STUDIES OF TARGETED THERAPIES AGAINST HUMAN T LYMPHOTROPIC VIRUS TYPE-1 ADULT T-CELL LYMPHOMA IN PRECLINICAL ANIMAL MODELS

DISSERTATION

Presented in Partial Fulfillment of the Requirement for the Doctor of Philosophy Degree in the Graduate School of The Ohio State University

By

Bevin Zimmerman, B.A., D.V.M.

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The Ohio State University
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Dissertation Committee:

Dr. Michael D. Lairmore, Advisor

Dr. Stefan Niewiesk

Dr. Lawrence Mathes

Dr. Paul Stromberg

Approved by:

__________________________
Advisor

Veterinary Biosciences Graduate Program
ABSTRACT

Human T lymphotropic virus type 1 (HTLV-1) infects 20 million people worldwide. It is the causative agent of adult T-cell leukemia/lymphoma (ATL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). ATL is a refractory T-cell malignancy with a poor prognosis due to its highly aggressive nature and resistance of neoplastic cells to conventional chemotherapies. Herein, the impact of multiple novel therapeutics was investigated in vitro in multiple HTLV-1 cell lines, in an immunodeficient mouse model of ATL, and a rabbit model of early infection.

The proteasome inhibitor bortezomib (PS-341 or Velcade) and the heat shock protein inhibitor 17-(Allylamino)-17-demethoxygeldanamycin (17-AAG) exhibited efficacy against multiple cell lines in vitro. In Chapter 2, we document the efficacy of PS-341 and 17-AAG alone and in combination in a preclinical model of ATL. We demonstrate that the combination of PS-341 and 17-AAG synergistically promoted cell death in ATL cell lines and reduced tumor burden in mice during cycles of drug treatments. Positive effects of 17-AAG required continuous drug treatments suggesting requirements for treatment protocols in human subjects.
Histone deacetylase inhibitors (HDACi) have shown efficacy against a variety of cancers. In Chapter 3, we tested the histone deacetylase inhibitors valproic acid and OSU-HDAC42 in our ATL mouse model. Both compounds reduced proliferation of ATL cell lines by promoting apoptosis and histone hyperacetylation. To further test the efficacy of this approach we evaluated OSU-HDAC42 for survival in an ATL NOD/SCID mouse model. Our data provide new directions for the treatment of ATL and support the further development of this class of drug against HTLV-1-associated lymphoid malignancies.

In Chapter 4, we tested the effects of the histone deacetylase inhibitor, valproic acid (VPA), on HTLV-1 proviral load in the rabbit model of early infection. Our data demonstrated that VPA can be safely administered in the rabbit model and altered proviral copy numbers in infected rabbits when compared to controls. This model will be useful to test other HDACi for their effects on HTLV-1 gene expression.

In this thesis, we investigated targeted therapeutics in a preclinical, NOD/SCID ATL mouse model and in a rabbit model of HTLV-1 infection. Our data indicated new directions in the development of targeted therapies against ATL and provide new directions for the design of treatment protocols for future studies.
Dedicated to my parents Michael and Margaret Zimmerman and my husband, Raymond Kinney
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VITA

November 10, 1976……………… Born- Buffalo, New York

1997……………………………….. B.A. (Biology)

State University of New York at Buffalo

2003……………………………….. Doctor of Veterinary Medicine

North Carolina State University, Raleigh

2003- present…………………….. Veterinary Anatomic Pathology Resident

Graduate Research Associate

Department of Veterinary Biosciences

The Ohio State University, Columbus, OH

PUBLICATIONS


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CHAPTER 1

LITERATURE REVIEW

1.1 HTLV-1 Epidemiology and Disease Association

A clustering of T-cell leukemias and lymphomas in Japan and other parts of the world with strikingly similar characteristics such as neoplastic T-cells with multilobulated nuclei (also called “flower cells”) led to the discovery of the first retrovirally induced human neoplasia. Poiesz et al., detected retroviral particles and reverse transcriptase activity in samples from a patient with cutaneous T-cell lymphoma confirming the first human retrovirus, human T-lymphotropic virus type 1 (HTLV-1) (1). Similar retroviral particles were detected in Japanese patients with Adult T-cell Leukemia/Lymphoma (2).

Human T-lymphotropic virus type-1 (HTLV-1) is a member of the deltaretroviridae, a family of retroviruses which includes both simian T lymphotropic virus and bovine leukemia virus. Based on epidemiology studies it has been estimated that approximately 15 to 20 million HTLV-1 carriers exist throughout the world, with endemic foci in Japan, the Caribbean, and Africa (3). HTLV-1 is spread through contact with bodily fluids containing infected cells most often from mother to child through breast milk and
parenterally via blood transfusion. After prolonged latency periods (20-60 years), approximately 5% of HTLV-1 infected individuals will develop either adult T-cell leukemia/lymphoma (ATL), or other lymphocyte-mediated disorders such as HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). ATL is an aggressive T-cell malignancy with a poor prognosis and survival times typically less than one year (4). HAM/TSP is a chronic inflammatory disease that results in progressive neurologic dysfunction and damage to the thoracic spinal cord. The factors favoring the development of ATL or HAM/TSP are not completely understood, but have been linked to host (e.g., immune response) and virus factors (e.g., viral load). It has also been demonstrated that the route of exposure to the virus may determine the clinical outcome of HTLV-1 infection. Individuals subjected to mucosal exposure are more likely to develop ATL while blood borne exposure favors the development of HAM/TSP (5). In the following sections, HTLV-1 and its disease outcomes will be detailed in context to therapeutic interventions and animal models of ATL.

1.2 The HTLV-1 Genome

HTLV-1 is a complex retrovirus comprised of a diploid, single stranded, positive sense, nine kilobase, RNA genome. Cell infection begins with the viral protein envelope binding its cellular receptor. In some instances, glucose transporter-1 (GLUT-1) appears to act as the cellular receptor (6).
However, GLUT-1 is not necessary for infection of all cells as it is not present on some target cells (7). Surface heparin proteoglycan and neuropilin have been proposed as HTLV-1 receptors depending on the context of the infection (8-11). Regardless of the receptor used, the virion fuses with the cell membrane and releases the viral genome into the cytoplasm of the cell. The viral genome is then reverse transcribed by the viral RNA dependent DNA polymerase. The result is a double stranded DNA provirus that integrates randomly into the host cell genome using virally encoded integrase (IN).

During the integration process, both the 5' and 3' ends of the retrovirus are duplicated to form the 5' and 3' long terminal repeats (LTR). The 5' and 3' LTRs are identical, although the 5'LTR directs synthesis of the viral genome while the 3'LTR extends transcription into the adjacent host DNA. The LTR is comprised of U3, R, and U5 regions and acts as the viral promoter. In addition, the LTR is responsible for polyadenylation of mRNAs, initiation and termination of transcription and strand transfer. The U3 region of the LTR contains the Tax Response Element (TRE). TRE binds multiple transcription factors, including viral Tax and cellular cyclic adenosine monophosphate response element binding proteins (CREB), CREB-2, ATF-1 and ATF-2, c-Fos, c-Jun, p-300 and CBP binding protein (12).

The integrated provirus is transcribed into both unspliced and spliced mRNA using the host cell’s DNA polymerase II. The mRNAs are used as
both the genome of new virions and as templates for the translation of both structural and nonstructural proteins.

1.2.1 Structural Gene Products

The HTLV-1 genome encodes structural, regulatory, and nonstructural genes. The structural genes include gag, pol, and env typical of all retroviruses. The Gag protein is translated from the unspliced full length mRNA p55. It is post translationally modified by myristylation targeting Gag to the inner plasma membrane. Gag is cleaved into three structural proteins, matrix (MA or p19), capsid (CA or p24) and nucleocapsid (NC or p15), all are components of the viral core. The matrix protein is localized to the inner surface of the viral envelope and is critical to virion release and cell to cell transmission.

Derived from the full length mRNA through ribosomal shifting, the pol gene encodes the reverse transcriptase (RT), integrase (IN), protease (PR), and RNase H proteins (13). Reverse transcriptase is an RNA dependent DNA polymerase that transcribes the viral RNA into DNA that can be integrated into the host genome. Integrase serves to integrate the DNA provirus into the host cell chromosome. Protease is necessary for proteolytic cleavage of primary translation products. The RNase H removes the RNA strand from the DNA-RNA hybrid that is synthesized from viral RNA using the reverse transcriptase.
Envelope protein is first synthesized as a 68 kDa precursor protein from the singly spliced viral mRNA. It is then cleaved into surface unit (SU) and transmembrane (TM) subunit; SU noncovalently associates with TM, which anchors it to the cell surface. The envelope is necessary for attachment and viral entry.

1.2.2 Regulatory and Nonstructural Gene Products

The pX region of the viral genome, located 3' to env and 5' to the 3’-LTR is unique and contains genes coding for regulatory or accessory gene products, which are generated by alternate splicing of RNA transcripts. This region of the viral genome encodes the transactivating protein Tax and regulatory protein Rex, as well as p30, p12, p13 and the newly discovered antisense encoded HBZ (12).

Tax is a 353 amino acid, 40 kDa transactivator protein that acts a dimer to promote cell transformation in vitro (14-18) and in transgenic mice (19-21). It is also known to alter the expression of numerous cellular genes known to contribute to malignant transformation and progression (22). Tax regulates expression of cell cycle regulators, apoptosis regulating genes, DNA repair genes, and tumor suppressor genes (including p53). NF-kB is a well known transcription factor, induced by Tax, which is constitutively active in ATL cells (23). Alteration of this transcription factor leads to alterations in cell cycle, apoptosis, and cellular differentiation. Tax protein expression
levels in ATL are low or undetectable. It is thought that the virus plays a role in the early expansion of cells, but upon transformation, it is no longer required for leukemic cell growth. Tax is also a primary antigenic target of cytotoxic T-lymphocyte (CTL) responses against HTLV-1 (24). Rex is a 27 kDa phosphoprotein encoded by ORF III (25). It localizes to the nucleolus of infected cells and is essential for viral replication. Rex is necessary for the export of unspliced and singly spliced viral mRNAs from the nucleus to the cytoplasm (25).

The pX region also encodes for several other accessory or nonstructural gene products. Protein products p12, p30 and p13 are encoded from open reading frames I and II (26). These proteins have several roles including early viral spread and control of virus replication. Open reading frame II encodes for both p13 and p30. p13 is encoded by singly spliced RNA of ORF II and localized to the mitochondria, while p30 is encoded by a doubly spliced mRNA and localizes to the nucleolus (12;27). p30 is a nonstructural protein encoded by the open reading frame II (ORF II). It serves as a negative regulator of viral expression (28) and may play a role in the virus’ ability to evade the host immune response. Also of note, it acts as a transcriptional regulator and, more importantly, is necessary for maintenance of in vivo infection (29). Recently, we have shown that p30 alters cell cycle regulation (30) and may play a role in viral integration through interactions with ATM kinase and double stranded DNA breaks (Datta et al., submitted).
Open reading frame I encodes p12. p12 is a 12 kDa protein that does not appear necessary for replication in vitro (31). This viral protein accumulates primarily in the golgi and endoplasmic reticulum (32). By interacting with calcineurin, p12 can activates nuclear factor of activated T-cells (NFAT) (33). In addition, p12 may interfere with the assembly of major histocompatibility complex I (MHC-1) and its trafficking to the cell membrane (34).

HTLV-1 basic leucine zipper factor (HBZ) is a protein encoded by the antisense strand of HTLV-1 provirus. It is conserved in ATL cells in which 5’ deletions and mutations often affect the structural proteins and prevent production of infectious virions (35). In vitro, HBZ down regulates viral transcription by its interaction with CREB-2 (36). It has also been shown that HBZ is not necessary for cellular transformation in vitro, however, in the rabbit model, HBZ enhanced infectivity and increased viral persistence (37). If HBZ is abrogated using siRNA cells in culture have reduced proliferation and HTLV-1-transformed cells have significantly reduced tumor size when transplanted in mice (38).

1.3 HTLV-1 Associated Diseases

1.3.1 Adult T-cell Leukemia/Lymphoma

The acute form of ATL is an aggressive T-cell malignancy caused by HTLV-1. It is characterized by several distinct clinical parameters including a
leukemic phase of increased circulating CD4+ CD25+ T-cells (39). Patients with the acute form of ATL have a poor prognosis independent of therapy with mean survival time is less than one year (4). Adult T-cell leukemia/lymphoma was first described in 1977 (4;40). A geographic clustering of patients with particularly aggressive T-cell malignancies and several common characteristics led the authors to coin the term “adult T-cell leukemia” to describe the disease. Common characteristics of the original 16 patients described include adult onset disease, a chronic leukemia with a rapidly fatal progressive course, lymphadenopathy, hepatosplenomegaly, lobulated neoplastic T-cells, and geographic distribution (4;40). Cells from patients from these original cases were cocultured with human umbilical cord lymphocytes and cell lines MT-1, MT-2, and MT-4 were generated (41). These cell lines were instrumental in the discovery of HTLV-1 and are still used in HTLV-1 research.

