the JCPyV strains from PML cases in cerebrospinal fluid (CSF) and/or blood often exists as a mixture of viral variants, with up to 10 variants in one patient. These variants could often be derived from each other by single duplication or deletion events, indicating a process in which the virus continuously adapts to its cellular environment (57). *In vitro* cell studies indicate that rearrangements enhance early viral gene expression and support higher replication rates in glial cells, increasing cytopathology (58). The first *in vitro* rearrangement of archetype JCPyV was recently demonstrated in COS-7 cells, green monkey kidney cells that constitutively express the SV40 large TAg protein. Two characteristic point mutations, T37G transversion in Spi-B binding site and G217A transition in NF-1 binding site were detected 28 days post transfection (59). Although it's thought that the archetype virus usually gives rise to various PML-type regulatory regions by deletion and/or duplication, these cells were transfected for a period much shorter than that of the persistence of archetype strains in humans (19). Taken together, these data suggest a sequential series of DNA replication-driven NCCR recombination events after reactivation resulting in increased glial cytopathogenesis leading to PML (Figure 4).

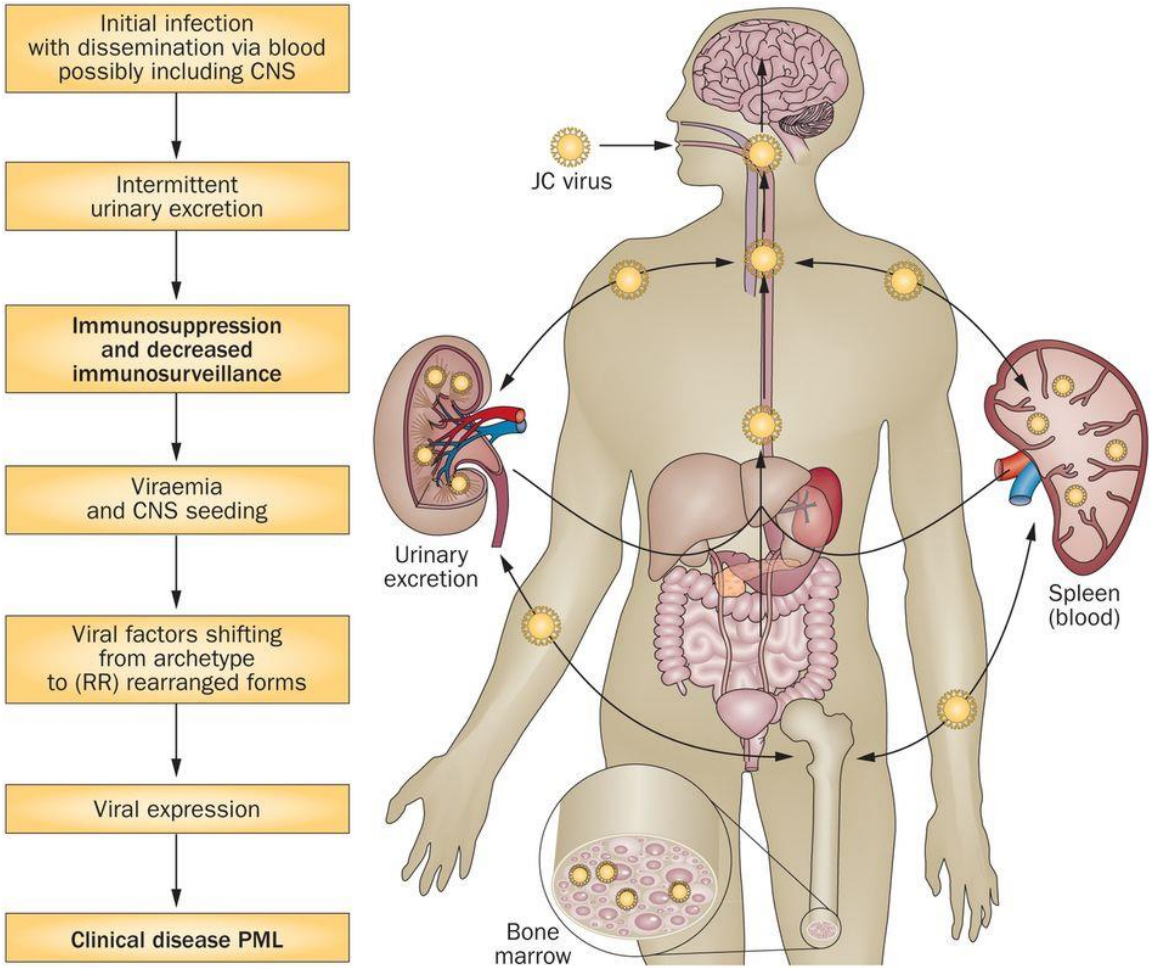

Figure 4. JCPyV dissemination and PML pathogenesis. The events hypothesized to occur during the JCPyV life cycle and pathogenesis of PML are shown. Transmission of infectious virions is thought to occur through inhalation or ingestion of sewage-contaminated material of archetype JCPyV. It is then though to enter the bloodstream through the epithelium of the tonsils, upper respiratory tract, or gastrointestinal tract to establish primary viremia. The transmission and dissemination of JCPyV is not well understood, but it is thought that virus can exist as free virions and/or as white blood-cell associated virus. JCPyV is thought to spread to the kidney, spleen, bone marrow, and other secondary sites of infection via hematogenous route. In the kidneys, JCPyV can maintain low level replication in the epithelium of the kidney tubules where it can shed virions via the apical face of the kidney epithelium (60). This shedding is thought to lead to viruria

and transmission of infectious virions via urine, completing the JCPyV lifecycle. JCPyV may undergo hematogenous spread from secondary sites of infection in association with B cells, including the brain, where rearranged JCPyV DNA can be detected in healthy, immunocompetent individuals in absence of viral protein expression (61). In the environment of immunosuppression or immunomodulation, JCPyV can become reactivated which results in PML (62).

CLINICAL AND PATHOLOGICAL FEATURES OF PML

Primary infection with JCPyV is typically subclinical. PML is a rare clinical entity, which usually develops only in individuals who have underlying changes to the immune system, including immune suppression and immunomodulation. Clinical features of PML vary according to the site of demyelination and are non-specific. The most affected regions of the brain are the periventricular and sub-cortical regions of the parieto-occipital and frontal lobes (63, 64). Common presenting symptoms include cognitive deficits, gait disorders, limb weaknesses, speech disorders, and visual impairments .

PML pathogenesis can be split into three phases: the first phase being a primary clinically inapparent infection; second phase persistent and latent peripheral infection in the urinary tract, bone marrow, and spleen; and the third induced by immunologic and molecular alterations of the viral NCCR resulting in reactivation of virulent JCPyV . The histopathological characteristics of PML consist of multifocal microscopic and macroscopic demyelinating lesions that tend to coalesce in the subcortical white matter near the grey-white matter junction. Oligodendrocytes support productive lytic infection and when infected their nuclei become enlarged and fill with JCPyV eosinophilic inclusion bodies. So-called “bizarre” astrocytes appear enlarged, with multiple or mutilobulated hyperchromatic nuclei which can resemble neoplastic cells (Figure 5) (68).

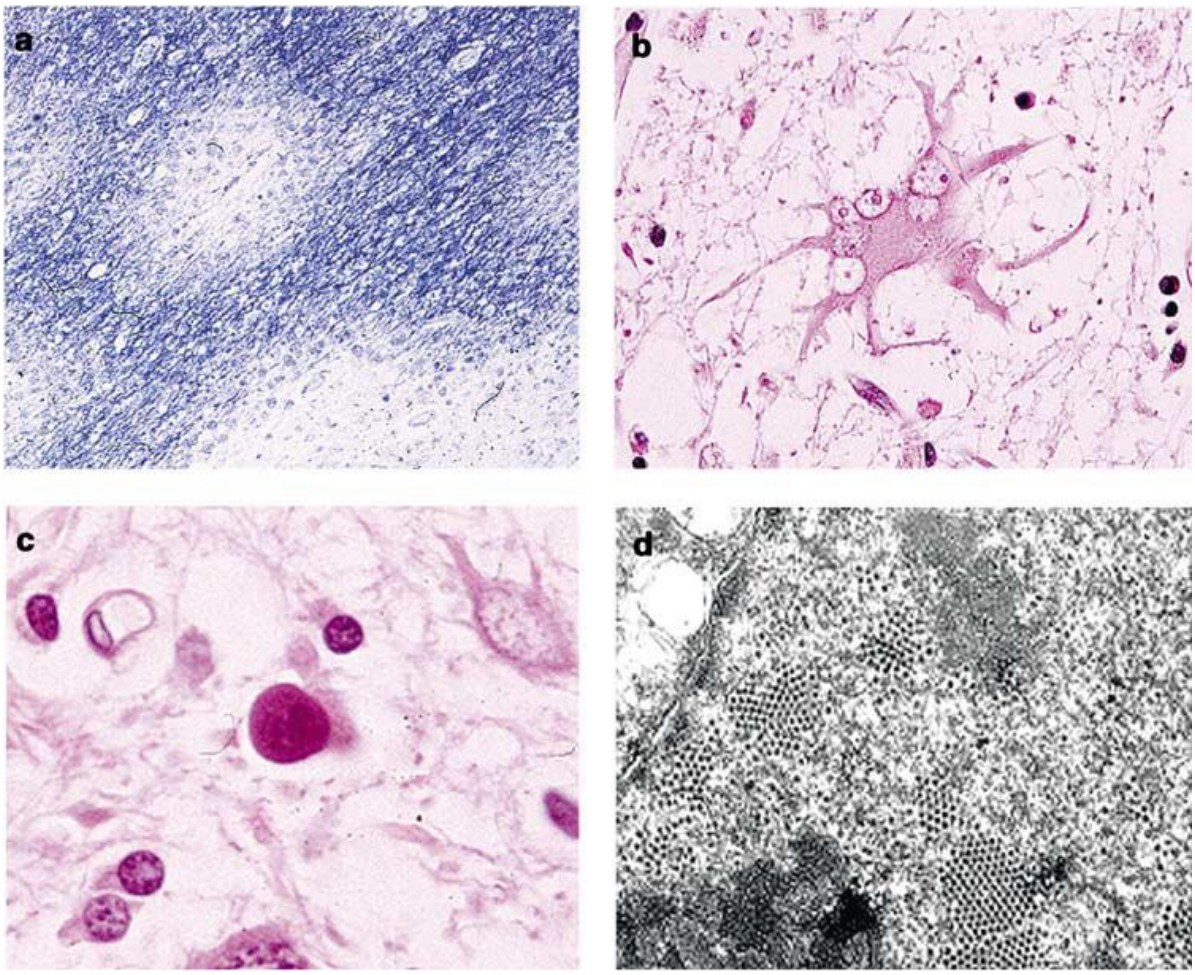

Figure 5. Histological characteristics of PML. (a) White shows areas of demyelination or plaques observed at low magnification in paraffin-embedded sections of PML brain tissue stained with luxol fast blue for myelin. (b) Bizarre, multinucleated astrocytes frequently observed in PML lesions. (c) Residual JCPyV-infected oligodendrocytes harboring intranuclear eosinophilic inclusion bodies may be observed in demyelinated plaques. (d) Electron microscopy of oligodendrocyte inclusions reveal 45 nm icosahedral viral particles in nucleus consistent with JCPyV virions (69).