The diagnostic criteria for ATL include the demonstration of a T-cell malignancy, HTLV-1 antibodies in the patient’s sera, and demonstration of monoclonal integration of HTLV-1 provirus in the leukemic cells by Southern blot assay (42). Since the original description of ATL, lymphoproliferative diseases associated with HTLV-1-infection has been classified into four clinical subtypes. The two most aggressive forms are the acute and lymphomatous forms (43). These forms are characterized by humoral hypercalcemia of malignancy (HHM) with subsequent bone lysis,
lymphadenopathy, visceral and skin involvement. The lymphomatous form is distinguished by its normal peripheral white cell count, while in the acute form, ATL cells are found in the peripheral blood. The smoldering and chronic forms are the more indolent clinical subtypes. Criteria for diagnosing smoldering ATL include a normal leukocyte count, lack of hypercalcemia, and skin or lung involvement without evidence of other visceral disease. In chronic ATL patients, their peripheral blood leukocyte count is typically elevated, they lack hypercalcemia, but may have histologic evidence of involvement of the liver, spleen or lungs (39;44).

The molecular pathogenesis from HTLV-1 infection to ATL development has not been fully elucidated. The randomly integrated provirus persists for many years and during this phase, infected subjects typically remain asymptomatic. Effective replication of the virus relies on reverse transcription and clonal expansion of infected cells (45). During the premalignant phase, there is a progression from polyclonal to monoclonal T-cells in circulation. Monoclonal T-cell expansion is believed to be promoted by an increasing ability of malignant T-cells to escape immune elimination and become less dependent on cell growth factors such as interleukin-2 (IL-2). Also during this phase, there is an accumulation of somatic mutations both within the provirus and within the flanking regions. Most often, the 5’ portions of the virus are deleted or mutated and functional infectious virions may not be produced (45). Tax, in the initial phases of transformation, acts
as a transcriptional transactivator and causes the simultaneous activation of multiple cellular pathways that disrupt the normal cell cycle and death pathways, leading to the transformation of the T-cells. Transformed T-cells eventually grow autonomously and the expression of Tax appears to be no longer required for maintenance of the malignancy.

1.3.2 Neurodegenerative and Other Diseases Associated with HTLV-1

The neurologic disease HAM/TSP is a degenerative neurologic condition first described by Gessain et al. (46) and Osame et al. (47). The disease is characterized by lymphocytic meningitis with demyelination and degeneration of the spinal cord. It most commonly occurs in the lumbar spine (48) in patients typically younger than those affected by ATL. Transfusion with HTLV-1 contaminated blood has been associated with a more rapid onset of development of HAM/TSP versus ATL (49;50). The pathogenesis of HAM/TSP is thought to involve both molecular mimicry and autoantigens (51;52). The autoantigen was identified from immunoglobulin G of HAM/TSP patients as the heterogeneous nuclear ribonucleoprotein-A1 (hnRNP-A1). This immunoglobulin also cross reacted with HTLV-1 Tax, indicating molecular mimicry (51). Cytotoxic T-cells (CTLs) directed against Tax are found in high levels in the blood and within the central nervous system (53). As the disease progresses, the composition of the inflammatory component of
the spinal cord lesions progresses from CD4+, CD8+ T-cells, B cells, and macrophages to a lesion comprised predominantly of CD8+ T-cells (54).

1.4 Animal Models of HTLV-1 Infection

Multiple animal models are available to study the infection and transmission of HTLV-1 (55). Rabbits (56;57), some nonhuman primates (58;59) and rats (60;61) can all be infected with HTLV-1 and have been utilized to monitor the virus spread, determine immune responses against the infection and in the development of vaccines against the viral infection.

1.4.1 The Rabbit Model of HTLV-1

Rabbits are the model used most often to study early infection events, viral transmission, and the host immune responses against the virus infection. Rabbits can be consistently infected; however, they remain, in the majority of cases, asymptomatic. The first reports of infectivity in rabbits were in the 1980s when MT-2 cells were inoculated into rabbits (56). The rabbit model was used to confirm the modes of transmission in humans, as well as early studies of the immune response against the infection or to develop vaccines against HTLV-1 (62-67). Early studies utilizing the rabbit model of HTLV-1 provided important data on the number of cells capable of transmitting the virus infection (64) and effective means to prevent the transmission of the virus (64;68-71).
Infection of rabbits with HTLV-1 parallels the asymptomatic infection of the virus in humans. Rabbits inoculated with HTLV-1-infected cell lines derived from patients with ATL or HAM/TSP demonstrate the heterogeneity in the biological response to HTLV-1 infection (57). Rabbits vaccinated with synthetic peptides verified immunodominant epitopes of the viral envelope protein (Env) (72;73) and defined regions of Env important for antibody dependent cell-mediated cytotoxicity (74). An infectious molecular clone of HTLV-1 was used to immortalize human peripheral blood mononuclear cells (PBMC) to create the ACH cell line, which was then used to infect rabbits (75;76). Subsequently, ACH clones with mutations within the open reading frames encoding the HTLV-1 accessory or regulatory proteins were inoculated into rabbits to demonstrate the necessity of these accessory proteins for establishment of infection and maintenance of proviral loads (29;37;77-79).

Sporadic reports of associated diseases in infected rabbits are reported, but are of limited value due to the inconsistent expression of disease in infected animals (80;81). An “ATL-like disease” following intraperitoneal or intravenous injection of HTLV-1 transformed cells has been reported, but this required a minimum of $1 \times 10^8$ cells in the inoculum, and death occurred within the first few weeks of inoculation (82-84). These studies failed to demonstrate if the leukemic cells were from the inoculum. Other case reports of clinical disease in HTLV-1 infected rabbits include
uveitis (80), cutaneous lymphoma (85;86), and thymoma (87). In each of these, clinical disease developed after one to several years following exposure to the virus limiting the use of rabbits as a disease model.

1.4.2 The Nonhuman Primate Model of HTLV-1

Cynomolgus macaques (Macaca fascicularis) and squirrel monkeys (Saimiri sciureus) can be experimentally infected with HTLV-1. The monkeys seroconvert, but typically do not demonstrate any clinical signs of disease (88-90). The squirrel monkey has been used to demonstrate that reservoirs of HTLV-1 include PBMCs, lymph nodes, and spleen (91). Generally, the nonhuman primates remain asymptomatic, however, there are two reports of malignant lymphoma in macaques that have HTLV-1 specific antibodies (92;93) and a single report of arthritis, uveitis and polymyositis (94).

Pig-tailed macaques (Macaca nemestrina) inoculated with a pig-tailed macaque cell line persistently infected with the ACH HTLV-1 molecular clone developed clinical disease including rash and lymphadenopathy, hypothermia, lethargy, lymphopenia, diarrhea, and arthropathy (95). The nonhuman primate is a useful model for vaccine studies. Gag and Env vaccines and naked DNA vaccines have all been tested in the nonhuman primate model (96;97).
1.4.3 The Rat Model of HTLV-1 Infection and Disease

The first reference to reproducible experimental infection of Rats with HTLV-1 was in 1991 (61). Rats have not been used exclusively, in part, due to considerable differences in the response of various rat strains to HTLV-1 infection (60;98;99). Wistar-King-Aptekman-Hokudai (WKAH) rats have been used as a model of HAM/TSP. This rat strain, when infected by HTLV-1, develops spastic paraparesis with degenerative thoracic spinal cord and peripheral nerve lesions several months following inoculation (98;99). The lesions (neurologic degeneration with a paucity of lymphocytes) are different than those observed in human HAM/TSP (100). The use of the immunodeficient rat has allowed for some studies of ATL (101). Adoptive transfer of T-cells and Tax-specific peptide vaccines have been used to study the role of immune responses against HTLV-1 infection in some rat models (102;103). A protective effect was achieved with each of these systems. Some protective effects have been reported against tumor formation in nude rats with Tax-specific small interfering RNAs (siRNA) (104).
1.4.4 The Mouse Model of HTLV-1 Infection and Disease

The need for useful animal models to study the histology, tumorigenesis and to test preclinical efficacy of potential therapeutic agents led to the development of several xenograft and transgenic mouse models. When compared to other available animal models, mouse models are small, comparatively inexpensive, and their genetics are easily manipulated. One caveat is that genetically normal, immunocompetent murine cells are not efficiently infected with HTLV-1 and do not develop a natural course of disease (105;106). The mouse model, while not an exact replica of the human course of disease, demonstrates multiple features in common with the human disease. Xenograft mouse models display multiple organ engraftment with ATL cell lines or patient samples (Table 1.1) (107-115). Large atypical lymphocytes are evident histologically in peritoneum, liver, spleen, and multiple organ types depending on the inoculum and the cell type. Xenograft mouse models can also display biochemical characteristics similar to ATL patients. These include PTHrP expression and increasing levels of serum IL-2Rα and β-2 microglobulin that correspond to increasing tumor burden. Transgenic mouse lines helped establish Tax as an oncoprotein and provided new information about the pathogenesis of ATL (116).
1.4.4.1 Xenograft Mouse Models of ATL

Transplantation of human cancer cells into immunodeficient mice has been in practice since the late 1960’s (117). Use of the xenograft provides the ability to examine human cells in a more physiological relevant context than \textit{in vitro} systems allow.

The SCID mouse, developed in 1983 (118), is commonly used as a model. This mouse contains a spontaneous nonsense mutation in the gene for the protein kinase DNA activated catalytic polypeptide (Pkrdc) on chromosome 16. The Pkrdc enzyme is necessary for the efficient recombination of the B and T-cell receptors. Without this enzyme, no mature B and T-cells develop. The SCID mouse retains normal macrophage, antigen presenting cell, and natural killer cell function. SCID mice are used extensively in human stem cell and tumor cell engraftment studies. Engraftment of ATL cells and cell lines within the SCID mouse has been shown to be cell line dependent (Table 1.1) (107;109). Cells that engraft and are recovered, maintain the genotype and phenotype of the original inoculate (107-109). Studies in the SCID mouse also demonstrated that the intact HTLV-1 provirus does not appear necessary for neoplastic cell growth (119). RV-ATL cells, an HTLV-1 positive leukemic cell line derived from a patient
sample, were reported to establish tumors readily and this cell line was propagated through mice (111). Whole body irradiation or administration of antibodies to abrogate natural killer cell function proved necessary to establish engraftment of non leukemic cells lines such as SLB-1 cells (111;120;121). MT-2 cells developed tumors at the site of injection in SCID mice treated with anti-asialo GM-1 antibody (122). Utilizing the knowledge of the immune functions of the SCID mouse in combination with the engraftment of various types of cell lines, Stewart et al., (123) demonstrated that the natural killer cells of the SCID mouse mediate the lysis of HTLV-1 expressing cell lines suggesting that the absence of HTLV-1 expression in the ATL lines allows these cells to evade immune surveillance.

The SCID/beige mouse is a double mutant mouse in which the SCID mutation is retained, but these mice have an additional beige/beige mutation in the lysosomal trafficking gene found on chromosome 13. This results in defective in B and T-cell function with lack of natural killer cell activity and defective granulocyte function. The RV-ATL cell line was reported to engraft in 75% of the SCID/beige mice while transformed cells (HT-1-RV, SLB-1, MT-2, ACH, and ACH.p12) were unable to establish engraftment (111).

SCID mice used in xenograft studies have been found to display “leakiness.” Leakiness allows for spontaneous rearrangement of antigen receptors and development of functional lymphocytes in aged mice (124). To combat the leakiness, the SCID mouse was crossed onto the NOD/Lt
background. NOD (non obese diabetic) mice are a model used to study the development of autoimmune mediated insulin dependent diabetes mellitus. The resultant NOD/SCID mice lack functional B and T-cells, have low natural killer cell activity, absence of complement activity (due to a lack of complement component 5 from the NOD/Lt background), and impaired macrophage and antigen presenting cell function. When compared to the SCID mouse and the SCID/bg mouse, the NOD/SCID mouse was reported to be more susceptible to engraftment with the HTLV-1 transformed cell lines (111). Sublethal whole body irradiation of the NOD/SCID mice one day prior to inoculation improved engraftment and tumorigenesis, as well as decreased time to clinical signs. Tumor engraftment was described as a lymphoblastic lymphoma with tumor development in the peritoneal cavity, spleen, and mesenteric lymph nodes. Lymphoblasts had large irregular nuclei and large prominent nucleoli. Abnormal mitotic figures were also observed. Tumor cells invaded and displaced multiple abdominal organs (111).