MECHANISMS OF EPIGENETIC REGULATION

Epigenetics are the heritable changes in gene expression that do not involve changes to the underlying DNA sequence (70, 71). Epigenetics responds to cellular cues and environmental stimuli and are regulated by post-translational covalent modifications on chromatin . Epigenetic events occur regularly and can be influenced by several factors including age, environment, lifestyle, and disease status (72). There are at least three known systems that allow for epigenetic DNA modulation: DNA methylation, histone modification, and non-coding RNA (Figure 6) . A variety of enzymes are responsible for regulation of epigenetic processes and these proteins are referred to as writers, erasers, or readers. Writers covalently add functional groups, erasers remove groups, and readers modify chromatin or exist as adaptor proteins required for gene expression. Some examples of writers include DNA methylase (DNMT), histone acetyl transferases (HAT), and well-studied erasers are histone deacetylases (HDAC) (72). DNMT adds a methyl group to the fifth carbon position of a cytosine residue, typically on a 5'-CpG-3'sequence, preventing transcription factor binding and attenuating transcription (72). HAT catalyzes the transfer of an acetyl group to the histone lysine residue which changes the overall charge between the DNA-histone complex resulting in a relaxation of the chromatin. This leads to increased transcriptional access and gene expression (72). HDACs are a class of enzymes responsible for the removal of acetyl groups, which lead to a tighter binding of histones to DNA and decreasing transcriptional access (72). HAT and HDAC can also add or remove acetyl groups from proteins other than histones, notably transcription factors (73).

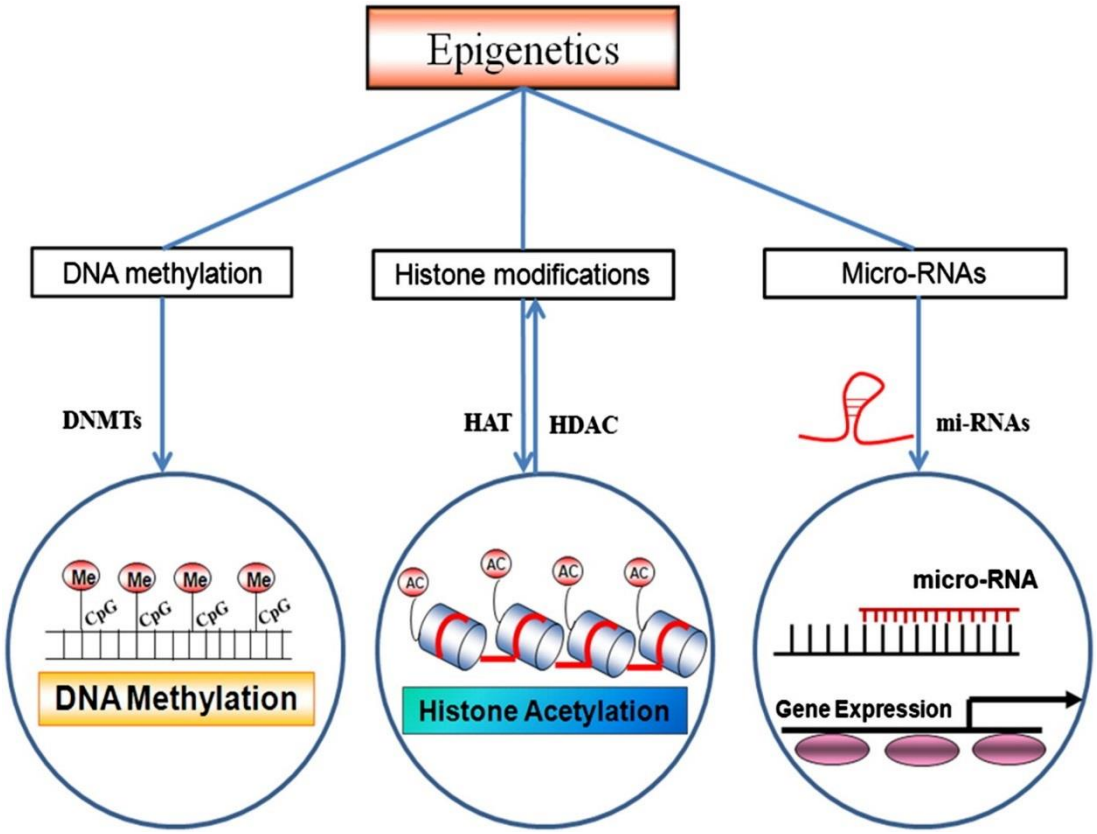

Figure 6. Mechanisms of epigenetic regulation. Histone modifications together with DNA methylation and micro-RNAs are dynamic processes that lead to chromatin remodeling and modulate gene expression without changing the underlying DNA sequence. DNA methylation at CpG sequences prevent binding of transcription factors and associated proteins to attenuate gene expression. Histones can undergo eight known modifications including acetylation, methylation, phosphorylation, sumoylation, and ubiquitination, however acetylation remains the most understood mechanism. Histone acetylation is modulated by histone acetylases (HATs) and histone deacetylases (HDACs). Acetylation alters the overall charge on the histone and weakens the DNA-histone interaction, leading to increased transcriptional access. Micro-RNAs are involved in post-transcriptional control of genes, binding complementary mRNA sequences and preventing translation (74).

EPIGENETIC INHIBITORS

The importance of epigenetic-modulating enzymes in gene regulation has prompted the use of inhibitors for various medical reasons, including cancer therapies, allograft rejections, and neurodegenerative diseases (74). Next generation sequencing has demonstrated that more than 50% of cancers harbor mutations in enzymes involved in chromatin organization (75). One advantage of epigenetic therapies is that they act on the transcriptional level, theoretically enabling repression of certain genes or transcriptional reactivation of epigenetically silenced genes. Additionally, the inhibition of one epigenetic regulator could have the same effect simultaneously on different cell processes if these pathways are targeted with a specific drug. These factors, in addition to the reversible nature of epigenetic treatments, make epigenetic targets a promising alternative for diseases which are difficult to treat using conventional approaches. Current inhibitors act on three epigenetic levels: DNA methylation, histone methylation or acetylation, and chromatin reading (Figure 7) (74). To date, there are two DNMT inhibitors (DMNTi) and four HDACi approved by the FDA to treat myelodysplastic syndromes (MDS)/acute myeloid leukemia (AML), and lymphomas respectively (75).

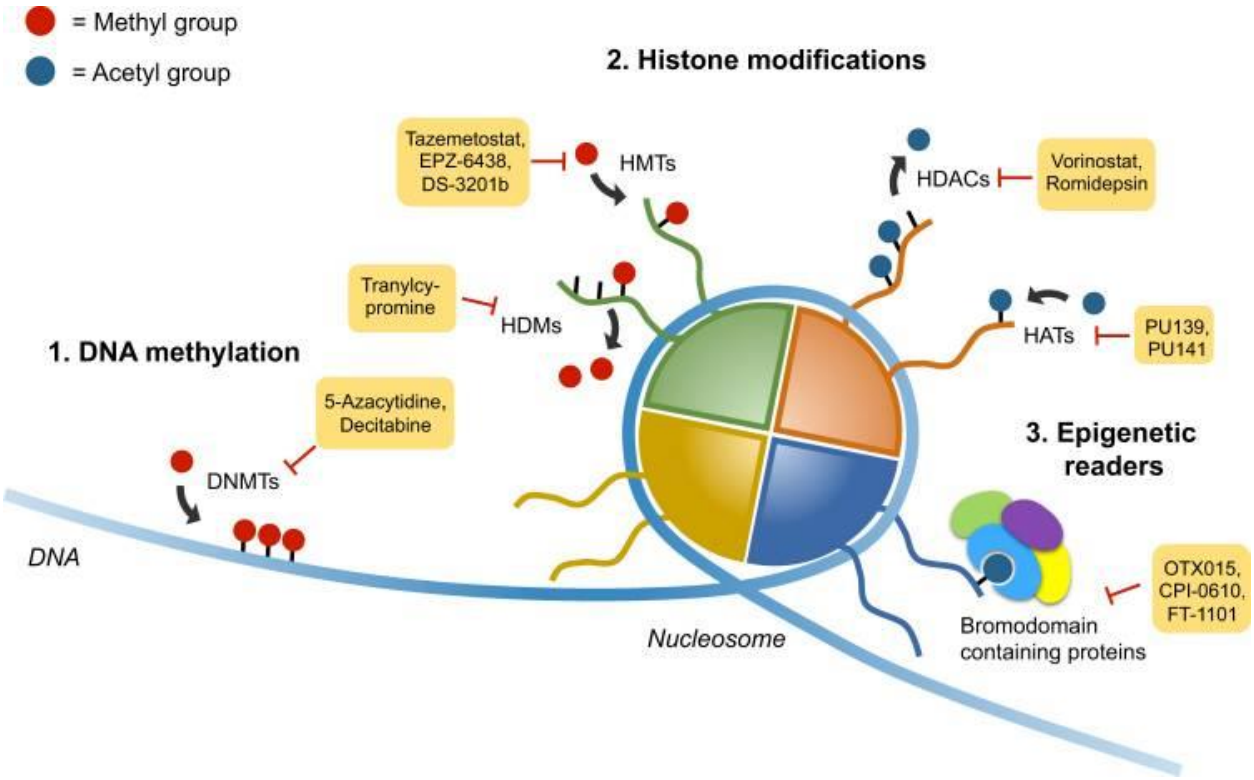

Figure 7. Epigenetic inhibitors and their targets. Current epigenetic inhibitors act on three different epigenetic levels: DNA methylation, histone methylation or acetylation, and chromatin reading. This figure shows the most promising drugs and target proteins involved in these processes, with some under clinical trials or approved for treatment of certain malignancies (76, 77).

HISTONE DEACETYLASE INHIBITORS

In the human genome, 18 HDAC family members have been identified and classified into four groups based on their homology to yeast HDACs. Class I HDACs are primarily located in the nucleus of the cell, whereas Class IIA is localized to the cytoplasm but can be shuttled between the cytoplasm and nucleus depending on their phosphorylation status . The remaining HDAC classes are mostly distributed in the cytoplasm and mitochondria. HDACs can modify other cellular proteins in addition to histones, and HDACi can influence a variety of processes including cell cycle arrest, angiogenesis, immune modulation, and apoptosis by targeting non-histone proteins. HDACi are grouped into four major structural classes: hydroxamic acid derivatives, including vorinostat and panobinostat; benzamide derivatives like mocetinostat; short chain fatty acids such as valproic acid; and natural inhibitors like trichostatin A, isolated from *Streptomyces hygroscopicus* (74). Most HDACi's target multiple classes of HDACs and are recognized as pan-HDAC inhibitors (79). In addition to their application in cancer therapies, HDACi have recently been investigated for a possible role in achieving a sterilizing cure for human immunodeficiency virus (HIV) infection. The most significant barrier to an HIV cure is the presence of replication-competent provirus in resting CD4 T cells. If the cells remain transcriptionally silent, viral proteins are not produced, and the cell is invisible to the immune system and unresponsive to antiretroviral therapy. HIV latency is partially controlled by the activity of HDACs, which repress proviral transcription (80, 81). HDACi are aimed at activating HIV from latency to induce viral replication and allow immune-mediated elimination to occur, known as the "shock and kill" method. However, these HDACi may exert unwanted effects due to their non-specific inhibition. Notably, HDACi treatment caused defects in T-cell development and distorted CD8+ T cell activity, possibly reducing the potential of these cells to effectively eliminate reactivated cells in patients . Additionally, whether these HDACi can cause reactivation of other latent viruses in human patients have not been evaluated.

VIRAL EPIGENETICS

Epigenetic regulation is now recognized as a ubiquitous mechanism to control gene expression and there are multiple examples in which viral proteins interact with the epigenetic machinery to modulate host and viral gene transcription . These interactions can be classified in two general ways: host epigenetic regulation of viral proteins or viral regulation of host epigenetics, both of which contribute to viral infection. An increasing body of evidence shows that histone modifications have critical roles in modulating viral gene expression and controlling viral life cycles, especially for those viruses exhibiting two distinct life cycles: latent and lytic productive stages (84). HIV has numerous well-documented interactions with the host-epigenetic machinery. An unbiased interaction study between HIV and host acetylation factors found a high degree of interplay: 19 of 26 HATs, 6 of 18 HDACs, and 16 of 46 bromodomain-containing proteins bind to diverse HIV proteins (82). Protein and chromatin acetylation have documented roles in HIV viral fusion and entry, HIV transcription, and HIV latency (82).