Further immunodeficiency was produced with development of the NOD/SCID mouse containing a targeted mutation in the Beta-2 microglobulin gene. Beta-2 microglobulin is a protein necessary for the presentation of antigens via major histocompatibility class (MHC) I. These mice lack all the immune functions that their less immunodeficient NOD/SCID predecessors lack with the addition of complete abolition of NK cell. ATL cells derived directly from patients were able to engraft in these mice (113). The
percentage of mice with tumor engraftment improves and time to clinical signs decreases in mice with this modification when compared to NOD/SCID mice.

NOD/SCID gamma c null (NOG) mice are homozygous for the SCID mutation and a targeted disruption of the interleukin (IL)-2Rγ gene (125). The gamma chain is common to the receptors for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. NOG mice are easily transplanted with human cells that would not normally transplant with the same efficiency in the more immunocompetent mouse models (126). NOGs lack B and T-cell development as well as NK cell function. NOG mice were found to have a severe reduction in IFN-γ production from dendritic cells (126). Mice with this combined immunodeficiency can be used to transplant cells directly from ATL patients (127). These NOG mice can also be used in the proliferation of PBMCs from asymptomatic carriers (128).

The degree of engraftment of ATL cells and their relationship to gene expression has been studied in the NOG mouse. Tumor suppressor lung cancer 1 (TSLC1) is aberrantly expressed in acute ATL cells and some cell lines. The mice inoculated with TSLC1 expressing ED-40515 cells formed larger tumors than their non TSLC1 expressing counterparts (115). The immunodeficient mouse is also becoming a model of early infection in which PBMCs and lethally irradiated MT-2 cells are inoculated in the NOG mouse and infection of the human PBMCs ensues (114).
Inoculation of MET-1 cells into NOD/SCID mice provide a model system for slowly developing T-cell leukemia with multiple organ involvement (129). In a comparative study by our group, leukemic mice had an increase in serum calcium levels that correlated with expression of receptor activator of nuclear factor κB (RANK) ligand on leukemic cells, and secretion of PTHrP and IL-6 (Parrula et al., epub ahead of print 2009 May 9). MET-1 cells expressed both the adhesion molecules CD11a (LFA-1α) and CD49d (VLA-4α) and produced or induced expression of matrix metalloproteinases 1, 2, 3, and 9.

In addition to investigating the early events, transformation, and tumor behavior; paraneoplastic syndromes are an important component of disease that can be addressed using mouse models. Humoral hypercalcemia of malignancy (HHM) is an important paraneoplastic syndrome that occurs frequently in ATL. It has clinical implications with regard to organ damage and chemotherapy. Mouse models of ATL that demonstrate HHM include the SCID mouse injected with human lymph node cells (130) and SCID/bg mice injected with RV-ATL cells (131).

1.4.4.2 Transgenic Mouse Models of HTLV-1

Tax, the 40 kDa transactivating protein of HTLV-1, has been shown to alter gene function. Tax has been shown to promote cell transformation in vitro (14-18). Tax has a multitude of cellular targets, the most important of
which are NF-κB and serum response factor. Through these signaling pathways, Tax can cause alteration of expression in genes involved in apoptosis, cell cycle, proliferation, and DNA repair. Tax in transgenic models is sufficient to cause oncogenesis, however, leukemia and lymphoma are rare. Serendipitously, the mouse models often recapitulate other chronic conditions associated with HTLV-1.

Transgenic mice expressing the tax gene under the control of the LTR promoter developed multicentric mesenchymal tumors of the nose, ear, mouth, tail, and foot (Table 1.2) (20). These tumors were characterized by a typical spindle cell component with infiltration of granulocytes. While this transgenic system is not appropriate for the study of ATL, it proved that the tax gene is, in fact, an oncogene. The close association of the mesenchymal tumors with peripheral and cranial nerves lead to their identification as neurofibromas and it was concluded that this model may be useful in the study of neurofibromatosis, an inherited disorder of humans (19). In addition to the mesenchymal tumors described above, these transgenic mice developed a wasting disease characterized by degeneration of oxidative muscle fibers. As such, this transgenic mouse may also serve to help understand the aspects of HTLV-1 associated myopathies (132). LTR-tax mice showed both gross and microscopic evidence of skeletal abnormalities. Bones were grossly thicker and more fragile, while histologically they exhibited high bone turnover characterized by increases in osteoclasts and
osteoblasts. These lesions were without evidence of PTHrP expression (133). The LTR-tax mice also develop a salivary and lacrimal exocrinopathy with lesions similar to Sjögren syndrome of people (134). Lastly, tax transgenic mice under the LTR promoter were shown to have ankylosing of multiple joints (135) and have potential benefits as models of other human diseases such as rheumatoid arthritis (136). Joint lesions similar to those observed in the LTR-tax mice were observed in mice constructed with tax under the control of the CD4 enhancer/promoter (135).

Combining the LTR-tax mice with LTR-βgal (β galactosidase) mice generated a bitransgenic mouse in which the transactivator protein acts on the LTR to increase expression of βgal. The enzyme was detected in bone, muscle, cartilage, exocrine glands, and mesenchymal tumors (137). This further supported the notion that the tax transgene was active in multiple tissues that developed lesions in this mouse model.

Leukemia and lymphoma have been reported in tax transgenic mice in which the transgene expression was restricted to the thymus by the Lck promoter (138). This thymus derived, pre-T-cell tumor was associated with constitutive NF-κB activation. This model verified that Tax expression alone is sufficient to cause leukemia and lymphoma.

The development of the tax transgenic mouse under the control of the granzyme B promoter restricted expression of Tax to CD4+, CD8+, Natural Killer cells, and lymphokine-activated killer cells. These mice developed a
large granular lymphocytic leukemia characterized by splenomegaly, lymphadenopathy, and masses on the ears, legs, and tail (21). Similar to ATL, these mice had humoral hypercalcemia of malignancy and osteolytic bone lesions associated with metastasis (139). When these mice are crossed with an interferon gamma knock out strain, there is enhanced rate of tumor development (140).

Transgenic mice using a tetracycline (Tet)-off system controlling wild type Tax, a Tax mutant incapable of activating NF-κB, Tax incapable of activating NF-κB or CREB were generated and crossed with EμSRα tTA mice to drive Tax expression specifically in the lymphocyte compartment (141). The Tax transgenic mice in which the ability to activate NF-κB was maintained developed a fatal dermatologic disease characterized by infiltration of Tax positive T-cells into the dermis and epidermis. Addition of doxycycline (suppression of Tax expression) resulted in the resolution of lesions. Although suggestive of cutaneous lymphoma observed in some ATL patients, the infiltrates in the skin were oligoclonal or polyclonal not monoclonal as in ATL (141).

Several transgenic mice strains were generated with Tax under the regulatory control of CD-3ε promoter enhancer sequence. These mice developed mesenchymal tumors at wound sites and mammary and salivary adenomas (142).
1.5 Treatment of Adult T-cell Leukemia

The strength of the mouse model lies in the ability to use it to test the preclinical efficacy of therapeutics. This tool provides insight to clinicians before the development of phase I clinical trials. Xenograft mouse models have been used to test conventional and targeted therapeutics as well as monoclonal antibodies (Table 1.3).

The finding that NF-κB is constitutively active in ATL led to several models to investigate the outcome of therapy that utilizes the blockade of NF-κB through multiple different mechanisms. These have proven especially promising in reducing tumor size of subcutaneous xenografts. In vivo preclinical efficacy has been studied with Arsenic trioxide (143), proteasome inhibitors (144), all trans retinoic acid (ATRA) (145), curcumin (146), and ritonovir (147).

The proteasome inhibitor, PS-341, inhibits the degradation of the inhibitor of NF-κB, IκBα. It was shown to inhibit NF-κB activation in ATL cells and Tax transgenic tumor cells in culture and in mouse transplantation studies. In several of these studies the majority of the cell death was found to be due to apoptosis. An inhibitor NF-κB DNA binding activity, BAY 11-7082, was also
shown to block NF-kB activity and resulted in tumor regression in ATL transplanted NOG mice.

The expression of markers on the cell surface of ATL cells implanted in mice has made them an excellent target for preclinical trials with monoclonal antibodies directed against IL-2Rα (110;148), CD52 (149;150), and CD2 (150). The outcome of the mouse studies may be predictive of successful therapy for human patients. A complete response has been reported in a human with alemtuzumab (anti-CD52) (151).

Treatment of ATL-bearing mice with a humanized anti-CD2 monoclonal antibody led to tumor regression (150). The activity of the monoclonal antibody was most likely due to antibody-dependent cellular cytotoxicity, since expression of $Fc_\gamma$ receptors on neutrophils and monocytes was required for activity. Treatment of ATL bearing NOD-SCID mice with an alpha-emitting radionuclide, bismuth 213, conjugated to an antibody to the IL-2 receptor proved to be highly effective in inducing tumor regression (152). The activity of bismuth 213 in this model was greater than that of unconjugate antibody or radionuclide, or antibody conjugate to $\beta$-emitting radionuclide yttrium 90.

Flavopiridol, an inhibitor of cyclin-dependent kinases, was tested for its therapeutic efficacy alone and in combination with humanized anti-Tac antibody (HAT), which recognizes CD25, in a murine model of human ATL using MET-1 leukemic cells (148). Either flavopiridol, given 2.5 mg/kg body weight daily for 5 days, or HAT, given 100 $\mu$g weekly for 4 weeks, inhibited
tumor growth and prolonged survival of the leukemia-bearing mice (148). Collectively these studies provide hope that ATL transplant and Tax transgenic models will provide new directions in the development of effective therapies against HTLV-1-induced lymphoproliferative diseases including ATL.

1.5.1 Proteasome Inhibitors

The 26S proteasome is a protease complex comprised of a 20S catalytic subunit and multiple regulatory subunits. It functions in the nonlysosomal breakdown of ubiquitinated proteins in an ATP dependent manner. The catalytic domains, contained within the 20S subunit include six active sites of tryptic, chymotryptic and caspase activity. At each end of the proteasome, there is a 19S cap which functions in removal of ubiquitin and unwinding of the target protein (153). Proteins targeted to the proteasome are numerous and include Bcl-2 family members, cell cycle proteins, and IκBα, to name a few.

Proteasome inhibition results in multiple effects that result in cancer cell death. These pleiotrophic effects are reviewed in Nencioni et al. (154). These effects include NF-κB inhibition, accumulation of p53, antiangiogenesis, effects on cdc25, a shift to proapoptotic Bcl-2 family members, JNK stabilization, and deregulation of cyclins, as well as increased reactive oxygen species.
1.5.1.1 Bortezomib (PS-341)

Bortezomib (PS-341 or Velcade) is a 26S proteasome inhibitor that inhibits the rate limiting chymotryptic activity of the proteasome (154). Inhibition of the proteasome confounds cellular signaling by the accumulation of normally short lived proteins within the cell. These activities result in increased cell death and decrease in tumor burden in multiple tumor types including multiple myeloma (155;156) and mantle cell lymphoma (157). In addition, the cellular transcription factor NF-κB, which is constitutively active in ATL cells (23) is blocked through inhibition of the proteasome. HTLV-1 infected cells exhibit decreased cell viability, and increased apoptosis in the presence of PS-341 (158). Tan et al., (129) reported an increase in phosphorylated IκBα and ubiquitination when HTLV-1 cells were treated with PS-341. Growth inhibition, increased apoptosis measured using Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and evidence of Poly ADP ribose polymerase (PARP) cleavage, G2/M cell cycle arrest, and accumulation of p21 demonstrated were shown by Nasr et al. (159) in several HTLV-1 cell lines. In vivo, in xenograft mouse models, administration of PS-341 resulted in increased apoptosis in tumor cells (160). In a separate study, PS-341 decreased tumor burden in ATL xenograft mice (158). However, PS-
341 was not found to be beneficial alone but enhanced efficacy was noted in combination with humanized anti-Tac (CD25 α chain) monoclonal antibody (129).

1.5.2 Heat Shock Protein Inhibitors

Heat shock protein 90 (Hsp 90) is one of the most abundant proteins in cells. It functions as an ATP dependent chaperone that assists in the folding of cellular proteins. Hsp90 exists in two isoforms (Hsp90α and Hsp90β) (161). The client proteins of Hsp90 are numerous and are summarized in Zhang et al., (162). Targets of particular interest in cancer therapy include Bcr-Abl, Akt, and Her-2/neu. Hsp90 is upregulated in ATL cells (163) and is important to cancer cells because it also assists with the folding of mutated proteins. Hsp90 is upregulated in response to chemotherapeutics to help the cell overcome/ survive therapy. The addition of heat shock protein inhibitors overcomes resistance to other chemotherapeutic agents (164).