The effect of active histone markers in virus lytic infection or reactivation from latency has been observed in multiple viruses but have been best characterized in Kaposi's sarcoma-associated herpesvirus (KSHV) (85), Epstein-Barr virus (EBV) (83), human cytomegalovirus (86), and HIV (86-88). Epigenetic interactions have also been well documented within the polyomavirus family. SV40 has been extensively studied as a model virus, and it has been demonstrated that SV40 undergoes almost all of the same forms of epigenetic regulation as cellular chromatin, including nucleosome location, histone modifications, and miRNAs. However, polyomaviruses do not appear to utilize DNA methylation as a form of epigenetic regulation due to their small genome size and relative absence of CpG target sites for methylation. Many polyomaviruses, including SV40 and JCPyV, contain a miRNA in the late message strand which can be cleaved to yield an miRNA which inhibits expression of the early genes. These viral miRNAs shares sequence homology with certain cellular miRNAs and it has been suggested that the viral miRNAs could

dysregulate cellular genes (90). Interestingly, it has recently been shown that SV40 chromatin from virus particles before infection and after infection results in a shift in nucleosome location, exposing the early and late start sites which were previously bound by the nucleosome (91). Although other polyomaviruses have not been studied to the extent of SV40, they have similar replication strategies and genomes and likely utilize epigenetics in comparable ways to regulate infection.

EPIGENETICS AND JCPyV

Recently, epigenetic events have been implicated in regulatory control of JCPyV early and late gene transcription. Transfection of Mad-1 JCPyV into TC620 human oligodendroglioma cells treated with HDACi trichostatin A (TSA) and sodium butyrate stimulated JCPyV early and late gene transcription (92, 93). Further transfection experiments utilizing mutations in the archetype NCCR showed the protein acetylation events affect the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) p65 protein's binding to the NCCR , but not with DNA methyltransferase inhibitors, suggesting that viral gene expression is regulated by events that involve protein acetylation. The effect was found to be mediated via the κB element, a site located within the NCCR responsible for: **1)** binding transcription factors NF-kB p65, CCAAT/Enhancer binding protein beta (C/EBPβ), nuclear factor of activated T-cells 4 (NFAT4), and Rad51, and **2)** mediating stimulation by tumor necrosis factor alpha (TNF-α) . Further site-directed mutagenesis studies showed that protein acetylation events at specific lysine residues in NF-kB p65 impaired or enhanced transactivation of JCPyV early promoter transcription. This suggests acetylation regulates NF-kB p65 activity toward Mad-1 JCPyV at the level of p65 binding to the JCPyV NCCR and activation of early viral protein transcription (94).

A separate study supported these findings by investigating the role of Brd4, an epigenetic reader protein that recognizes acetylated lysine residues and specifically binds to acetylated NF-kB p65. A Brd4 inhibitor blocked transcription of early genes and reactivation of infection, further

implicating epigenetic regulation by histone acetylation as a critical step in regulation of early Mad-1 JCPyV virus transcription (42). Although in healthy individuals, JCPyV exists as a persistent latent infection in viral reservoirs, in immunocompromised patients the virus undergoes rearrangements and reactivation into a more pathogenic virus which leads to PML (1). The effects of epigenetic drug-modulated immunosuppression on JCPyV latency and reactivation are not well understood. Thus, there is a ***gap*** in our understanding of the effects of epigenetic drug-modulated immunosuppression on the reactivation and rearrangement of archetype JCPyV.

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CHAPTER 2

THESIS SCOPE

Background and rationale

The mechanisms which control JCPyV reactivation and PML pathogenesis have not been defined, although host immune status has been implicated as a critical factor. However, even within immunocompromised patients only a subset of these individuals will develop PML, indicating a more nuanced modulatory role of the immune system in PML development. Epigenetics is a rapidly expanding field whose systems have critical regulatory roles in various cellular processes including cell cycle arrest, angiogenesis, immune modulation, and apoptosis. With the advent of new epigenetic therapies, it is important to understand the effect of epigenetic control on the replication and reactivation of JCPyV for risk stratification and potential therapeutics. Since epigenetic drug-modulated immunosuppression of JCPyV latency and reactivation are not well understood, there is a ***gap*** in our understanding of the effects of epigenetic drug-modulated immunosuppression on the reactivation and rearrangement of archetype JCPyV.

Long-term goal, Objective, and Hypothesis

Our ***long-term goal*** is to delineate the natural history of archetype JCPyV infection, reactivation, and rearrangement for evidence-based approaches to improve treatment for PML. The ***objective*** of this research is to determine the epigenetic regulation of archetype JCPyV. The ***aims*** of the proposed research are to determine the effects of HDACi on urine-derived archetype JCPyV replication kinetics *in vitro* and JCPyV viruria and NCCR sequences in HIV-infected individuals on uninterrupted combination antiretroviral therapy (cART) treatment with HDACi panobinostat. We ***hypothesize*** that archetype JCPyV infection is regulated epigenetically by histone modifications to maintain viral latency and that HDACi treatment will increase JCPyV replication kinetics.

Specific Aims

Specific Aim 1: **Determine the effect of HDACi trichostatin A (TSA) on the replication kinetics of archetype JCPyV *in vitro*.**

Specific Aim 1a: Investigate the effect of TSA on the replication kinetics of urine-derived archetype JCPyV in primary human brain cortical astrocytes (HBCA).

Specific Aim 1b: Investigate the effect of TSA on the replication kinetics of urine-derived archetype JCPyV replication in primary renal proximal tubule epithelial (RPTE) cells.

Hypothesis: Based on published literature, we *hypothesize* that treatment with TSA will increase the replication kinetics of urine-derived archetype JCPyV in HBCA and RPTE cells.

Approach:. First, cytotoxicity will be assessed using an MTS assay. HBCA or RPTE cells will be seeded at 20,000 cells/well in 96-well plates maintained at 37°C, 5% CO_2. At 80% confluency, cells will be treated with 0, 50, 100, 200 nM TSA. Cell viability will be assessed at 1, 5, 10, 15, and 20 days post treatment. The susceptibility of HBCA and RPTE cells to urine-derived archetype JCPyV infection will be monitored by detection and analysis of JCPyV DNA, RNA, protein, and infectious virions. Cells will be seeded at $1.5x10^5$ cells/well in a six-well plate and infected with 41 HA units of JCPyV/well (1). Quantitative analysis of JCPyV genome copies and RNA transcripts will be conducted using qPCR and qRT-PCR assays, respectively, using JCPyV TAg and VP1-specific primers at 12 hours, 1, 3, 5, and 10 days after infection, as previously described (2). qPCR gene copies will be normalized to GAPDH. JCPyV VP1 protein will be detected by immunofluorescence assay (IFA) in archetype JCPyV-infected cells at 15 and 25 days after infection (3). NCCR sequence analysis will be conducted in infected cells at days 1 and 10 following archetype JCPyV infection. To demonstrate the production of infectious archetype JCPyV virions, naïve cells will be infected with supernatant from previously infected cells collected

at day 15 or 20 after infection. Viral DNA and RNA will be quantitated by qPCR as described above.

Data Analysis: All experiments will be performed in duplicate and repeated at least once. For qPCR and qRT-PCR analysis, average cycle threshold (C_T) values will be log transformed, mean centered, and autoscaled in order to draw statistical comparison as previously described (4). qPCR and qRT-PCR data will be from two biological replicates. Error bars will indicate 95% confidence intervals, and asterisks will indicate Welch-modified t test p values of less than 0.05, 0.01, and 0.001 as previously described (2).

Specific Aim 2: Determine the effect of HDACi panobinostat treatment on viruria and NCCR sequence of JCPyV in HIV-infected individuals on uninterrupted cART.

> **Specific Aim 2a:** Determine the effect of panobinostat treatment on JCPyV viruria in HIV-infected individuals on uninterrupted cART.
>
> **Specific Aim 2b:** Determine mutations in JCPyV NCCR sequence isolated from HIV-infected individuals on uninterrupted cART treated with panobinostat.

Hypothesis: Based on the published literature, we *hypothesize* that panobinostat treatment will increase JCPyV excretion in the urine than in baseline untreated measurements. We also hypothesize that the panobinostat-treated HIV-infected individuals may have mutations and rearrangement events within the NCCR region of the archetype JCPyV.

Approach: HIV-infected individuals who were treated with 20 mg oral panobinostat while on uninterrupted cART will be screened for JCPyV by PCR and the NCCR region from JCPyV-positive urine samples were sequenced. Randomized blinded patient urine samples from the CLEAR cohort (clinical trial # NCT01680094) (5) will be first screened for JCPyV DNA by conventional PCR. DNA will be extracted from urine samples using QIAprep Spin Miniprep kit using previously described protocols. Conventional PCR of the NCCR region primers (JRR 25

and JRR 28) and VP1 region primers (JLP 15 and JLP 16) will be used as previously described [50-51]. JCPyV DNA-positive samples will undergo quantitative analysis of JCPyV genome copies by qPCR specific for TAg and VP1 normalized to GAPDH, as previously described (4). Additional NCCR sequence analysis will be conducted for JCPyV-positive urine samples as previously described (6).

Data Analysis: All experiments will be performed in duplicate and repeated at least once. For qPCR analysis, average cycle threshold (C_T) values will be log transformed, mean centered, and autoscaled in order to draw statistical comparison as previously described (4). Error bars will indicate 95% confidence intervals, and asterisks will indicate Welch-modified t test p values of less than 0.05, 0.01, and 0.001 as previously described (2). NCCR from panobinostat-treated JCPyV-infected HIV-positive individuals will be sequenced and analyzed for mutations, deletions, and or duplications compared to archetype JCPyV NCCR.

Significance

JCPyV remains an elusive virus to study, partly due to the difficulty of propagating the archetype virus *in vitro* and the lack of an animal model of disease. Nearly 40 years after the discovery of JCPyV, there remains no therapeutic treatment for the devastating disease PML or vaccine for prevention of JCPyV infection. The widespread seroprevalence of JCPyV in the general population further emphasizes the need to understand the molecular mechanisms that underly JCPyV infection and PML development. Further, with the new wave of immunomodulatory and epigenetic drugs in development and coming to market, understanding JCPyV infection in the context of nonspecific pharmaceutical immune modulation will be critical. This study is significant in that it utilizes a primary cell model to investigate the effect of HDACi on JCPyV replication *in vitro,* as well as in patient samples. Insight into these mechanisms may contribute to developing preventative or therapeutic interventions for PML and JCPyV.

Innovation

Epigenetics is an important aspect to consider in the context of viral infection as it has been implicated as a major factor in viral latency. Elucidating the molecular mechanisms involved in epigenetic events will drive development of therapeutic agents to disrupt viral latency and increase immune surveillance. One well-known example in which this type of approach has been utilized is in development of new HIV therapeutics. Due to the integration of HIV into the host genome and complexity of viral latency, curative therapy has long thought to be unfeasible. However, recently viral reactivation from latently infected cells has become a promising therapeutic approach to eradicate HIV. Using treatment to reactivate HIV from its latent reservoirs followed by cART to kill the activated virus is one potential approach to achieve a sterilizing cure. In JCPyV, transcription of rearranged JCPyV early genes in transfected oligodendroglioma cells can be enhanced with the use of HDACi . Based on the published literature, this effect is due to the acetylation of transcription factor NF-kB p65.

Although these studies examined HDACi modulation of JCPyV, it has always been in the context of transfection utilizing the rearranged virus rather than infection with the archetype virus. This is primarily due to the complexity in isolating and cultivating live archetype JCPyV from infected individuals, as well as difficulty in identifying a primary cell culture model. The ***innovation*** of this study will be the use of archetype JCPyV isolated from a healthy individual to infect primary cell cultures treated with epigenetic inhibitors. This model system was developed in our laboratory and is more biologically relevant than transfections as it utilizes the natural course of JCPyV attachment to host cell receptors, internalization, and replication. Additionally, JCPyV viruria and NCCR sequences have not been assessed *in vivo* in individuals who were treated with HDACi. Understanding the molecular mechanisms underlying the effects of epigenetic inhibitors on JCPyV infection and replication *in vitro* and *in vivo* will drive development of more effective therapeutics for PML.

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CHAPTER 3

HISTONE DEACETYLASE INHIBITOR TSA INCREASES JCPYV REPLICATION KINETICS IN VITRO

Histone deacetylase inhibitor trichostatin A increases JCPyV replication kinetics *in vitro*

Running title: Trichostatin A increases archetype JCPyV replication *in vitro*

Michellei C. Fisher[1,2], Nelson B. Lazaga[1,2], and Vivek R. Nerurkar[1,2,*]

[1]Department of Tropical Medicine, Medical Microbiology and Pharmacology, [2]Pacific Center for Emerging Infectious Diseases Research, John A. Burns School of Medicine, University of Hawaii at Manoa, Honolulu, Hawaii 96813

*Corresponding author: Vivek R. Nerurkar, Ph.D. John A. Burns School of Medicine, University of Hawaii at Manoa, 651 Ilalo Street, BSB 320G, Honolulu, HI, 96813,

Phone: (808) 692-1668, Fax: (808) 692-1984, E-mail: nerurkar@hawaii.edu

Abstract

The human polyomavirus JC (JCPyV) is the causative agent of progressive multifocal leukoencephalopathy (PML), a demyelinating disease of the brain. Although archetype JCPyV infection is common in the healthy population, PML occurs almost exclusively in individuals with immunodeficiencies or on immunomodulatory medication, particularly HIV-1/AIDS patients and patients on monoclonal antibody regimens. After primary infection and dissemination, replication is controlled by the immune system, causing an asymptomatic infection with occasional viruria. However, when immune function is perturbed, JCPyV can re-emerge as a rearranged virus, with deletions and/or duplications in the non-coding control region (NCCR). This rearranged JCPyV virus can undergo lytic replication in the oligodendrocytes and astrocytes, causing PML. Therefore, a crucial question in understanding PML pathogenesis is the nature of the molecular mechanisms which maintain JCPyV latency. Epigenetic regulation is now recognized as a ubiquitous mechanism to control gene expression. The JCPyV genome structure closely resembles host chromatin, and it is possible that these mechanisms modulate JCPyV replication. Histone deacetylase inhibitors (HDACi) have been demonstrated to increase rearranged JCPyV replication in a transfection model. We used patient-derived archetype JCPyV to infect primary human brain cortical astrocytes (HBCA) and renal proximal tubule epithelial cells (RPTE) treated with HDACi trichostatin A (TSA). Treated HBCA ad a four-log fold increase in genome copy numbers at 12 h post infection, while RPTE cells showed a 20 to 30-fold increase at day 10. TSA-treated HBCA showed a slight increase in RNA transcripts, while RPTE cells demonstrated a 30 to 60-fold increase in RNA transcripts 10 days post infection. TSA treatment also rescued VP1 protein production in HBCA and increased VP1 production two-fold in RPTE cells, although infectious virions were only detected in RPTE cells. To our knowledge, this is the first study to show the effect of HDACi on archetype JCPyV replication in an *in vitro* infection model. Taken together, our data demonstrates that TSA treatment in primary HBCA and RPTE cells increases

both early and late archetype JCPyV replication in a cell-specific manner. Further studies are needed to understand the mechanism of regulation in archetype JCPyV replication.

Introduction

Human polyomavirus JC (JCPyV) is the causative agent of progressive multifocal leukoencephalopathy (PML), a demyelinating disease of the brain. PML was originally recognized as a rare complication of hematological malignancies or systemic inflammatory disorders, however incidence increased rapidly during the AIDS epidemic (1). AIDS is the most frequent condition associated with PML (2), with approximately 6% of patients developing AIDS related PML (3). A resurgence of PML occurred in the 2000s as a result of the use of immunomodulatory compounds, including monoclonal antibodies natalizumab, efalizumab, and rituximab, for the treatment of the autoimmune conditions, such as multiple sclerosis, Crohn's disease, severe forms of plaque psoriasis, hematologic malignancies, and rheumatoid arthritis (4). Currently, there is no treatment for PML or vaccine against JCPyV.

JCPyV is a neurotropic human polyomavirus belonging to the genus *Orthopolyomavirus* in the family *Polyomaviridae*. It was first isolated in 1971 from the brain of a patient suffering from PML (5). Infection with JCPyV produces a self-limiting infection in immunocompetent individuals with intermittent viruria (6-8). The route of JCPyV transmission and the primary sites of replication are unknown. JCPyV is detected in human sewage, so transmission via urine to oral or respiratory route and primary replication in tonsillar tissue has been proposed . Primary JCPyV infection is cleared by the immune system to where viremia is undetectable and is thought to persist in the kidneys.

JCPyV has a naked icosahedral capsid and a circular double-stranded DNA genome of approximately 5.1 kb . The viral genome is functionally divided into an early region encoding large and small T antigen proteins; a late region (2.3 kb) encoding viral capsid proteins VP1, VP2 and VP3, and the accessory agnoprotein; and a non-coding regulatory region (0.4 kb) referred to as the noncoding control region (NCCR). Based upon the structure of the NCCR, two types of JCPyV have been identified: the archetypal form, which is predominantly detected in kidney and urine of people with and without PML (8); and the rearranged form which is predominantly detected in

brain, tonsils, and lymphocytes of individuals with PML (9,10) . The archetype JCPyV NCCR, which consists of six regions, designated A to F, displays minimal sequence variation, whereas the NCCR of JCPyV isolated from PML patients are highly variable and contain rearrangements such as deletions, duplications, tandem repeats, and insertions in the NCCR . It is thought that all other rearranged forms of JCPyV arise from the archetype form within the patient, and most likely arise during immunosuppression or immunomodulation (10,11).

JCPyV is a DNA virus with no transcription factors in the infectious virion, therefore it is highly dependent on host and cellular transcription machinery to initiate the early phase of transcription. Similar to human DNA, JCPyV DNA is tightly wound around host-derived positively-charged histone proteins in a chromatin-like complex (12-13). This complex is subject to regulation by epigenetics, which include histone modifications that alter the chemical charge of the DNA-histone complex modulating access to transcription machinery. Histone acetylation is a well-studied histone modficiation in which histone acetylases (HAT) add acetyl groups to the histones. This causes the charge on the histones to become more neutral, weakening the association of the DNA-histone complex and allowing for a more open chromatin conformation, generally associated with an increase in transcription. This process is reversed by histone deacetylases (HDAC), which remove the acetyl groups and is generally associated with transcription repression.

Recently, epigenetic events have been implicated in regulatory control of JCPyV early and late gene transcription. Transfection of Mad-1 JCPyV into TC620 human oligodendroglioma cells treated with HDACi trichostatin A (TSA) and sodium butyrate stimulated JCPyV early and late gene transcription (14). Further transfection experiments utilizing mutations in the archetype NCCR showed the protein acetylation events affect the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) p65 protein binding to the NCCR , but not with DNA methyltransferase inhibitors, suggesting that viral gene expression is regulated by events that involve protein

acetylation. The effect was found to be mediated via the κB element, a site located within the NCCR responsible for: **1)** binding transcription factors NF-kB p65, CCAAT/Enhancer binding protein beta (C/EBPβ), nuclear factor of activated T-cells 4 (NFAT4), and Rad51, and **2)** mediating stimulation by tumor necrosis factor alpha (TNF-α) . Further site-directed mutagenesis studies showed that protein acetylation events at specific lysine residues in NF-kB p65 impaired or enhanced transactivation of JCPyV early promoter transcription. This suggests acetylation regulates NF-kB p65 activity toward Mad-1 JCPyV at the level of p65 binding to the JCPyV NCCR and activation of early viral protein transcription (15). A separate study supported these findings by investigating the role of Brd4, an epigenetic reader protein that recognizes acetylated lysine residues and specifically binds to acetylated NF-kB p65. A Brd4 inhibitor blocked transcription of early genes and reactivation of infection, further implicating epigenetic regulation by histone acetylation as a critical step in regulation of early Mad-1 JCPyV transcription (16).

To date, experiments with JCPyV have routinely used the rearranged Mad-1 JCPyV in transfection models due to the difficulty in isolating and culturing archetype JCPyV. The novelty of this study is to investigate the role of epigenetics in archetype JCPyV infection, using a patient-derived archetype JCPyV and an infection model in primary cells. We used two different *in vitro* models, renal proximal tubule epithelial cells (RPTE) and primary human brain cortical astrocytes (HBCA), as the kidneys are a hypothesized place of latency and the brain is the area of disease pathogenesis. We demonstrated that treatment with TSA increased archetype JCPyV DNA and RNA and stimulated JCPyV late protein production. Further, we demonstrated infectious virion production in RPTE cells but not HBCA. Together, we demonstrated that HDACi TSA increases JCPyV replication kinetics *in vitro*.

Results

TSA is not cytotoxic to HBCA and RPTE cells

To ensure cell viability would not be affected by treatment, a cytotoxicity assay was performed with multiple epigenetic inhibitors (Supplementary Fig. 1). Two HDACi, TSA and suberoylanilide hydroxamic acid (SAHA), were purchased from Sigma Aldrich. HBCA and RPTE cells were cultured with varying concentrations of TSA and SAHA for 20 days and cell viability was assessed by an MTS assay on days 1, 5, 10, 15, and 20. The physiological concentrations of TSA and SAHA used were based on previous reports which utilized 200 nM TSA and 5 mM SAHA which resulted in a 10-fold increase in Mad-1 JCPyV luciferase transcription reporter activity respectively (16). SAHA was highly cytotoxic at 5, 10, and 20 µM concentrations to HBCA and RPTE cells, resulting in a 50% reduction in cell viability at higher concentrations just one day after treatment (Supplemental Fig. 1A). Subsequent experiments with SAHA at 0.625, 1.25, and 2.5 µM showed moderately cytotoxic effects, with a 30% reduction in viability at day 20 (Supplemental Fig. 1B). TSA at 50, 100, and 200 nM showed no difference in cytotoxicity in both HBCA and RPTE cells (Supplemental Figure 1C). Therefore 200 nM TSA was used for all subsequent experiments.

JCPyV early and late transcription are enhanced by HDACi TSA

In at least two independent experiments, the replication kinetics of archetype JCPyV in 200 nM TSA-treated primary HBCA and RPTE cells was monitored from day 1 to 10 after infection. The viral titer of the urine-derived archetype JCPyV virus was determined using an HA assay (Supplementary Fig. 2). $1.5x10^5$ cells were inoculated with 41 HA units of archetype JCPyV and quantitative analysis of JCPyV TAg and VP1 genome copies and RNA transcripts were conducted by qPCR and qRT-PCR. At 12H after infection, the total JCPyV TAg ($2.58x10^8$) and

VP1 ($2.58x10^8$) genome copies recovered from treated HBCA showed a four-log fold increase in TAg ($1.34x10^4$) and VP1 ($1.23x10^4$) compared to control untreated cells (Fig. 1A). At day 10, the total mean genome TAg ($4.08x10^9$) and VP1 ($1.31x10^{10}$) copies recovered from each 35 mm plate seeded with HBCA were approximately two-fold (2.58) and two-log fold ($1.27x10^2$) higher than untreated cells. Treated HBCA showed no fold increase in TAg transcripts but a larger (11.1) fold increase in VP1 transcripts. In treated RPTE cells, total JCPyV TAg ($8.71x10^{12}$) and VP1 ($4.37x10^{11}$) genome copies recovered at day 10 post infection showed a fold increase of 36.9 and 13.5 compared to control RPTE cells, respectively (Fig. 1B). Treated RPTE cells showed a fold increase of 61.6 and 96.2 for TAg and VP1 RNA transcripts at day 10 post infection, respectively. NCCR sequence analysis confirmed no change in sequence from infecting dose (ID) to day 10 post infection (Supplementary Fig. 3).