Inhibitors of heat shock proteins result in the accumulation of misfolded or normally short lived proteins within the cell. The accumulated proteins may trigger apoptosis or the proteins are tagged with ubiquitin and directed for degradation via the ubiquitin-proteasome pathway.
1.5.2.1 17-(Allylamino)-17-demethoxygeldanamycin (17-AAG)

17-(Allylamino)-17-demethoxygeldanamycin (17-AAG), a derivative of the benzoquinone ansamycin geldanamycin, is a heat shock protein 90 inhibitor. 17-AAG binds to the ATP binding pocket at the amino terminus of Hsp 90 (165). Disruption of the heat shock protein-client complex results in ubiquitination of the client protein with direction to the proteasome for degradation. Hsp 90 inhibition affects multiple signaling pathways, including Akt and chimeric oncoproteins like Bcr/Abl and Her2Neu. It has been shown that 17-AAG will sensitize cells to other therapies (166). Hsp90 is often upregulated in malignancies, including ATL (163) and this makes 17-AAG an attractive mechanism of pharmacologic disruption.

Administration of 17-AAG alone in a multiple myeloma model has shown a decrease in tumor burden (166). Incubation of ATL cell lines and PBMCs isolated from patient samples with 17-AAG resulted in a decrease in cell survival and increase in apoptosis through the NF-kB, AP-1, and Akt signaling pathways (163). 17-AAG is currently in clinical trials alone or in combination in advanced solid malignancies (167). In ATL cell lines, administration of 17-AAG resulted in inhibition of growth and increased
apoptosis as well as decreases in survivin, cdk6, cdk 4, cyclin D1, Akt and phosphorylated Akt (163).

1.5.3 Histone Deacetylase Inhibitors

Transcriptional regulation is controlled in part by the acetylation of histones and non histone proteins. The acetylation of lysine residues on histones was first discovered in 1968 (168) but was not linked to control of DNA transcription until the mid 1990s (169). Acetylation of histones results in the relaxation of chromatin and the promotion of transcription.

Histone deacetylases (HDACs) are enzymes that promote the removal of the acetyl groups from the lysines. This results in the restoration of positive charge, tightening of DNA (negatively charged) around the histone core, and decrease in transcription of affected genes. Currently, there are at least 18 HDACs that are classified into six different groups. These HDACs control the activation and repression of proteins and may affect non histone proteins as well. Non histone proteins affected by HDACs include hormone receptors, chaperones, viral proteins, and cytoskeletal proteins.

Inhibitors of the HDAC enzymes (HDACi) and their role in cancer therapy have been recently reviewed in multiple journals (170-173). HDACi promote the acetylated state of the histone and relax chromatin structure. HDACi’s are divided into several classes including short chain fatty acids,
hydroxamic acids, benzamides, and cyclic peptides. The classes differ in their potency, but are generally not specific for HDAC isoenzymes (172).

Recently, HDACi have been used as targeted therapies in cancer research. As reviewed in Xu et al., (171) HDACi have been shown to induce apoptosis, induce cell cycle arrest, disrupt Hsp90 and the aggresome, inhibit angiogenesis, and induce mitotic cell death, including autophagic cell death and senescence. It is through these pleiotropic effects that HDACi have shown efficacy in vitro against prostate cancer (174;175).

In addition, the HDACi suberoylanilide hydroxamic acid (SAHA or Vorinostat) is approved for the treatment of cutaneous T-cell lymphoma. Depsipeptide (FR901228) has been used in the treatment of peripheral and cutaneous T-Cell lymphoma (176). Several other first generation HDACi are currently in phase I and phase II clinical trials (177).

The role of the HDACi in viral gene expression is slowly being unraveled. Trichostatin A (TSA) has been shown to increase bovine leukemia virus (BLV) LTR expression in vitro. In sheep and cattle peripheral blood mononuclear cells (PBMCs), BLV viral gene expression was increased in response to TSA (178).

1.5.3.1 Valproic Acid

Valproic acid, an eight carbon branched chain fatty acid, has been used in the treatment of epilepsy since 1978 (179). Recently, it has been
shown to have efficacy in the treatment of cancer through blocking the hyperacetylation of histones 3 and 4 (180). Valproic acid alone has been shown to inhibit prostate cancer cell growth (181), medulloblastoma (182), and multiple myeloma (183). Valproic acid has been shown to enhance the sensitivity of cells to chemotherapeutic agents (184;185).

In addition to the potential effects on neoplasia (ATL in the subsequent chapters), valproic acid has effects on the expression of viral genes. Epstein Barr Virus (EBV), a human herpesvirus, is often latent in the majority of people infected. When exposed to valproic acid, EBV gene expression was increased as was sensitivity of lytic gene expressing cells to chemotherapy (184). In the deltaretroviruses, particularly BLV, valproic acid treatment of isolated sheep PBMCs resulted in increased levels of BLV gene expression and a decrease in leukocyte numbers in leukemic sheep (186).

1.5.3.2 OSU-HDAC42

OSU-HDAC42 is a phenylbutyrate derived HDACi that has been included in the Rapid Access to Intervention Development Program through the National Cancer Institute (175). The modifications to the phenylbutyrate improved the potency over the original compound from the nanomolar range to the submicromolar range (187). There are multiple non-histone related affects of OSU-HDAC42. These include Akt dephosphorylation and disrupting protein phosphatase 1 (188).
In a prostate cancer model, OSU-HDAC42 was shown to decrease cell viability, decreased phospho-Akt, Bcl-xL, and Survivin (174). In vivo, a xenograft mouse model had decreased tumor burden (174) and decreased tumor levels of phospho-Akt and Bcl-xL in response to the drug (175).

1.6 Summary

Despite repeated attempts to develop effective therapies against ATL, the prognosis for the disease remains poor. Continued progress in understanding the multiple genetic and cellular aberrations that occur in the progression of HTLV-1 to ATL will provide many new therapeutic targets to explore. Translating knowledge from the laboratory bench to appropriate animal models and subsequently to cancer patients provides hope. The thesis chapters herein document a series of studies using reproducible animal models to test the efficacy of novel treatments against ATL. Our data provides new directions in the design of targeted therapy against this lethal viral-induced cancer.
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Figure 1.1 The HTLV-1 genome including all transcripts and their protein products. HTLV-1 full length provirus followed by all viral transcripts and their corresponding proteins. This includes the HBZ protein which is encoded by the antisense RNA. Open reading frames are indicated by roman numerals and colored boxes indicate the protein coding region.
Table 1.1 Engraftment of ATL cell lines in immunodeficient mice
Engraftment is defined as having evidence of growth within the mouse line, using either serum biomarkers or histopathology.

<table>
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<th>Mouse strain</th>
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**Table 1.2 Transgenic mouse models of adult T-cell leukemia/lymphoma**
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**Table 1.3 Preclinical efficacy studies utilizing mouse models of ATL**

Endpoints for studies included tumor burden (measured histologically, volumetrically or biochemically) or survival.
CHAPTER 2

COMBINATION THERAPY OF BORTEZOMIB (PS-341) AND 17-(ALLYLAMINO)-17-DEMETHOXYGELDANAMYCIN (17-AAG) DECREASES TUMOR BURDEN IN A MOUSE MODEL OF ADULT T-CELL LEUKEMIA/LYMPHOMA

2.1 ABSTRACT

The proteasome inhibitor bortezomib (PS-341 or Velcade) and the heat shock protein inhibitor 17-(Allylamino)-17-demethoxygeldanamycin (17-AAG) alone and in combination exhibit efficacy against multiple tumor types in vitro. In this study, we document the efficacy of PS-341 and 17-AAG alone and in combination in a preclinical model of adult T-cell leukemia/lymphoma (ATL). ATL is a refractory CD4+/CD25+ T-cell malignancy with multiple clinical forms caused by the retrovirus human T-lymphotrophic virus type 1 (HTLV-1). ATL patients have a poor prognosis due to the highly aggressive nature of the cancer and the resistance of neoplastic cells to conventional chemotherapies. Consistent cellular alterations within these cells, such as NF-κB activation, make targeted therapeutics a viable alternative to conventional chemotherapeutic protocols. We utilized a NOD/SCID ATL mouse model to test multiple dose regimens of PS-341 and 17-AAG alone and in combination.
Tumor burden was quantified by measuring serum IL-2Rα as a biomarker and by quantifying and grading tumor engraftment. Our data indicate that the combination of PS-341 and 17-AAG synergistically promoted cell death in ATL cell lines in vitro and reduced tumor burden during cycles of drug treatments. Upon removal of drug therapy, the differences observed between the treatment groups were minimized. Using this model, PS-341 and 17-AAG targeted therapy offers promise to decrease tumor burden, but our data indicates that the additive effects of 17-AAG are transient and require continued presence of the drug.

2.2 INTRODUCTION

Adult T-cell leukemia/lymphoma (ATL) is a refractory CD4/CD25+ CD8- T-cell malignancy caused by human T-lymphotrophic virus type 1 (HTLV-1) (1). Persistent HTLV-1 infections are established following transmission from infected mothers to their children, through breast feeding, and horizontally, through transfer of contaminated whole blood products. In approximately 1 to 5% of persistently infected individuals, clinical disease may manifest as ATL or other lymphocyte mediated disorders including the neurologic disease tropical spastic paraparesis (HAM/TSP) (2).

The mechanism of carcinogenesis induced by HTLV-1 infection is not completely understood, but it is thought to occur following viral induced cell dysregulation and accumulation of other transformation events. ATL is
classified into four primary clinical subtypes. The two most aggressive forms are the acute and lymphomatous (3). These forms are associated with humoral hypercalcemia of malignancy (HHM), bone lysis, lymphadenopathy, visceral and skin involvement. The lymphomatous form typically is associated with a normal peripheral blood leukocyte cell count, whereas in the acute form, ATL cells are found in high numbers in the peripheral blood. The more indolent clinical subtypes are the smoldering and chronic forms.

HTLV-1 is a complex retrovirus that encodes viral structural and enzymatic proteins from the gag, pol, and env genes as well as a pX region that encodes four open reading frames (ORFs) immediately prior to the 3’ long terminal repeat (LTR) (4). The most well characterized product of these ORFs is the transactivator oncoprotein Tax. Tax is a 40 kDa protein that acts as a transcriptional transactivator to increase viral transcripts. Tax also acts to upregulate several cellular gene products that result in dysregulation of cell cycle and promotion of cell survival (3). These alterations include upregulation of NF-κB through several mechanisms, including the phosphorylation of IKKγ or IKKα and IKKβ, ultimately leading to an increase in the phosphorylation of IκBα. Phosphorylation of IκBα targets it for ubiquitination and degradation via the proteasome. Degradation of IκBα exposes NF-κB for translocation to the nucleus where it acts to upregulate transcription of several prosurvival genes. Tax can also act through the
noncanonical pathway by binding to the p50 subunit of NF-κB and by enhancing the phosphorylation of protein kinase B (Akt).

Conventional chemotherapies against ATL have produced inconsistent or limited results in treatment outcome and prolongation of mean survival times (1). A combination of Cyclophosphamide, Adriamycin, vincristine, and prednisolone is typically the first line of therapy, but is of limited efficacy. The use of nucleoside analogs, interferon, zidovudine, topoisomerase inhibitors, and monoclonal antibodies has produced more encouraging results; however the prognosis for ATL patients remains poor. Bone marrow transplant has been reported to “cure” ATL (5). This is complicated by patient age, condition, and likelihood of immunologic match and therefore is of limited use.

Bortezomib (PS-341 or Velcade) is a 26S proteasome inhibitor that inhibits the rate limiting chymotryptic activity of the proteasome. Inhibition of the proteasome confounds cellular signaling by the accumulation of normally short lived proteins within the cell. These activities result in increased cell death and decrease in tumor burden in multiple tumor types including multiple myeloma (6;7) and mantle cell lymphoma (8). In addition, inhibition of the proteasome blocks the function of the cellular transcription factor NF-κB, which is constitutively active in ATL cells (9;10). HTLV-1 infected cells in culture have decreased cell viability, and increased apoptosis in the presence of PS-341 (11). Tan et al., (12) reported an increase in phosphorylated IκBα and ubiquitination when HTLV-1 cells were treated with PS-341. In vivo, in a
Tax transgenic mouse model, administration of PS-341 resulted in increased apoptosis in tumor cells (13). In a separate study, PS-341 decreased tumor burden in ATL xenograft mice (14). However, PS-341 exhibited enhanced efficacy in combination with a humanized anti Tac (HAT) monoclonal antibody (12).

A derivative of the benzoquinone ansamycin geldanamycin (17-AAG) is a heat shock protein 90 inhibitor. 17-AAG binds to the ATP binding pocket at the amino terminus of heat shock protein (Hsp) 90 (15). Disruption of the heat shock protein-client complex results in ubiquitination of the client protein with direction to the proteasome for degradation. Heat shock protein 90 inhibition affects multiple signaling pathways, including Akt and chimeric oncoproteins such as Bcr/Abl and Her2Neu and sensitizes cells to other therapies (16). In addition, Hsp90 is often over expressed in malignancies, including ATL (17) and this makes 17-AAG an attractive mechanism of pharmacologic disruption.

Administration of 17-AAG alone in a multiple myeloma model has shown a decrease in tumor burden (16). Incubation of ATL cell lines and peripheral blood derived mononuclear cells (PBMC) isolated from ATL patient samples with 17-AAG resulted in a decrease in cell survival and increase in apoptosis through the NF-kB, AP-1, and Akt signaling pathways (17). 17-AAG is currently in clinical trials alone or in combination in advanced solid malignancies (18;19). In ATL cell lines, administration of 17-AAG resulted in
inhibition of growth, increased apoptosis and decreases amounts of Survivin, cdk6, cdk 4, cyclin D1, Akt and phosphorylated Akt (17).

Herein, we demonstrate that the combination of PS-341 and 17-AAG caused decreased growth of ATL cell lines and significantly diminished the tumor burden in a xenograft NOD.Cg-Prkdc^{SCID} (NOD/SCID) mouse model. Our data indicate that targeted and synergistic therapy offers promise to abate ATL, but indicates that additive effects of 17-AAG requires continued treatment.

2.3 MATERIALS AND METHODS

Cell lines

The HTLV-1 infected cell lines C8166-45 (20), MT-2 (21), and HTLV-1 negative Jurkat cells (clone E6-1; American Type Culture Collection catalog number TIB-152) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 10% penicillin/streptomycin (100 μg/mL), and 10% glutamine (0.03 mg/mL) at 37°C in 5% carbon dioxide. MET-1 cells are an HTLV-1 positive cell line derived from a patient with ATL (22). These cells, like patient ATL cells, are unable to proliferate in vitro, but can be expanded by passage through NOD/SCID mice. MET-1 cells were expanded by inoculating $2 \times 10^7$ cells intraperitoneally (IP) into each mouse and harvesting at optimal tumor growth, typically at 5-6 weeks post inoculation. Spleen, lymph nodes and inoculation site masses were harvested from the mice at
necropsy. Tissues were minced and passed through a 100 μm cell strainer to create a single cell suspension. Isolated cells were then frozen in 90% fetal bovine serum and 10% DMSO or propagated in NOD/SCID mice to produce tumors for experimental procedures.

**Animals**

Female immunodeficient NOD/SCID (NOD.CB17-Prkdc^{scid}/J), (Jackson Laboratory, Bar Harbor, ME), were maintained under specific pathogen-free conditions in animal facilities in the College of Veterinary Medicine at The Ohio State University. Mice were kept in individually ventilated cages at 20 ± 2°C in accordance with the *Guide for Care and Use of Laboratory Animals*. This includes 12 hour light/dark cycle, free choice standard rodent chow, and water ad lib. All procedures were approved by The Ohio State University Institutional Laboratory Animal Care and Use Committee.

Mice were injected IP or subcutaneously with 2 x 10^7 MET-1 cells in 1 ml calcium/magnesium free phosphate buffered saline. Mice were monitored visually daily and weighed regularly. At 19 days post inoculation, serum was drawn and tested for serum IL-2Rα levels (R&D Systems). On Day 20, mice began a treatment regimen (Table 2.1). In the first phase of the experimental protocol mice were divided into four groups. The first group received vehicle only (100 μl of normal saline and 200 μl of Intralipid/10%DMSO/2.5% Tween 20). The second group received 0.43 mg/kg PS-341 in normal saline in a
total volume of 100 µl. The third group received 40 mg/kg 17-AAG in Intralipid/10% DMSO/2.5% Tween 20. The fourth group received combination therapy of PS-341 and 17-AAG. All mice received injections IP on days 20, 23, 27, 30, 41, 44, 48, and 51. Serum was drawn via the anterior facial vein every two weeks. Mice were euthanized using carbon dioxide inhalation on day 68.

In phase II, the mice were divided into three groups, eliminating the 17-AAG alone treatment group. To ascertain differences in survival between treatment groups, mice were continually monitored after completion of treatment regimen until such time as euthanasia criteria were met. Mice were euthanized by CO₂ inhalation upon developing signs of morbidity (rapid weight loss, rough hair coat, lethargy or persistent recumbence, labored breathing, difficulty with ambulation, or obtaining food and water).

Compounds

PS-341 (bortezomib) was provided by Millennium Pharmaceuticals. 17-AAG (Kosan Biosciences) was provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute. PS-341 was diluted in normal saline. 17-AAG was diluted in Intralipid/10% DMSO/2.5% Tween 20.
**Growth Inhibition Assays**

Forty thousand cells were plated in each well of a 96 well plate and incubated with media only, 1, 2.5, 5 nM PS-341, 1000 nM 17-AAG, or a combination of the increasing doses of PS-341 and 1000 nm 17-AAG. After 24, 48, and 72 hours, 20 µl of MTS reagent (Cell Titer 96® AQueous assay kit from Promega) was added to each well. The plate was incubated for two hours at 37° C. After this incubation period, the absorbance of the formazan (resulting from the chemical reduction of the MTS) was measured at 490 nm. Color change within the wells correlate to the number of metabolically active cells.

**Western Blot Assays**

Cells (2 x 10^6) were incubated with 5 nM PS-341, 1000 nM 17-AAG or both 5 nM PS-341 and 1000 nM 17-AAG for 48 hours. Cells were washed once in PBS and lysed. Aliquots of protein (20 µg) were run on SDS polyacrylamide gels. Protein was transferred to a nitrocellulose membrane and blocked overnight at 4° C in 1% BSA in 5% nonfat dry milk in 0.1% Tween-20 in Tris buffered Saline (TBST). Membranes were incubated with
primary antibodies in 5% milk in TBST. Poly (ADP-ribose) polymerase (PARP) antibody (Oncogene Catalog #AM30-100UG) was diluted at 1:100. Akt (Stressgen BioReagents cat# KAP-PK004) and phosphorylated Akt (Cell Signaling cat# 9271) were both dilute 1:1000. Ubiquitin (BD Pharmingen cat# 550944) was diluted to 1:10,000. Mouse Monoclonal antibody to beta-actin AC-74 (Sigma) was diluted 1:20,000. Membranes were then incubated with 1:1000 dilutions of corresponding secondary antibodies. Bound antibodies were detected with a chemiluminescent detection system (Cell Signaling Technologies #7003: 20X LumiGLO and 20X peroxide).

Histology and Immunohistochemistry

In phase I, all mice were euthanized on day 68. A routine necropsy was performed on all mice and organs were weighed and fixed in 10% neutral buffered formalin for 24 hours. Representative sections of tissues of interest were embedded in paraffin, sectioned, and stained with hematoxylin and eosin or stained with Ki67 (KiS5 clone) monoclonal antibody with ARK kit.

Serum IL-2Ra

Whole blood was collected from the anterior facial vein prior to the start of treatment and periodically throughout the study (minimum time between samples of 14 days). Immediately post collection, samples were centrifuged to separate the serum. Aliquots of serum were frozen at -80° C. To assay for
the presence of the human serum interleukin 2-receptor alpha chain (sIL-2Rα), a specific proliferation/tumor marker of human lymphocytes, the R&D systems IL-2sRα Duo Set ELISA kit was used. Samples were diluted 1:500 in reagent diluents and assayed according to manufacturer's protocol.

**Statistical Analysis**

Two way ANOVA models were used for the data analyses of cell growth, body and organ weights, cell counts and sIL-2Rα. Treatment group, time and their interaction were included in the models. Contrasts of treatment group means of interests, such as the combination group versus no treatment group, combination group versus PS-341 group at each time point were calculated and p-values were adjusted for multiplicity by Holm's step down (23) or Dunnet's method (24). Engraftment as measured by histologic grading was analyzed using Fisher's exact test. Kaplan-Meier curves were compared using the log-rank test for survival analyses. Cox regression models were performed to compare the effects of combination therapy PS-341 and sIL-2Rα on survival time.
2.4 RESULTS

17-AAG and PS-341 synergistically inhibit growth of Jurkat T-cell and MT-2 ATL cell lines

PS-341 and 17-AAG alone or in combination were first tested for their ability to inhibit the growth of ATL cell lines, HTLV-1 positive T-cell lines MT-2 and C8166 or the HTLV-1 negative Jurkat T-cell line (Fig. 2.1). Concentrations of PS-341 and 17-AAG were chosen based on previous literature on the effects in ATL cells (17;25). The combination of 1000 nm of 17-AAG and 2.5 nM PS-341 produced a statistically significant decrease in growth in the MT-2 cell line and Jurkat T-cell lines (p<0.001). Similar results were seen at 48 and 72 hours. Using these concentrations, we were not able to cause a statistically significant decrease in growth in C8166 cells. At 24 hours, in all three cell lines, a significant decrease in growth was not achieved with 1000 nM of 17-AAG alone or with increasing concentrations of PS-341 alone.
The combination of PS-341 and 17-AAG diminishes T-cell signaling through inhibition of Akt and the proteasome

To test the influence of PS-341 and 17-AAG alone or in combination on the signaling pathways important for T-cell proliferation, cell lysates of MT-2 cells treated with 5 nM PS-341 and 1000 nM 17-AAG for 48 hours were probed with multiple antibodies to test for inhibition of the proteasome and Hsp90 signaling pathway (Fig. 2.2). Our data indicated a decrease in PARP, phosphorylated Akt (p-Akt), Akt, as well as evidence of proteasome inhibition with increased ubiquitination of proteins. Thus, the combination of these two drugs appeared to target critical components of Tax-mediated cell proliferation and further justified in vivo studies in our mouse model of ATL.

Treatment with PS-341 alone and in combination with 17-AAG reduced tumor burden in organs

An established NOD/SCID mouse model of ATL (22) was used to test the in vivo efficacy of PS-341 alone and in combination with 17-AAG. In phase I, 20 mice were divided into four groups, and treated with different regimens of drugs or control vehicle (Table 2.1). Body weights of the mice did not differ between any treatment groups when compared to vehicle only
treatment. However, the liver and spleen weights in the animals receiving both PS-341 and 17-AAG were significantly lower than those in the vehicle only group (p=0.0013, 0.0354 respectively). In this ATL animal model, the liver and the spleen are typically heavily infiltrated with tumor, thus a significantly reduced spleen or liver weights suggested less tumor cell infiltration of these organs (confirmed by histologic examination below).

**Neoplastic infiltrates in organs were reduced in mice treated with PS-341 alone and in combination with 17-AAG**

To visualize and quantify the tumor burden in mice at the end of our study, routine hematoxylin and eosin staining was performed on paraffin embedded sections of collected tissues. Large neoplastic lymphocytes with characteristic MET-1 cell morphology were present in the liver, spleen, pancreas, brain, kidney, and heart (summarized in Table 2.2). The engraftment was widespread throughout the sections, but the size of the individual foci of neoplastic accumulations varied (Fig. 2.3). Statistical comparison indicated that tumor-bearing mice treated with PS-341 alone or in combination with 17-AAG had reduced tumor engraftment in systemic organs. Tumor-bearing mice treated with 17-AAG alone did not have significant differences in organ infiltration compared to vehicle treated control mice.

To compare the number of mitotically active cells in neoplastic foci between groups, we stained paraffin embedded sections of lung, liver, and
spleen with an antibody specific for the human proliferation marker Ki67.
Liver and lung sections from tumor-bearing mice treated with PS-341 in combination with 17-AAG had reduced number of Ki67 positive foci compared to vehicle control mice (Fig. 2.3). There was no difference in the number of Ki67 positive foci between the liver and lung section of PS-341 only or 17-AAG only when compared to the vehicle treatment. No statistically significant difference was observed in the spleen tissue from any of the treatment groups.