TSA effect on VP1 and virion production

In two independent experiments, we examined VP1 protein production by IFA. At day 15 post infection, approximately 2% of HBCA (Fig. 2A) and 13% RPTE cells (Fig. 2B) expressed JCPyV VP1 protein using IFA, compared to 0% of HBCA and 6% of RPTE untreated archetype JCPyV infected cells. To demonstrate production of infectious archetype JCPyV virions, naïve HBCA and RPTE cells were infected with virus isolated from previously infected HBCA and RPTE cells collected on days 20 and 15 after infection (Fig. 2C). Naïve HBCA showed no detectable DNA replication or RNA transcripts. DNA replication and RNA transcripts in infected naïve RPTE cells were comparable to those observed in archetype JCPyV-infected RPTE cells.

Discussion

JCPyV has been known to be the causative agent of PML for more than 50 years, yet little is known about the basic mechanisms which maintain JCPyV latency and persistence. Although these mechanisms have not been clearly defined, it is known that PML primarily affects immunocompromised individuals. The current model suggests immune perturbance leads to decreased immunosurveillance and poor JCPyV specific immune control. This is thought to result in an increase in JCPyV viral replication, which generates JCPyV variants, which will differ in replication capacity and cellular tropism based on their NCCR sequences. It is thought that some of these high-replication capacity variants lead to viremia and migrate to the brain resulting in PML (11). Archetype JCPyV has been demonstrated to infect B cells and transmigrate through the blood brain barrier in an *in vitro* model (18), supporting this model of pathogenesis.

Epigenetic regulation has been implicated in control of JCPyV latency due to the structural similarity of the JCPyV genome to cellular chromatin. In the last ten years, several papers have implicated histone acetylation in modulating rearranged JCPyV replication. These studies have demonstrated that histone acetylation regulates NF-kB p65 activity toward Mad-1 JCPyV and activates early viral protein transcription (15). However, to date these experiments have been performed using the rearranged JCPyV isolate Mad-1 in transfection models. There are several drawbacks to these models: first, that the behavior of the prevalent archetype virus cannot be observed, limiting the potential to understand the factors which influence the rearrangement to the highly pathogenic rearranged JCPyV; second, that the transfection model is artificial and does not mimic engagement of the host-cell surface receptors and entry steps and their downstream effects; and third, that the immortalized cell types used do not resemble those that would be infected *in vivo.*

For the first time, we demonstrate that HDACi increases archetype JCPyV replication in an infection model and in a cell-dependent manner. We demonstrate an increase in JCPyV DNA

and RNA at late time points as well as a doubling of VP1 positive cells and production of infectious virions in TSA-treated RPTE cells. Further, we demonstrate an increase in JCPyV DNA and RNA at early time points in TSA-treated HBCAs and rescue of VP1 protein production, but not infectious virions. The kidneys are a site of latency for archetype JCPyV as virus is detected in the kidneys and is excreted in urine (8). Therefore, we expected a productive infection in RPTE cells. Our data supports this hypothesis, and replication in RPTE cells was further driven with TSA treatment. The brain is the site of pathogenesis for PML, and archetype JCPyV has not been detected in the brain. Therefore, we did not expect a productive replication of archetype JCPyV in HCBA cells. However, with TSA-treatment, the rescue of VP1 production indicates the possibility that archetype JCPyV could productively replicate in HBCAs under specific conditions.

Most HDACis target multiple classes of HDACs and are recognized as pan-HDAC inhibitors (17). In addition to their role as cancer therapeutics, HDACi have recently been investigated for a possible role in achieving a sterilizing cure for HIV infection. HDACi are aimed at activating HIV from latency to induce viral replication and allow immune-mediated elimination to occur, known as the “shock and kill” method. However, these HDACi may exert unwanted effects due to their non-specific inhibition. Notably, HDACi treatment caused defects in T-cell development and distorted CD8+ T cell activity, possibly reducing the potential of these cells to effectively eliminate reactivated cells in patients as well as being ineffective at controlling other infections (17). Whether these HDACi can cause reactivation of other latent viruses in human patients have not been evaluated. Our data strongly demonstrates the possibility of these LRAs in reactivating JCPyV, a ubiquitous polyomavirus for which the HIV-positive population is at a particular risk. This further highlights the need to study the effect of these drugs on other latent viruses to improve risk stratification for patients.

Our data demonstrate the importance of histone modulation on JCPyV early and late transcriptional control. These epigenetic mechanisms should be investigated further, to identify

possible therapeutic targets and develop effective treatment for PML, as well as mitigating risk in patients administered HDACis.

Conclusions

We have demonstrated that TSA treatment in archetype JCPyV infected primary HBCA and RPTE cells increases both early and late replication in a cell-specific manner. Treated HBCA had a higher fold increase in genome copy number, while RPTE cells demonstrated a larger increase in RNA transcripts. TSA treatment also rescued VP1 protein production in HBCA and increased production in RPTE cells, although infectious virions were only detected in RPTE cells. To our knowledge, this is the first study to show the effect of HDACi on archetype JCPyV replication in an infection model. Further studies are needed to understand the exact mechanism of regulation in archetype JCPyV replication within HBCA and RPTE cells. Differentiating the mechanisms which control JCPyV latency in glial and nonglial cells is crucial to understanding JCPyV reactivation and PML disease pathogenesis.

Materials and Methods

Cell culture

Primary HBCA were purchased from Cell Systems Corporation and maintained as previously described . Primary RPTE cells (Cat #4100) were purchased from Sciencell. HBCA and RPTE cells between passages P6 and P9 were used in all experiments.

Virus

Archetype JCPyV was isolated from the urine of healthy volunteers after obtaining written informed consent and study approval by UH-CHS by former graduate student Dr. Nelson Lazaga. Urine collected from healthy individuals were stored at 4°C and processed as previously described (18). DNA was extracted using Qiagen QIAprep Spin Miniprep Kit according to manufacturer's protocol. Urine-isolated JCPyV was then quantitated by real-time PCR (qPCR) (19) and confirmed by sequencing the NCCR as previously described (18). To generate archetype virus stock, COS-7 cells were infected with urine-derived JCPyV and harvested at day 35 after infection. Virus isolation and purification was conducted as previously described (20). Virus was then quantitated by HA assay (20) and qPCR prior to infection experiments and confirmed by NCCR sequence analysis before and after infection.

HA assay

VP1 is the major capsid protein of the JCPyV and it is responsible for the attachment to cells and agglutinates human type O erythrocytes (20). Human type O erythrocytes were centrifuged at 2,500 rpm for 10 min at 4^{o}C, washed twice in Alsever's buffer (20 mM sodium citrate, 72 mM NaCl, 100 mM glucose, pH 6.5 adjusted with acetic acid), and suspended in

Alsever's buffer at a final concentration of 0.5%. Serial two-fold dilutions of virus suspensions were prepared in Alsever's buffer. Fifty µL of viral suspension and an equal volume of RBC were added to each well of a 96-well "U" bottom microtiter plate and incubated at 4^{o}C for 3 to 6 hours, with a final volume of 100 µL. The final dilution of virus suspension that agglutinates red blood cells was considered the end point of the titration and read as the reciprocal of that dilution. The end point dilution is considered 1 hemagglutination (HA) unit, with the estimated ratio of infectious particles being approximately 10^4 to 1 HA unit .

JCPyV infection

$1x10^5$ HBCA or RPTE cells were seeded in tissue culture treated 35-mm plates to study viral kinetics. Additionally, $5x10^4$ cells were seeded in each well of a 24-well plate containing cover slips for immunofluorescence assay (IFA). At 80 to 90% confluency, cells were either mock-infected with medium only, or inoculated with 41 HA units of JCPyV per $1x10^5$ cells. Initial virus inocula were measured using qPCR, prepared at appropriate concentrations, and added into designated plates, wells, or flasks and returned to an incubator (37^{o}C with 5% CO_2) for 2 hour adsorption for archetype JCPyV. Each plate, well, or flask was then washed twice with 1X PBS to remove unadsorbed virus followed by replenishment of fresh medium. Wells, plates, and flasks were kept at 37^{o}C with 5% CO_2 until time of cell harvest at designated time points. Culture medium was changed every three days.

TSA treatment

HBCA and RPTE cells were treated after infection and followed by continuous treatment until designated harvest time points. Culture media with TSA was changed every three days. TSA was added to the culture media just before changing the media.

Cytotoxicity

Cytotoxicity was assessed using the Promega Cell Titer 96® AQ_{ueous} One Solution Reagent MTS assay, according to the manufacturer's protocol. Briefly, 20 µL of reagent was added into each well, incubated at 37°C, 5% CO_2 for 4 hours. Absorbance at 490 nm was read using a 96 well plate reader.

Reinfection of naïve cells

At 80 to 90% confluency, cells were infected with 41 HA units of archetype JCPyV per $1x10^5$ cells. Media was changed every two days and cell lysates were collected for DNA and RNA analysis. For the reinfection of naïve HBCA or RPTE cells, 15 or 20 days after infection, infected cells were subjected to virus isolation and purification as previously described (18). Initial virus inoculum was measured using qPCR and naïve HBCA or RPTE cells were reinfected, as mentioned above.

DNA and RNA extraction and quantitative analysis

Low molecular weight DNA and total RNA were extracted from mock- and archetype JCPyV-infected cells from 35-mm plates harvested at 12 hours, day 1, 3, 5, and 10 after infection or days 1, 5, 10, 15, and 20 after reinfection, as previously described (19). cDNA was synthesized from 1 µg of cellular RNA using Bio-Rad iScript cDNA synthesis kit following the instructions provided by the manufacturer. JCPyV DNA or cDNA was amplified using 2 µL of template DNA or cDNA, 10 pmol each of forward and reverse primers, and probe specific for JCPyV TAg and for VP1 genes in a final reaction volume of 20 µL as previously published by our group and normalized to GAPDH (19). qPCR was conducted using a Bio-Rad iCycler iQ™ Multicolor Real-

Time PCR Detection System. Analysis was conducted via Bio-Rad iCycler iQ™ Multicolor Real-Time PCR Optical System Software Version 3.1.

PCR amplification and sequence analysis

JCPyV NCCR was amplified using 2 µL of template DNA and primers JRR-25 and JRR-28 as described previously (21). PCR products were separated on a 2% agarose gel, visualized with ultraviolet light, and purified by QIAquick PCR purification column and sequenced for positive identification of archetype JCPyV.

Immunofluorescence assay

HBCA and RPTE cells were seeded on coverslips in 24-well pates ($5x10^4$ cells/well). Cells were either mock-infected with medium only or inoculated with 20.5 HA units of archetype JCPyV. Cell preparation and staining with various primary antibodies were conducted as previously described (18). Fluorescent cells were examined using an Axiocam MRm camera mounted on a Zeiss Axiovert 200 microscope equipped with the appropriate fluorescent filters and objectives.

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Figure Legends

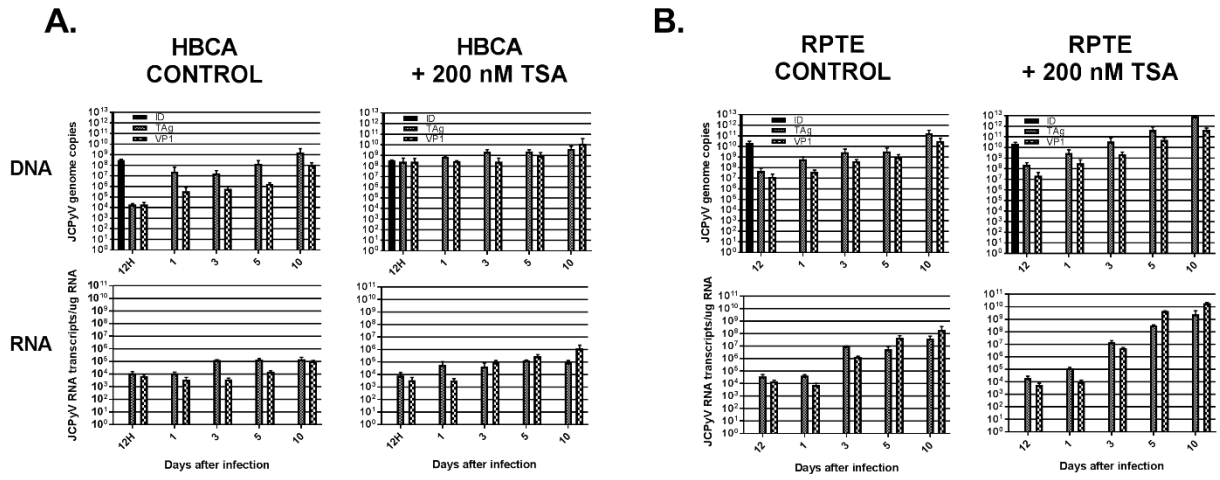


Figure 1. TSA treatment increases archetype JCPyV DNA and RNA expression in HBCA and RPTE cells. (A) HBCA or (B) RPTE cells were infected with 41 HA units of COS-7 cell propagated urine-isolated archetype JCPyV per well and incubated for 2 hours. Immediately following infection, cells were washed with PBS and fresh media with or without 200 nM TSA was added. Treatment continued until cells were harvested at indicated time points. DNA and RNA were extracted and viral TAg and VP1 genome copies and RNA transcripts were quantitated by qPCR and qRT-PCR, respectively.

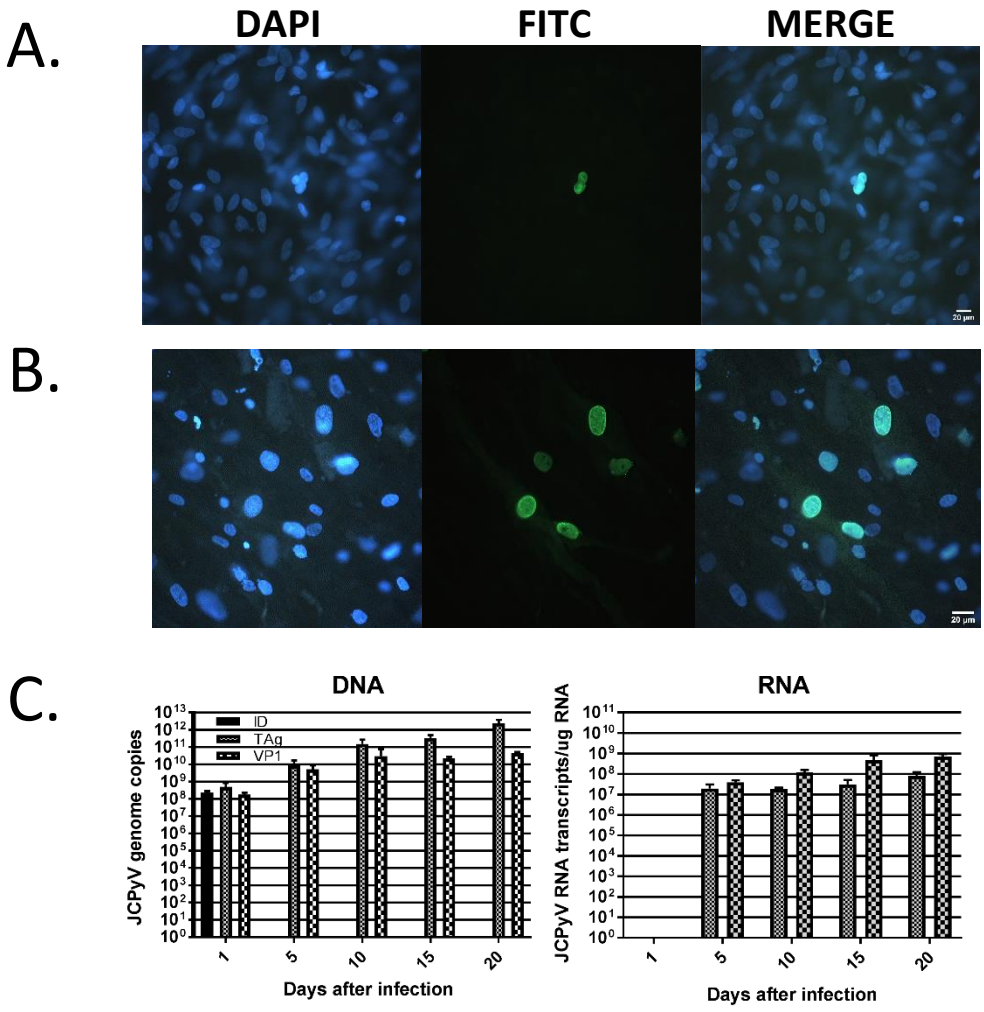
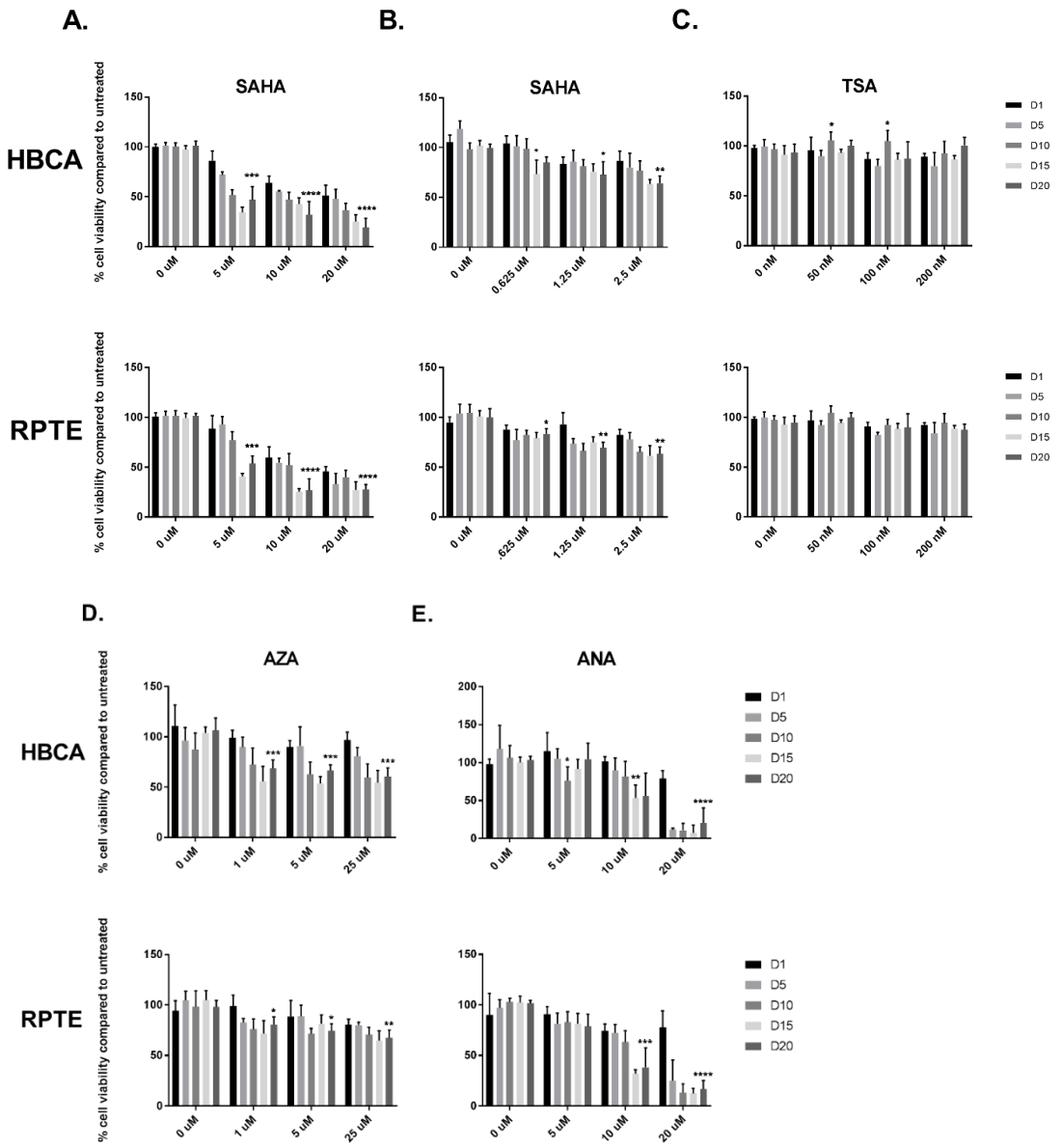
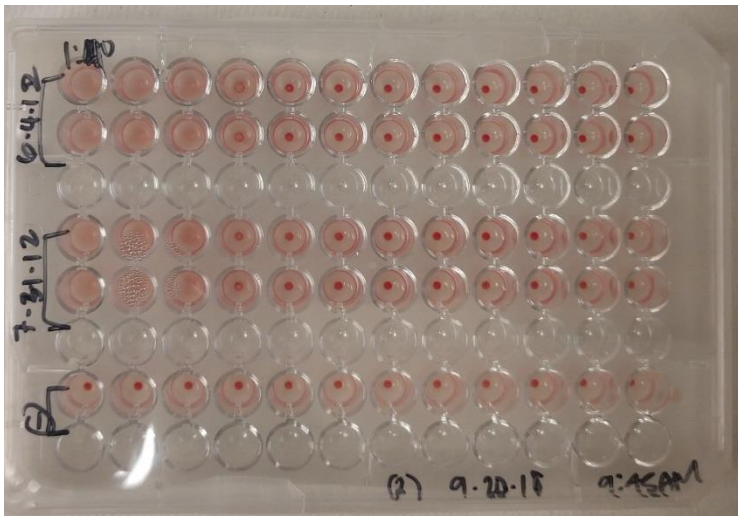


Figure 2. VP1 protein and reinfection of naïve HBCA and RPTE cells. Immunofluorescence staining was conducted on (A) HBCA and (B) RPTE cells infected with archetype JCPyV at day 15 after infection and cells were stained using anti-VP1 mouse monoclonal Ab (green) and DAPI (blue), scale bar 20 µm. (C) Naïve HBCA and RPTE cells were infected with 41 HA of archetype JCPyV isolated from previously infected HBCA and RPTE cells. Reinfection of isolated archetype JCPyV in RPTE cells TAg and VP1 genome copies and RNA transcripts are shown. There was no detectable DNA or RNA in the reinfected HBCA.



Supplemental Figure 1. Cytotoxicity of various epigenetic inhibitors in HBCA and RPTE cells: HBCA or RPTE cells were treated with (A) 5, 10, 20 µM SAHA, (B) 0.625, 1.25, 2.5 µM SAHA, (C) 50, 100, 200 nM TSA, (D) 1, 5, 25 µM AZA, and (E) 5, 10, 20 µM ANA. At the indicated time points cell viability was assayed using Promega's CellTiter 96® AQueous ONE Solution Cell Proliferation Assay System following the manufacturer's protocol. Absorbance at 490 nm was recorded using a 96-well plate reader.