**Serum biomarker IL-2Rα was reduced mice treated with PS-341 alone and in combination with 17-AAG**

To monitor tumor burden noninvasively in mice, we measured serum IL-2Rα, a biomarker that has been effectively used to correlate with ATL tumor proliferation (26). Tumor-bearing mice treated with PS-341 and 17-AAG in combination had significantly lower sIL-2Rα levels than the vehicle only group (p<0.0001). The PS-341 alone treatment group also had significantly lower sIL-2Rα when compared to the vehicle only group (p=0.046). Tumor-bearing mice treated with 17-AAG alone exhibited a trend toward a difference in the sIL-2Rα levels (p=0.0571), but were not significantly different compared to the vehicle control group (Fig. 2.4A).
Additive effects of 17-AAG on survival required continuous treatment

To establish if combinational therapy of PS-341 with 17-AAG prolonged the survival of tumor-bearing mice, additional groups of mice were inoculated with ATL cells and allowed to survive post drug treatments until they met euthanasia criteria. A Kaplan Meier curve was generated and compared between groups (Fig. 2.4B). Median survival times were 64, 85, and 105 days respectively for the vehicle control, PS-341 and 17-AAG combination, and PS-341 alone groups. A statistically significant difference was found between treatment groups (p=0.03). However, the median survival time for the mice treated with the combination therapy was not different than the mice treated with the PS-341 alone.

2.5 DISCUSSION

Our data from both in vitro analysis and in an established ATL mouse model indicated that the combination therapy of PS-341 and 17-AAG is effective to abate the growth of ATL cell lines and reduce tumor burden in vivo. The addition of 17-AAG to PS-341 resulted in growth inhibition and induction of apoptosis of ATL cells indicated by decrease in PARP, Akt, p-Akt, and evidence of both Hsp90 and proteosome inhibition with increased ubiquitination. We are the first to demonstrate that treatment with PS-341 in combination with 17-AAG reduced the tumor burden of established ATL cells in a mouse model. Our histologic examination of mice on drug treatment
revealed significant difference in engraftment when both drugs were combined. Reductions in the expression of the proliferation marker, Ki67, confirmed these data in both the liver and the lung in the combination therapy group when compared to the vehicle only group. Importantly, our survival studies in ATL engrafted mice indicated that the differences observed between the treatment groups were minimized upon removal of drug therapy. Thus, while this combination of targeted therapy offers promise to decrease tumor burden, our results indicate that additive effects of 17-AAG are transient and require continued presence of the drug.

The acute form of ATL is a refractory malignancy with a median survival time of less than one year regardless of therapy (27). Adult T-cell leukemia/lymphoma-derived cells have multiple ways of surviving and persisting in presence of chemotherapy. While, HTLV-1 is clearly linked to the development of ATL, active virus replication is not required to maintain tumor growth. The outgrowth of ATL cells is believed to be initiated by key viral proteins such as Tax, but is more dependent upon genetic or epigenetic changes of the host cell for eventual selection of clonally expanded malignant lymphocytes. Recently, the expression of HBZ, a unique antisense encoded viral protein or its mRNA has been correlated with ATL cell survival (28;29). Over expression of the multidrug resistance protein has been reported in ATL (30;31) further complicating treatment options.
Various therapeutic approaches have been attempted against ATL, but with limited sustained success. These include chemotherapy regimens, a combination of zidovudine and interferon alpha-2a, antibody or antibody-radioconjugate therapy, stem cell transplantation, or targeted approaches with bortezomib or arsenic trioxide [reviewed in (1)]. Conventional chemotherapeutics have thus far failed to significantly increase the median survival time of ATL patients. As a result, new therapeutic approaches to abate this aggressive malignancy have focused on targeted therapies. In vitro and in vivo studies with targeted therapies have included monoclonal antibodies (alemtuzumab (32), Anti-Tac (33)), histone deacetylase inhibitors (34), and NF-κB inhibitors (13;35). While these studies have shown promise in experimental models, single agent therapies are rarely effective when translated to ATL patients (1).

Proteasome inhibition has been demonstrated to result in increased cell death and decrease in tumor burden in multiple tumor types including multiple myeloma (36;37) and mantle cell lymphoma (38). Myeloma cells respond to administration of PS-341, by upregulating heat shock proteins to assist in the folding and function of the proteins that would normally be targeted to the proteasome for degradation (16). Administration of 17-AAG alone in a multiple myeloma animal model decreased tumor burden (16). The combination of PS-341 and 17-AAG has been shown to produce synergistic effects, including increased chymotryptic activity in multiple
myeloma (16). In other cell lines, combination treatment has been shown to enhance protein ubiquitination and enhance antitumor activity (39).

The cellular transcription factor NF-κB, which is constitutively active in ATL cells (9) is blocked through inhibition of the proteasome (40). HTLV-1 infected cells in culture have decreased cell viability, and increased apoptosis in the presence of PS-341 (41). Tan et al., (12) reported an increase in phosphorylated IkBα and ubiquitination when HTLV-1 cells were treated with PS-341. Nasr et al., (42) reported growth inhibition, increased apoptosis measured by terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) and evidence of PARP cleavage, G2/M cell cycle arrest, and accumulation of p21 in several HTLV-1 cell lines treated with PS-341. The effect of PS-341 on the growth of tumors in a Tax transgenic mouse model revealed no consistent inhibition of NF-κB activation in vivo, however transplanted Tax tumors in Rag-1 mice showed consistent inhibition of tumor growth and prolonged survival (13). In a separate study, PS-341 decreased tumor burden in ATL xenograft mice (43). In the MET-1 ATL mouse model, PS-341 was not beneficial alone, however enhanced efficacy was noted in combination with humanized anti-Tac (CD25) monoclonal antibody (12).

Our studies reveal that PS-341 and 17-AAG, while effective at reducing established tumor burdens in short term studies, indicated a need for continuous drug pressure to maintain the additive benefits of both drugs in longer term survival studies. Mice were treated only eight times in both the
phase I and phase II studies. In phase I, the mice were euthanized shortly after the final treatment. Our design allowed us to test the effects of the continued pressure of the combination therapy. In phase II, the animals continued without treatment until they met euthanasia criteria. The removal of drug pressure for a prolonged time appeared to allow tumor cells to regain growth capacity and thus minimize the affects of the combination therapy when compared to the single agent therapy. The mouse model, while providing an excellent preclinical model in which to assay the effects of the compounds on the ATL cell growth, does not assay the effects of prolonged therapy in context to ATL patients or in the presence of a functional immune system. Despite the limitations of the model system, our results have implications for the design of human trials using this combination of drugs or when considering 17-AAG or derivatives as adjunct therapy to standardized protocols. Our data support further investigations to establish therapeutic dosing regimen and drug cycles of PS-341 and 17-AAG in both animal and human clinical trials.
2.6 REFERENCES


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<td><strong>Liver Weights (mean +/- SD, P value)</strong></td>
<td>1.45 +/-0.29</td>
<td>1.24 +/-0.08, p=0.10 80</td>
</tr>
</tbody>
</table>

ND= not determined- terminal study

Table 2.1 Summary of treatment groups and organ weights
### Table 2.2 Histologic ranking of tumor engraftment

<table>
<thead>
<tr>
<th>Mouse #</th>
<th>No treatment</th>
<th>PS-341</th>
<th>17-AAG</th>
<th>PS-341 + 17-AAG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Pancreas</td>
<td>+</td>
<td>+++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Spleen</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Brain</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td>+++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Adrenal</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

+ 0-5 cells per focus  
++ 6-10 cells per focus  
+++ 11-15 cells per focus  
++++ 16 or more cells per focus
Figure 2.1 The addition of 17-AAG significantly inhibits growth in both the MT-2 and Jurkat cell lines. MT-2, C8166, and Jurkat T-cells were grown in media only, 1, 2.5, or 5nM PS-341 with or without the addition of 1000nM 17-AAG. At 24, 48, and 72 hours, growth was measured by the addition of MTS reagent and absorbance at 490 nM.
Figure 2.2 Western blot of MT-2 cell whole cell lysate after 48 hours. Cells were treated with media only, 5nM PS-341 or 1000 nM 17-AAG or the combination of PS-341 and 17-AAG. Cells show decreased PARP, Akt and pAkt with increased ubiquitination.
Figure 2.3 Histologic representation of mouse tissues from phase I. Panels A and B are sections of lung stained with routine H&E. Histologic findings include fewer neoplastic round cells in the PS-341 and 17-AAG group when compared to the no treatment group. Panels C and D are Ki67 stained sections of lung from Phase I. Numbers of positive nuclei (brown staining) were counted in ten randomly selected high powered fields (400X magnification) for each tissue.
Figure 2.4 Serum IL-2Rα levels and survival curves of mice.  A. Serum IL-2Rα levels of mice at the end of Phase I study.  There is a statistically significant decrease in serum IL-2Rα levels in both the PS-341 and combination therapy groups while there is a trend toward a significant decrease in the 17-AAG treatment group.  B. Kaplan Meier curve for Phase II in vivo experiments.  While treatment with either PS-341 or combination therapy proved better than vehicle only treatment, treating with combination therapy did not improve survival over PS-341 alone.
CHAPTER 3

EFFICACY OF HISTONE DEACETYLASE INHIBITORS IN A MOUSE MODEL OF HUMAN T-LYMPHOTROPIC VIRUS TYPE 1 ADULT T-CELL LYMPHOMA

3.1 ABSTRACT

Human T-lymphotropic virus type 1 (HTLV-1) causes adult T-cell leukemia/lymphoma (ATL) a refractory CD4+/CD25+ T-cell malignancy. ATL patients have a poor prognosis due to the highly aggressive nature of the cancer and the resistance to conventional chemotherapies. The low penetrance and prolonged latency period of ATL suggests that genetic and epigenetic alterations precede the development of ATL. Histone deacetylase inhibitors (HDACi), which promote the relaxation of chromatin and the promotion of transcription have shown efficacy against a variety of cancers and are in phase I and phase II clinical trials. Herein, we tested if the histone deacetylase inhibitors valproic acid and the novel OSU-HDAC42 reduced the proliferation of ATL cell lines by promoting apoptosis and histone hyperacetylation. Both compounds reduced cell growth and elicited a dose dependent increase in cytochrome C and cleaved Poly (ADP-ribose) polymerase (PARP) indicating the induction of cell death by
apoptosis. To further test of the efficacy of this approach we evaluated a novel HDACi, OSU-HDAC42, for survival in an ATL NOD/SCID mouse model. A dietary formulation of OSU-HDAC42 prolonged survival of ATL engrafted mice compared to controls. Our data provide new directions for the treatment of ATL and support the further development of this class of drug against HTLV-1-associated lymphoid malignancies.

3.2 INTRODUCTION

Human T-lymphotropic virus type 1 (HTLV-1) infection is associated with an array of devastating malignancies and inflammatory disorders. Patients with the acute form of adult T-cell leukemia/lymphoma (ATL) present with a refractory CD4+/CD25+ T-cell malignancy. These patients have a poor prognosis due to the highly aggressive nature of the cancer and the resistance of neoplastic cells to conventional chemotherapies. HTLV-1 is estimated to have infected 15 to 20 million people worldwide (1). The virus is endemic in southern Japan (2), the Caribbean basin (3), central Africa (4), Central and South America (5;6), the Melanesian Islands in the Pacific basin, and in the aboriginal population in Australia (7). Typically, 1-5 % of HTLV-1-infected individuals develop ATL after a latent period of 20-30 years (8;9). ATL patients typically present with malaise, fever, lymphadenopathy, hepatosplenomegaly, jaundice, weight loss, and various opportunistic infections, such as *Pneumocystis carinii* (10). Based on the clinical course of the disease, four classifications have been used to categorize ATL
patients as acute, smoldering, chronic and lymphoma stages (11-13). Acute ATL is highly refractory to standard chemotherapeutic approaches (14) and patients have a higher incidence of hypercalcemia of malignancy, in association with lytic bone lesions, elevated lactate dehydrogenase (LDH), and the presence of soluble form of interleukin-2 (IL-2) receptor in their serum (15).

Due to the low penetrance and prolonged latency period (up to 70 years), the hypothesis that genetic and epigenetic alterations precede the development of ATL has been postulated. Transcriptional regulation at the chromosomal level is controlled in part by the acetylation of histones and non-histone proteins (16;17). Acetylation of histones results in the relaxation of chromatin and the promotion of transcription. This is controlled, in part, by histone deacetylases (HDACs) that promote the removal of the acetyl groups from lysines on histones. This results in the restoration of positive charges, tightening of DNA (negatively charged) around the histone core, and decreases in transcription of affected genes. Currently, there are eighteen HDACs classified into six different groups (18). These HDACs control the activation and repression of proteins and may also functionally influence non-histone proteins such as hormone receptors, chaperones (heat shock proteins), viral proteins, and cytoskeletal proteins (18).