Supplemental Figure 2. HA assay to determine infectious dose. Type O negative, JCPyV seronegative blood was incubated with increasing dilutions of virus stocks. The final dilution of virus suspension that agglutinates red blood cells was considered the end point of the titration and read as the reciprocal of that dilution. The end point dilution is considered 1 hemagglutination (HA) unit, with the estimated ratio of infectious particles being approximately 10^4 to 1 HA unit. A negative control was performed to ensure there was no self-agglutination.

	Mad-1	CY	HBCA ID	HBCA D10 I	RPTE ID	RPTE D10 I
Mad-1		85.8	85.8	85.8	85.8	85.8
CY			100	100	100	100
HBCA ID				100	100	100
HBCA D10 I					100	100
RPTE ID						100
RPTE D10 I						

Supplemental Figure 3. NCCR sequencing of archetype JCPyV infection experiment. DNA was extracted from infecting dose (ID) and final collection day (D10 I). Samples underwent PCR and sequencing for the NCCR region.

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Author Contributions

MCF, NBL, and VRN designed the experiments. MCF conducted the experiments. MCF and VRN analyzed the results and wrote the manuscript.

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CHAPTER 4

HISTONE DEACETYLASE INHIBITOR PANOBINOSTAT INCREASES JCPyV VIRURIA IN HIV-POSITIVE CART-ADHERENT PATIENTS

Histone deacetylase inhibitor panobinostat increases JCPyV viruria in HIV-positive cART-adherent patients

Running title: Panobinostat treatment increases JCPyV viruria

Michellei C. Fisher[1,2], Ole S. Søgaard[3], Lishomwa C. Ndhlovu[1], and Vivek R. Nerurkar[1,2*]

[1]Department of Tropical Medicine, Medical Microbiology and Pharmacology, [2]Pacific Center for Emerging Infectious Diseases Research, John A. Burns School of Medicine, University of Hawaii at Manoa, Honolulu, Hawaii 96813, [3]Department of Infectious Diseases, Aarhus University Hospital, Denmark

*Corresponding author: Vivek R. Nerurkar, Ph.D., John A. Burns School of Medicine, University of Hawaii at Manoa, 651 Ilalo Street, BSB 320G, Honolulu, HI, 96813:

Phone: (808) 692-1668, Fax: (808) 692-1984, E-mail: nerurkar@hawaii.edu

Abstract

Combination antiretroviral therapy (cART) controls replication of HIV-1 by affecting different stages of the viral life cycle. The use of cART has led to a prolongation of the lifespan of HIV-positive individuals and an improvement in quality of life, transforming HIV infection from a life-threatening disease into a chronic infectious disease. Despite the efficacy of cART, it is unable to completely eliminate infected cells and treatment is lifelong. One of the main obstacles to curative HIV treatment is the existence of proviral DNA in memory $CD4^+$ T cells, which retain the potential to produce new viruses. A new approach to curative treatment is the "shock and kill" method, which reactivates these latent cells with latency reversal agents (LRA) and uses cART to eliminate reservoirs. However, the ability for these histone deacetylase inhibitors (HDACi) ability to reactivate HIV suggests the possibility of reactivation of other latent viruses as well. In this study we examined whether treatment with HDACi affects JCPyV viruria. We analyzed randomized, blinded patient samples from the CLEAR cohort (clinical trial #NCT01680094), in which HIV-positive cART-adherent patients were treated orally with HDACi panobinostat for eight weeks. We detected JCPyV DNA in the urine of 4/15 (27%) of patients, and NCCR sequencing confirmed homology to the archetype JCPyV. We demonstrate that 3 out of 4 (75%) of patients had a statistically significant increase in JCPyV genome copies during treatment than at baseline measurements. This increase was abrogated in post-treatment follow up urine samples. Taken together, these findings suggest that HDACi may increase JCPyV viruria in patients. This study emphasizes the need to understand the effects of these global HDACi on other latent viruses to improve risk stratification and minimize complications for LRA treatments.

Introduction

Combination antiretroviral therapy (cART) controls replication of HIV-1 by affecting different stages of the viral life cycle (1). The use of cART has led to a prolongation of the lifespan of HIV-positive individuals and an improvement in quality of life, transforming HIV infection from a life-threatening disease into a chronic infectious diseases (2, 3). Despite the efficacy of cART, it is unable to completely eliminate infected cells and treatment is lifelong.

One of the main obstacles to curative HIV treatment is the existence of proviral DNA in memory $CD4^+$ T cells. These cells present a major difficulty for HIV eradication because once reactivated, these latently infected cells are a potential source of new viruses (4). Although HIV integrates into the host DNA it cannot express itself significantly, and in the absence of adequate stimuli latent reservoirs are stable and resistant to multiple treatment regimens (5). Recently, studies have identified drugs which are able to reverse latency without activating T cells and causing the production of new virions (6). This new strategy combines these latency reversal agents (LRA) with cART to activate production of virus by latently infected cells and eliminate them with cART. LRA include disulfiram and histone deacetylase inhibitors (HDACi) such as vorinostat (suberoylanilide hydroxamic acid or SAHA) or panobinostat (LBH589, Farydak™) (7,8). The role of HDAC is to remodel chromatin leading to a transcriptionally repressed state. HDACi impede that process, causing the chromatin to remain in a conformationally active state (9).

The ability of HDACi to reactivate HIV suggests the possibility of reactivation of other latent viruses as well. One virus in which reactivation is a concern is human polyomavirus JC (JCPyV), etiological agent of the demyelinating disease, progressive multifocal leukoencephalopathy (PML). PML was originally recognized as a rare complication of hematological malignancies or systemic inflammatory disorders, however, a dramatic 50-fold increase in the incidence in the last 30 years occurred as a result of the acquired immunodeficiency syndrome (AIDS) epidemic (10). AIDS is the most frequent condition associated with PML (11), with approximately 6% of patients

developing AIDS related PML (12). Recently, studies have demonstrated that HDACi increase Mad-1 JCPyV replication *in vitro,* but lack of an animal model prevents studies *in vivo* (13). Due to the relationship between PML and AIDS, determining if unintentional reactivation of JCPyV occurs with LRAs is a crucial determinant for risk-stratification in future treatments.

To our knowledge, this is the first study to examine whether treatment with HDACi affects JCPyV viruria. We detected JCPyV DNA in the urine of 4 of 15 (27%) of patients, and NCCR sequencing confirmed homology to the archetype JCPyV. We demonstrate that patients treated with panobinostat had higher detectable amounts of JCPyV viruria than their baseline urine. This increase was abrogated in post-treatment follow up urine samples. Taken together, these findings suggest that HDACi may increase JCPyV viruria in patients. Additional studies are warranted on the effects of LRAs on latent human viruses to minimize the risk of unintentional reactivation.

Results

Study design and participants

Fifteen patients were enrolled in the CLEAR cohort and all completed the panobinostat dosing regimen (Fig. 1). All participants were white males with a median age of 47 years (range: 28-53). The median time since HIV diagnosis was 81.4 months (range: 33.4-340) and median days from diagnosis to cART initiation was 540 (range: 0-6574). Patients were on cART a median of 43.4 months (range: 30.5-191.7) and had a median of 38 months (range: 26.3-169.6) with HIV RNA <50 copies per mL. Patients had a median nadir CD4 350 cells per µL (range: 130-710) and median baseline CD4 count of 935 cells per µL (range: 615-1990). Patients received oral panobinostat (20 mg) three times per week for 8 weeks while continuing cART. This regimen was based on clinical safety and preclinical testing of the effect of panobinostat on HIV production in latently infected cells Urine samples were collected at before treatment (baseline), on-

panobinostat during the first (early panobinostat) and third (late panobinostat) treatment cycle, and four weeks post-panobinostat (follow up) (Fig. 2). CSF samples were taken at baseline and late panobinostat.

JCPyV detected in urine of panobinostat-treated patients

DNA was extracted from 1 mL of urine or 100 µL of CSF and conventional PCR of the JCPyV non-coding regulatory region (NCCR) and major viral capsid protein VP1 genes were performed and visualized on a 2% agarose gel. Four out of fifteen (27%) patient's urine tested positive for archetype JCPyV, and all four were positive at all time points measured (Table 1). JCPyV DNA was not detected in any of the CSF samples. All urine and CSF samples were positive for GAPDH gene. Positive urine samples underwent gel extraction and NCCR sequencing to compare baseline and follow up samples. Sequencing results showed no difference in NCCR gene between baseline and follow up samples, and NCCR sequences aligned with archetype JCPyV (GenBank accession number M35834) (Fig. 3).

Panobinostat increases archetype JCPyV viruria in panobinostat-treated patients

JCPyV positive urine samples underwent quantitative analysis of JCPyV genome copies by qPCR using primers and probes specific for early viral protein T antigen (TAg) and VP1, normalized to GAPDH. Three out of four (75%) patients showed a statistically significant increase in JCPyV genome copies per mL of urine during late panobinostat treatment compared to baseline. P values are as follows: PID 7 VP1 ($p = 0.0077$), PID 10 VP1 ($p = 0.0198$, $p = 0.0140$), PID 12 TAg and VP1 ($p < 0.0001$, $p < 0.0001$), PID 17 TAg and VP1 ($p = 0.0155$, $p = 0.0149$). This increase was abrogated in the follow up period.

Discussion

Histone deacetylase inhibitors make up the majority of LRAs in clinical trials (14). Therefore, understanding the effect of LRAs on latent viruses is crucial before implementing treatment regimens involving these drugs, especially in patients who are immunocompromised. JCPyV is of significance since 80% of PML cases occur in the HIV population (10). In this study, we examined JCPyV viruria in panobinostat-treated HIV-positive individuals on uninterrupted cART.

We demonstrate that 4/15 (27%) of the panobinostat-treated patients were positive by urine at all time points tested. JCPyV viruria varies depending on location, population, immune status, and ethnicity and can be intermittent with periods of little to no excretion (15, 16). In healthy populations, it's estimated that approximately 15-30% of individuals shed JCPyV compared to approximately 20-45% of HIV-infected individuals (17, 18). Our results are consistent with this data. JCPyV is not detected in the CSF until the onset of PML (16) and JCPyV positive CSF is used to confirm a PML diagnosis (19). None of the patients treated with panobinostat developed PML, therefore we did not expect to detect any JCPyV in the CSF.

JCPyV positive urine samples NCCR sequence aligned with archetype JCPyV sequence, which was expected as archetype JCPyV is excreted in the urine and is latent in the kidneys, whereas rearranged JCPyV is detected in the brain or CSF (19, 20). However, we did not detect any changes in NCCR sequence between baseline and follow up. This could be due to the nature of double-stranded DNA viruses, which are more structurally stable biomolecules and utilize host DNA replication machinery that have a low error rate, causing DNA viruses to mutate much slower than their RNA counterparts. Alternatively, it could be relatively the short treatment cycle of panobinostat (8 weeks), as JCPyV is a lifelong infection that presumably takes many years and specific immune-conditions to accumulate pathogenic mutations. Studies performed in our laboratory with cells transformed with SV40 TAg to drive JCPyV replication did not induce

rearrangements in archetype JCPyV until day 645 after infection. The effect on JCPyV NCCR sequence in longer HDACi treatment cycles should be assessed for pathogenic mutations.

Lastly, we demonstrate an increase in JCPyV viruria during late panobinostat treatment (third treatment cycle) compared to corresponding baseline samples. This increase is significant as the current model of PML pathogenesis implicates increased viral replication in the absence of a sufficient immune response as the source for the emergence of pathogenic viral variants. Studies have shown that the rearranged viruses within a PML patient are derived from the archetype virus detected in the kidney of the same patient and that the rearranged virus exists as a population of quasispecies in the PML brain, with up to 20 subtypes in one individual (19). These support the hypothesis that the archetype virus undergoes rearrangements in the host, further highlighting the importance of host immunity in PML pathogenesis. More studies are needed to elucidate the effect of global LRAs on latent viruses to improve risk stratification for serious complications.