The role of inhibitors of HDACs (HDACi) in cancer therapy has recently been reviewed (18-23). HDACi promote the acetylated state of the histone and relaxed chromatin structure. They are divided into several classes including short chain fatty acids, hydroxamic acids, benzamides, and cyclic peptides.
These classes differ in their potency, but are generally not specific for HDAC isoenzymes (19).

Recently, HDACi have been used as targeted therapies in cancer research. As reviewed in Xu et al. (18), HDACi have been shown to induce apoptosis, induce cell cycle arrest, disrupt Hsp90 and the aggresome, inhibit angiogenesis, and induces mitotic and autophagic cell death and promote senescence. It is through these pleiotropic effects that HDACi have shown efficacy in vitro against prostate cancer (24;25). In addition, the HDACi suberoylanilide hydroxamic acid (SAHA or Vorinostat) is approved for the treatment of cutaneous T-cell lymphoma. Depsipeptide (FR901228) has also been used in the treatment of peripheral and cutaneous T-cell lymphoma (26). Several other first generation HDACi are currently in phase I and phase II clinical trials (27).

Herein, we evaluated the histone deacetylase inhibitors valproic acid (VPA) and the novel OSU-HDAC42 for their ability to reduce the proliferation of ATL cell lines through apoptosis and histone hyperacetylation. Our data indicated that both compounds reduced cell growth and promoted a dose dependent increase in cytochrome C and cleaved Poly (ADP-ribose) polymerase (PARP). Finally, we tested the efficacy of a novel HDACi, OSU-HDAC42 for its ability to enhance the survival of NOD/SCID mouse model engrafted with ATL cells. We are the first to demonstrate that a dietary formulation of OSU-HDAC42 prolongs the survival of mice engrafted with ATL cells. Our findings support
further development of this class of drug against HTLV-1-associated lymphoid malignancies.

3.3 MATERIALS AND METHODS

Cell lines

The HTLV-1 infected cell lines C8166-45(28), MT-2(29), and HTLV-1 negative Jurkat cells (clone E6-1; American Type Culture Collection catalog number TIB-152) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 10% penicillin/streptomycin (100 μg/mL), and 10% glutamine (0.03 mg/mL) at 37°C in 5% carbon dioxide. MET-1 cells are an HTLV-1 positive cell line derived from a patient with ATL (30). These cells, like patient ATL cells, are unable to proliferate in vitro, but can be expanded by passage through NOD/SCID mice. MET-1 cells were expanded by inoculating 2 x 10^7 cells intraperitoneally (IP) into each mouse and harvesting at optimal tumor growth, typically at 5-6 weeks post inoculation. Spleen, lymph nodes and inoculation site masses were harvested from the mice at necropsy. Tissues were minced and passed through a 100 μm cell strainer to create a single cell suspension. Isolated cells were then frozen in 90% fetal bovine serum and 10% DMSO or passaged as above to produce tumors for experimental procedures.
Animals

Female immunodeficient NOD/SCID (NOD.CB17-Prkdcscid/J), (Jackson Laboratory, Bar Harbor, ME), were maintained under specific pathogen-free conditions in animal facilities in the College of Veterinary Medicine at The Ohio State University. Mice were kept in individually ventilated cages at 20 ± 2°C in accordance with the Guide for Care and Use of Laboratory Animals. This includes 12 hour light/dark cycle, free choice standard rodent chow, and water ad lib. All procedures were approved by The Ohio State University Institutional Laboratory Animal Care and Use Committee.

Mice were injected IP with 2 x 10⁷ MET-1 cells in 1 ml calcium/magnesium free phosphate buffered saline (PBS). Mice were monitored visually daily and weighed regularly. At 20 days post inoculation and every two weeks throughout the study, serum was drawn and tested for serum IL-2Rα levels (R&D Systems). On Day 21, mice began a treatment regimen. In the first phase of the experimental protocol mice were divided into two groups. The first group received vehicle only (methylcellulose/Tween 80). The second group received 50 mg/kg (S)-HDAC42 orally in methylcellulose/Tween 80 in a total volume of 10 µl/gram body weight by gavage.
In phase II, the mice were again divided into two groups. The mice were injected IP with $2 \times 10^7$ MET-1 cells. After the engraftment period of 21 days, the mice were fed a diet formulated by Research Diets, Inc (New Brunswick, NJ). The AIN-76A rodent diet with and without 208 ppm OSU-HDAC42 was fed to the treatment groups. Dietary intake was monitored by weight of diet consumed (24).

To ascertain differences in survival between treatment groups, mice were continually monitored until such time as euthanasia criteria were met. Mice were euthanized by CO$_2$ inhalation upon developing signs of morbidity (rapid weight loss, rough hair coat, lethargy or persistent recumbence, labored breathing, difficulty with ambulation, or obtaining food and water).

**Compounds**

OSU-HDAC42 was provided by The College of Pharmacy at The Ohio State University. It was diluted in DMSO to obtain a stock solution of 10 mg/ml then diluted in 10% FBS containing culture media for in vitro studies. For in vivo studies, the OSU-HDAC42 was prepared as a suspension in 0.5% methylcellulose and 0.1% Tween 80 in sterile water. The OSU-HDAC42 containing diet and control diet were prepared by Research Diets, Inc (New Brunswick, NJ). Valproic acid was obtained from Sigma (Saint Louis, MO) and prepared in PBS stock solution prior to dilution in 10% FBS containing media for in vitro studies.
Growth Inhibition Assays

Forty thousand cells were plated in each well of a 96 well plate and incubated with media only, 1, 2.5, 5 mM VPA or 0.5, 1, or 2.5 μM OSU-HDAC 42. Concentrations of OSU-HDAC42 and VPA were chosen based on the previous literature (24;25;31). After 24, 48, and 72 hours, 20 μl of MTS reagent (Cell Titer 96® AQ_{ueous}, Promega) was added to each well prior to incubation for two hours at 37°C. Absorbance was measured at 490 nm indicating the metabolically active cells.

Western blot assays

Cells (2 x 10^6) were incubated with media only, 1, 2.5, 5 mM valproic acid or 0.5, 1, or 2.5 μM OSU-HDAC 42 for 24 hours. Cells were washed once in PBS and lysed. Aliquots of protein (20 μg) were run on SDS polyacrylamide gels. Protein was transferred to a nitrocellulose membrane and blocked overnight at 4°C in 1% BSA in 5% nonfat dry milk in 0.1% Tween-20 in Tris buffered saline (TBST). Membranes were incubated with primary antibodies in 5% milk in TBST. The following antibodies were used: Poly (ADP-ribose) polymerase (PARP) (Oncogene Catalog #AM30-100UG) diluted at 1:100, cleaved PARP (AbCam #ab4830) diluted 1:1000, acetylated histone H3 (Millipore #06-599) diluted 1:3000, cytochrome C (AbCam #ab28137) diluted 1:500, and beta-actin AC-74 (Sigma) diluted 1:20,000. Membranes were then
incubated with 1:1000 dilutions of corresponding secondary antibodies. Bound antibodies were detected with a chemiluminescent detection system (Cell Signaling Technologies #7003: 20X LumiGLO and 20X Peroxide).

**Histology**

Standard necropsies were performed on all mice and organs fixed in 10% neutral buffered formalin for 24 hours. Representative sections of liver, lung, kidney, heart, spleen, and brain were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (Fig 3.4).

**Serum IL-2Ra**

Whole blood was collected from the anterior facial vein prior to the start of treatment and periodically throughout the study (minimum time between samples of 14 days). Immediately post collection, samples were centrifuged to separate the serum. Aliquots of serum were frozen at -80°C. To assay for the presence of the human serum interleukin 2-alpha chain (sIL-2Ra), a specific proliferation/tumor marker of human lymphocytes, the R&D systems IL-2sRa Duo Set ELISA kit was used. Samples were diluted 1:500 in reagent diluents and assayed according to manufacturer’s protocol.
Statistical Analysis

ANOVA models were used to test if there were significant differences between concentrations of compounds and if differences existed between different time points. The numbers of multiple comparisons were adjusted by Tukey’s method.

3.4 RESULTS

OSU-HDAC42 and valproic acid significantly inhibit growth of ATL cell lines

The histone deacetylase inhibitors OSU-HDAC42 and VPA were tested for their ability to inhibit growth of HTLV-1 positive cell lines prior to in vivo testing in our animal model of ATL. Direct testing of ATL cells in cell culture is difficult as these cells typically do not grow in culture and are unavailable. To provide surrogate models to test HDACi, we tested the HTLV-1 positive cell lines MT-2 and C8166, which were originally derived from ATL patients. The Jurkat cell line was used as the HTLV-1 negative T-cell control.

Valproic acid significantly inhibited growth in C8166 cells at 2.5 mM concentration (p<0.0001). In this cell line, we also demonstrated that increased duration of exposure significantly inhibited growth (Fig. 3.1A). When compared to 24 hours of exposure, 48 and 72 hour exposures significantly inhibited growth.
at all concentrations (p<0.0001). Valproic acid inhibited growth in MT-2 cells at a concentration of 1mM (p<0.0001). Increasing the duration of exposure did not further inhibit growth until 72 hours (p=0.0005). Growth of Jurkat cells was inhibited with 2.5 mM VPA (p<0.0001). Further significant decreases in Jurkat T-cell growth were observed at 72 hours of exposure (p<0.0001).

OSU-HDAC42 inhibited growth in all three cell lines tested. In the C8166 cell line, there was a statistically significant decrease in growth with 0.5 μM concentration and 48 hours duration (p=0.0011). In MT-2 cells, a statistically significant decrease in growth was observed with 0.5 μM OSU-HDAC42 (p<0.0001). Jurkat T-cells were significantly inhibited in growth at the 2.5 μM OSU-HDAC42 concentration (p=0.0001)(Fig 3.1B).

**OSU-HDAC42 and valproic acid induced apoptosis and hyperacetylation of histones**

Whole cell lysates of C8166 and MT-2 cells treated with VPA and OSU-HDAC42 for 24 hours were tested for hyperacetylation of histones and HDACi affects on non-histone target proteins by western immunoblot assay (Fig. 3.2). Our data indicated dose dependent increases in acetylated histone H3 in both MT-2 and C8166. In addition, dose dependent increases in both cytochrome C and cleaved PARP were observed, each indicative of apoptosis induced by each compound in both cell lines (Fig. 3.2A and B). Collectively our western immunoblot data indicated that both VPA and OSU-HDAC42 targeted the
appropriate pro-apoptotic pathways to limit cell proliferation and justified in vivo studies with our NOD/SCID ATL mouse model.

**OSU-HDAC42 prolongs survival in the ATL NOD/SCID mouse model with continued drug administration**

We utilized a previously established NOD/SCID ATL mouse model (30) to test the efficacy of OSU-HDAC42 in vivo (Fig 3.3). Following engraftment with MET-1 cells, we first divided 10 mice into two groups of 5 each and treated them by oral gavage with 50 mg/kg OSU-HDAC42 or vehicle alone respectively for three times per week for four weeks. Serum was obtained every 14 days to measure the serum biomarker for tumor progression (serum IL-2Rα). After one month, OSU-HDAC42 was discontinued and mice were monitored for development of removal criteria. Mice treated with OSU-HDAC42 tended to have lower serum IL-2Rα levels (Fig. 3.5A), but did not differ in time to removal (Fig. 3.5B).

During phase II of our study, we used a dietary formulation of OSU-HDAC42 and compared it to a commercially prepared vehicle only diet (Table 3.1). Mice were inoculated intraperitoneally with $2 \times 10^7$ MET-1 cells and allowed three weeks to engraft. At three weeks post inoculation, mice were given free choice access to either the control diet or the OSU-HDAC42 diet. Food consumption was measured by weight of food remaining in cage every other day and divided by the number of animals in the cage. The intake of food did not
differ between groups (Fig. 3.6). Mice treated with OSU-HDAC42 overall exhibited a trend to survive longer than the vehicle only treated group (Fig. 3.7).

3.5 DISCUSSION

Our data indicate that HDACi are an effective treatment for ATL. In vitro, both VPA and OSU-HDAC42 abate growth of ATL cell lines. Exposure of ATL cell lines to either HDACi resulted in hyperacetylation of histones. In addition, we demonstrated that both VPA and OSU-HDAC42 induced apoptosis. This was indicated by accumulation of cytochrome C and increased cleaved PARP. We are the first to demonstrate that OSU-HDAC42 was safe and efficacious in the ATL NOD/SCID mouse model. In addition, our feed formulation design demonstrated that oral dosing of OSU-HDAC42 appeared to be more effective compared to oral gavage in improving survival of the engrafted mice.