Conclusion

To our knowledge, this is the first study to examine archetype JCPyV viruria in HIV-positive individuals treated with an HDACi. We detected JCPyV in the urine of four out of fifteen (27%) patients, who shed JCPyV at all time points tested, however JCPyV was not detected in any CSF samples. JCPyV NCCR sequencing showed no difference between the initial and final urine samples and sequences aligned to the archetype JCPyV, as expected. We also demonstrated statistically significant increase in JCPyV genome copies in three out of four (75%) JCPyV PCR positive urine at late panobinostat treatment when compared to baseline measurements, and this increase was abrogated at follow up. This study emphasizes the need to study the effects of these

global HDACi on other latent viruses to improve risk stratification and minimize complications for LRA treatments.

Materials and Methods

Study design and participants

The phase 1/2 clinical trial was conducted at the Aarhus University Hospital in Denmark between October 1, 2012 and January 16, 2014 (CLEAR cohort clinical trial # NCT01680094). HIV-infected adults taking antiretroviral therapy with virological suppression (<50 copies per mL) for at least two years and CD4 counts above 500 cells per µL were enrolled. Exclusion criteria were co-infection with hepatitis B or C viruses, clinically significant cardiac disease including QTc prolongation, and current use of a protease inhibitor (due to drug interactions). Patients provided written and informed consent before any study procedures, and ethics committee approval was obtained. Patients received oral panobinostat (20 mg) three times per week (Mondays, Wednesdays, Fridays) every other week for 8 weeks while maintaining combination antiretroviral treatment. Patients had 13 follow up visits during treatment and samples were taken roughly eight hours after panobinostat dose. Randomized blinded patient urine and CSF samples were provided for this study.

PCR amplification and sequence analysis

JCPyV NCCR and VP1 genes were amplified using 2 µL of template DNA and primers JRR-25 and JRR-28 or JLP-15 and JLP-16 as described previously (21). PCR products were separated on a 2% agarose gel, visualized with ultraviolet light, NCCR product was purified by

QIAquick PCR purification column and sequenced for differences in NCCR. Sequence analysis was conducted using Clustal W program.

DNA extraction and quantitative analysis

Low molecular weight DNA was extracted from 1 mL of urine as previously described (21). Two µL of JCPyV DNA template, 10 pmol each of forward and reverse primers, and probe specific for JCPyV TAg and for VP1 genes in a final reaction volume of 20 µL were amplified as previously published by our group and normalized to GAPDH (22). qPCR was conducted using a Bio-Rad iCycler iQ™ Multicolor Real-Time PCR Detection System. Analysis was conducted via Bio-Rad iCycler iQ™ Multicolor Real-Time PCR Optical System Software Version 3.1. Statistical analysis was performed using GraphPad Prism version 7.04.

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Table 1. Participant characteristics. Randomized blinded patient samples baseline characteristics. Boxed individuals positive by PCR (1, 2).

	Sex	Ethnic origin	Age (years)	Months since HIV diagnosis	Days from HIV diagnosis to ART initiation	ART regimen	Months on ART	Months with HIV RNA <50 copies per mL	Nadir CD4 count (cells per µL)	Baseline CD4 count (cells per µL)	HLA class 1 A-alleles	HLA class 1 B-alleles	ATI?
1	Man	White	43	80·3	1167	TDF, FTC, RPV	41·4	35·7	390	710	01:01:01:01	08:01:57:01	Yes
2	Man	White	49	81·4	519	TDF, FTC, RPV	64·1	59·6	270	615	02:01:24:02	07:02:35:01	Yes
4	Man	White	50	33·4	1	TDF, FTC, RAL	33·3	27·0	210	955	01:01:02:01	08:01:15:01	Yes
5	Man	White	39	191·7	0	TDF, FTC, EFV	191·7	169·6	540	1615	01:01:68:01	08:01:35:01	No
6	Man	White	42	48·9	244	TDF, FTC, RPV	40·8	36·0	410	750	02:01:11:01	27:05:40:01	No
7	Man	White	41	175·6	4139	TDF, FTC, RAL	37·6	40·0	310	940	01:01:11:01	08:01:35:01	No
8	Man	White	51	165·6	1526	TDF, FTC, EFV	114·8	107·2	179	1525	01:01:68:01	08:01:40:01	Yes
9	Man	White	51	56·4	390	TDF, FTC, EFV	43·4	35·8	350	830	03:01:30:01	13:02:35:01	Yes
10	Man	White	47	340·0	6574	TDF, FTC, EFV	120·9	>24·0*	710	1220	01:01:11:01	08:01:27:05	Yes
12	Man	White	34	49·3	477	TDF, FTC, RPV	33·4	32·5	400	810	02:01:24:02	39:06:44:02	Yes
14	Man	White	49	38·9	5	TDF, FTC, EFV	38·8	31·9	130	935	03:01:03:01	07:02:44:02	No
15	Man	White	35	86·1	782	TDF, FTC, EFV	60·0	54·0	290	705	02:01:32:01	13:02:35:08	No
17	Man	White	49	280·8	3992	ABC, 3TC, AZT	147·7	146·8	623	1990	11:01:32:01	14:01:18:01	Yes
18	Man	White	28	48·5	540	TDF, FTC, EFV	30·5	26·3	370	1515	01:01:24:02	08:01:15:07	Yes
19	Man	White	53	267·4	5446	TDF, FTC, EFV	85·9	78·9	165	800	29:02:30:01	13:02:57:01	No
Median (range)	..	..	47 (28–53)	81·4 (33·4–340)	540 (0–6574)	..	43·4 (30·5–191·7)	38 (26·3–169·6)	350 (130–710)	935 (615–1990)	..	..	..

ART=antiretroviral therapy. TDF=tenofovir disoproxil fumarate. FTC=emtricitabine. RPV=rilpivirine. RAL=raltegravir. EFV=efavirenz. ABC=abacavir. 3TC=lamivudine. AZT=zidovudine. ATI=analytical treatment interruption. *Exact value not available, the patient had been away, returning to Norway in 2010.

Table 1: **Participant characteristics**

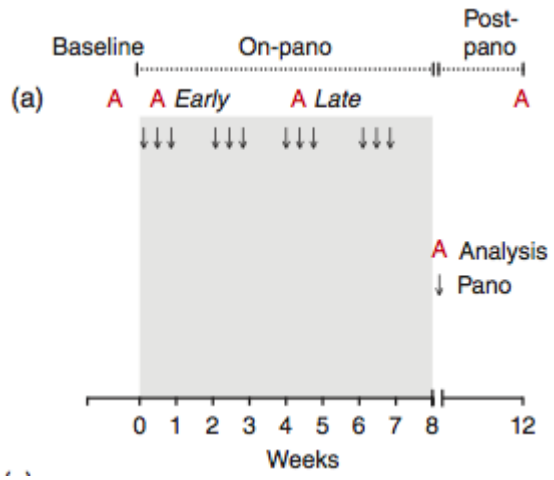

Figure 1. Panobinostat treatment schedule. Patients were treated with 20 mg oral panobinostat three times a week for eight weeks. *A* indicates points of analysis (28).

Table 2. Detection of JCPyV DNA in HIV positive cART adherent patients during panobinostat treatment

Patient ID	**Baseline**	**Early Panobinostat**	**Late Panobinostat**	**Follow up**
7	+	+	+	+
10	+	+	+	+
12	+	+	+	+
17	+	+	+	+
1	-	-	-	-
4	-	-	-	-
5	-	-	-	-
6	-	-	-	-
8	-	-	-	-
9	-	-	-	-
14	-	-	-	-
15	-	-	-	-
18	-	-	-	-
19	-	-	-	-

Percent Identity

Patient ID

	Mad-1	CY	7 B	7 F	10 B	10 F	12 B	12 F	17 B	17 F
Mad-1		88.94	91.86	91.86	92.21	92.21	92.21	92.21	91.9	91.9
CY			99.63	99.63	99.63	99.63	99.63	99.63	99.26	99.26
7 B				100	100	100	99.73	99.73	99.73	99.73
7 F					100	100	99.73	99.73	99.73	99.73
10 B						100	99.74	99.74	99.74	99.74
10 F							99.74	99.74	99.74	99.74
12 B								100	99.49	99.49
12 F									99.49	99.49
17 B										100
17 F										

Figure 2. JCPyV NCCR sequencing of JCPyV DNA in HIV positive cART adherent patients during panobinostat treatment. Amplification of the NCCR by PCR with JCPyV specific NCCR primers JRR-25 and JRR-28 and sequence analysis by Clustal W demonstrate percent identity of JCPyV positive patient IDs compared to archetype JCPyV (CY [GenBank #M35834] and Mad-1 JCPyV [GenBank #J02227].

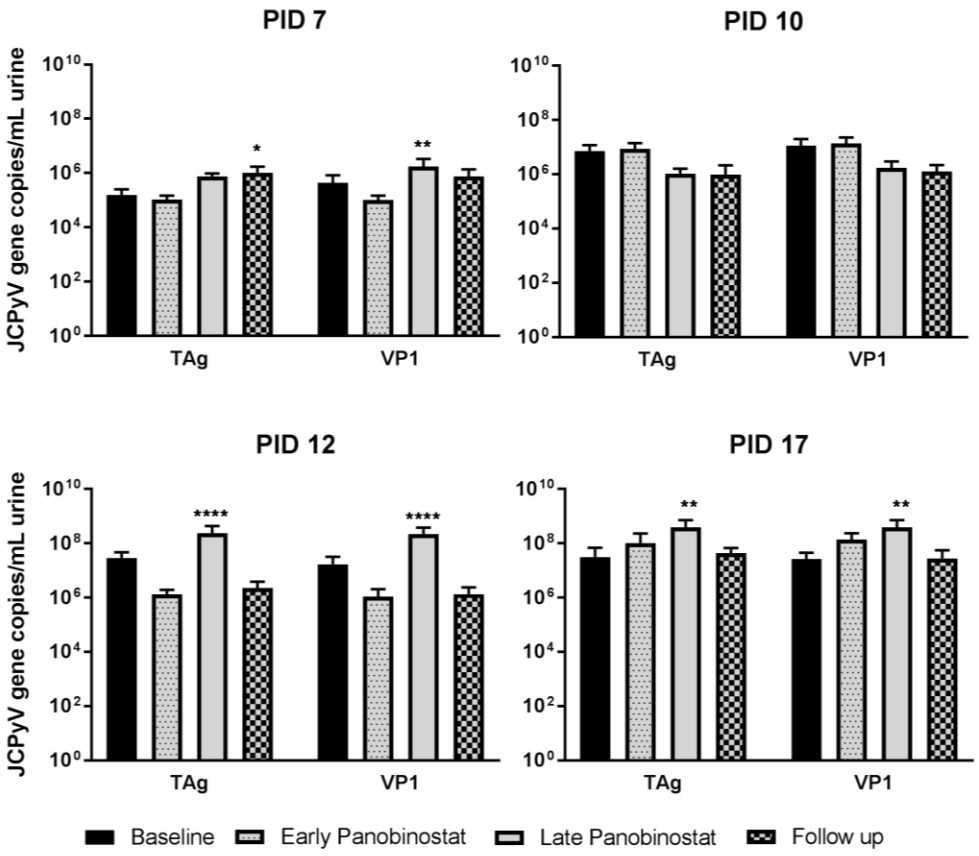

Figure 3. JCPyV TAg and VP1 gene copies in panobinostat treated HIV positive cART adherent patients. One mL of urine was pelleted, washed with PBS, and DNA was extracted. Viral TAg and VP1 gene copies were measured by qPCR and normalized to GAPDH. * $p<0.01$, **$p<0.001$, ****$p<0.0001$.

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Author Contributions

OSS provided the samples, MCF, LCN, and VRN designed the experiments. MF conducted the experiments. MF and VRN analyzed results and wrote the manuscript.

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