Acute ATL is a CD4+ CD25+ T-cell malignancy with a universally poor prognosis. HTLV-1 is linked to the development of ATL; however, active virus replication is not required to maintain tumor growth. In part, the outgrowth of ATL is dependent on genetic or epigenetic changes of the host cell for selection of clonally expanded malignant lymphocytes. Thus, novel treatments are needed against ATL to more selectively target key cancer cell pathways to induce cell death. Conventional chemotherapies against ATL have produced inconsistent or limited results against ATL (32). New therapeutic approaches have focused on targeted therapies. In vitro and in vivo studies with targeted therapies have
Histone deacetylase inhibitors promote the acetylated state of histones and relax chromatin structure leading to induction of apoptosis, cell cycle arrest, disruption of Hsp90 and the aggresome, inhibition of angiogenesis, mitotic cell death, and senescence (reviewed in (18)). In ATL cell lines, the histone deacetylase inhibitor FR901228 induced apoptosis and reduced tumor size in mice (37). In our study, we compared two HDACi (VPA and OSU-HDAC42) for their ability to abate ATL cell growth and reduce tumor burdens in a mouse model. Valproic acid (VPA) is an eight carbon branched chain fatty acid that has been used in the treatment of epilepsy since 1978 (21). Recently, it has been shown to have efficacy in the treatment of cancer through the hyperacetylation of histones 3 and 4 (38). VPA inhibits prostate cancer cell growth (39) medulloblastoma (40), and multiple myeloma (41) in vitro. OSU-HDAC42 is a phenylbutyrate derived HDACi included in the Rapid Access to Intervention Development (RAID) program through the National Cancer Institute (25). In a prostate cancer model, OSU-HDAC42 was shown to decrease cell viability, decreased phospho-Akt, Bcl-xL, and Survivin (24). In vivo, a xenograft mouse model had decreased tumor burden (24) and decreased tumor levels of phospho-Akt and Bcl-xL in response to the drug (25).

In our study, we demonstrated that OSU-HDAC42 while not improving survival in short term tumor engraftment studies did prolong survival when
provided as a continuous feed supplement. Thus, OSU-HDAC42 was required on a more continuous basis to demonstrate antitumor activity. Mice were only treated 12 times in phase I of the study. In phase I, the mice were allowed to progress until they met euthanasia criteria. Our phase II design allowed us to test the effects of the continued pressure of OSU-HDAC42. In phase II, the animals continued with in feed treatment until they met euthanasia criteria. The addition of continued drug pressure for a prolonged time appeared to prolong survival and prevent tumor cells from regaining growth capacity.

The mouse model, while providing an excellent preclinical model in which to assay the effects of the compounds on the ATL cell growth, does not assay the effects of prolonged therapy in context to ATL patients or in the presence of a functional immune system. Our results have implications for the design of human trials using OSU-HDAC42 as adjunct therapy to standardized protocols and support further investigations to establish more effective therapeutic dosing regimens and drug cycles of OSU-HDAC42 in both animal and human clinical trials.
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3.6 REFERENCES


Figure 3.1. Histone deacetylase inhibitors significantly inhibit growth in ATL cell lines. A. MT-2, C8166, and Jurkat T-cells were grown in media only, 1, 2.5, 5 mM valproic acid. At 24, 48, and 72 hours, growth was measured by the addition of MTS reagent and absorbance at 490 nM. B. MT-2, C8166, and Jurkat T-cells were grown in media only, 0.5, 1, 2.5 μM OSU-HDAC42. At 24, 48, and 72 hours, growth was measured by the addition of MTS reagent and absorbance at 490 nm.
Figure 3.1
Figure 3.2 Western blot of whole cell lysate after 24 hours. MT-2 cells were treated with media only, 1, 2.5, 5 mM valproic acid or 0.5, 1, 2.5 μM OSU-HDAC42. Cells showed a dose dependent increase in cleaved PARP, cytochrome C, and acetylated histone H3. C8166 cells were treated with media only, 1, 2.5, 5 mM valproic acid or 0.5, 1, 2.5 μM OSU-HDAC42. Cells showed a dose dependent increase in cleaved PARP, cytochrome C, and acetylated histone H3.
Figure 3.3 Linear schematic of dosing regimen. In phase I, mice were treated by gavage with 50mg/kg OSU-HDAC42 of vehicle three times per week for two weeks, rested one week and treated in a similar manner for two weeks. In phase II, the mice were started on OSU-HDAC42 or vehicle in diet at day 21. In both instances, the mice were allowed to survive until removal criteria were met.
Figure 3.4 Engraftment of MET-1 cells in multiple organs. Examples of NOD/SCID mice inoculated intraperitoneally with $2 \times 10^7$ MET-1 cells. Neoplastic round cells are present in the brain (meninges), stomach, heart, lung, kidney, and liver. All tissues magnified 200X, bar denotes 50 µm.
Figure 3.5 Oral gavage of OSU-HDAC42 in the NOD/SCID ATL mouse model. A. Serum IL-2Rα levels of mice during the Phase I study. Solid squares indicate values for mice administered vehicle only. Open circles indicate values for mice administered OSU-HDAC42. Serum levels of this biomarker do not differ significantly between the groups at any time. B. Kaplan Meier curve for Phase I in vivo experiments. The solid line represents control mice. Dotted line represents the OSU-HDAC42 treated. Treatment of OSU-HDAC42 by oral gavage did not significantly improve survival in mice.
Figure 3.6 Mice fed ad lib formulated diets did not differ significantly in intake. Mice were fed a diet containing OSU-HDAC42 or control diet ad lib. Average intake per mouse per day did not differ between the groups.
Figure 3.7 In feed OSU-HDAC42 in the NOD/SCID ATL mouse model. A. Serum IL-2Rα levels of mice during the Phase II study. Solid squares indicate values for mice administered vehicle only. Open circles indicate values for mice administered OSU-HDAC42. Serum levels of this biomarker do not differ significantly between the groups at any time. B. Kaplan Meier curve for Phase II in vivo experiments. The solid line represents control mice. Dotted line represents the OSU-HDAC42 treated. There is a trend toward prolonged survival in the treatment group.
CHAPTER 4
EFFECTS OF THE HISTONE DEACETYLASE INHIBITOR VALPROIC ACID
ON HUMAN T-LYMPHOTROPIC VIRUS TYPE 1 REPLICATION IN A RABBIT
MODEL OF INFECTION

4.1 ABSTRACT

Human T-lymphotropic virus type 1 (HTLV-1) is the etiologic agent of adult T-cell leukemia/lymphoma (ATL) and a number of lymphocyte mediated disorders including HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). The pathogenesis of HTLV-1-mediated disease is unclear. The virus establishes a lifelong infection with approximately 5% of infected subjects developing disease. It is known that disease outcomes are controlled by multiple viral and host determinants of gene expression. Following integration, the expression of the HTLV-1 provirus is dependent upon the local chromatin environment and influences upon the cell that mediated gene expression. Histone deacetylase inhibitors (HDACi), which promote the relaxation of chromatin and promote gene transcription have been shown to promote transcription of viral genes in vitro and offer new therapeutic strategies against
Herein, we tested the effects of the histone deacetylase inhibitor, valproic acid (VPA), on HTLV-1 proviral load in the rabbit model of early infection. We inoculated three groups of six rabbits intravenously with HTLV-1 infected cells. The first group of rabbits was treated with 70 mg/kg valproic acid subcutaneously three times per week during the first four weeks of infection. The second group of rabbits was treated with valproic acid during weeks four through eight of infection. The third group was treated as a control and given subcutaneous saline injections three times per week during weeks zero through four. Our data demonstrate that VPA can be safely administered in the rabbit model of HTLV-1 infection, but did not significantly alter viral parameters of infection. Our data indicated that VPA treatment caused a decrease in proviral load when administered during the first four weeks of infection and altered virus infection-associated lymphocytosis in treated rabbits. This model will be useful to test other combinations of HDACi for their effects on HTLV-1 gene expression in the rabbit model.

4.2 INTRODUCTION

Human T-lymphotropic virus type 1 (HTLV-1) is a deltaretrovirus that causes adult T-cell leukemia/lymphoma (ATL) and a variety of lymphocyte-mediated diseases (1). Disease outcomes associated with HTLV-1 infection appear to be controlled by both viral and host determinants of gene expression. After infection and integration into the host cell chromatin the HTLV-1 provirus is
dependent upon the local chromatin environment and epigenetic influences on local gene expression. Histone deacetylase inhibitors (HDACi), which promote the relaxation of chromatin and promote gene transcription, provide new therapeutic strategies against ATL, but their influence on virus replication in vivo is largely unknown. It is clear that HTLV-1 infected patients with high proviral load are more likely to develop HAM/TSP when compared to patients that develop ATL or remain asymptomatic (2). To fully understand how HDACi may be used therapeutically in infected patients, a more complete understanding of this class of drugs on HTLV-1 replication in vivo is needed.

Increased viral expression and corresponding proviral copy numbers in circulating lymphocytes has been associated with lymphocyte-mediated disease syndromes associated with HTLV-1 infection. HTLV-1 expression is controlled by both methylation and histone acetylation (3-7). Both methylation and histone acetylation cause transcriptional silencing of HTLV-1 at the 5'LTR (8). It has been estimated that approximately 9% of the HTLV-1 proviral load may be influenced by acetylation of histones (9). In addition, histone deacetylases such as HDAC1 decreased Tax mediated gene expression (10) and modulate HTLV-1 p30 transcriptional effects (4).

Valproic acid (VPA) is an eight carbon branched chain fatty acid that has been used in the treatment of epilepsy since 1978 (11). Recently, it has been shown to have efficacy in the treatment of cancer through the hyperacetylation of histones 3 and 4 (12). VPA inhibits prostate cancer cell growth (13)
medulloblastoma (14), and multiple myeloma (15) in vitro. During two months of treatment with the valproic acid (VPA), HAM/TSP patients exhibited a transient increase in proviral load followed by decreased proviral load by undetermined mechanism (16).

Herein, we evaluated the effects of VPA on HTLV-1 proviral load in the rabbit model of infection. We inoculated three groups of six rabbits intravenously with HTLV-1 infected cells. The first group of rabbits was treated with 70 mg/kg valproic acid subcutaneously three times per week during the first four weeks of infection. The second group of rabbits was treated with valproic acid as in group 1 during weeks four through eight of infection. The third group was treated as a control and given subcutaneous saline injections three times per week during weeks zero through four. Our data indicated that VPA treatment caused a decrease in proviral load when administered during the first four weeks of infection and altered virus infection-associated lymphocytosis in treated rabbits. Our study demonstrates that VPA can be safely administered in rabbits and establishes parameters to test HDACi for their effects on HTLV-1 gene expression in the rabbit model.
4.3 MATERIALS AND METHODS

Rabbit and human cell lines

A CD4+ rabbit lymphocyte line (R49) was used to establish HTLV-1 infection in New Zealand white rabbits as described (17). The derivation and infectious properties of the full-length, wild-type HTLV-1 proviral clone (ACH) and generation of the R49 cell line has been previously reported (17). Jurkat T-cells (clone E6-1; American Type Culture Collection catalog number TIB-152) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 10% penicillin/streptomycin (100μg/mL), and 10% glutamine (0.03 mg/mL) at 37°C in 5% carbon dioxide. Peripheral blood mononuclear cells (PBMC) were isolated from up to 10 ml of whole blood from the central auricular artery at each time point indicated. Percoll (Sigma-Aldrich Corp., St. Louis, MO) at 1.083 g/ml was used to isolate rabbit lymphocytes. Rabbit lymphocytes were cultured in RPMI 1640 (GIBCO BRL), 15% fetal bovine serum (FBS) (GIBCO BRL), 1% penicillin-streptomycin (GIBCO BRL), 1% sodium pyruvate (GIBCO BRL), 2% L glutamine (GIBCO BRL), and 250 μl of 2 mercaptoethanol.
Rabbit inoculation

Twenty, 12 week old, specific pathogen free, female New Zealand White rabbits were purchased from Harlan Laboratories (Indianapolis, IN) and housed and maintained individually. All procedures were approved by the Institutional Laboratory Care and Use Committee. After an acclimation period of one week, Rabbits were inoculated via the lateral auricular vein with either $1 \times 10^7$ R49 cells or $1 \times 10^7$ Jurkat cells (Fig. 4.1). The rabbits were regularly evaluated for overt signs of clinical disease. Seven to ten milliliters of whole blood was drawn from the median auricular artery for analysis at week 0, 1, 2, 4, 6, and 8. A complete blood count (CBC) and blood smear were performed on the whole blood prior to isolation of peripheral blood mononuclear cells (PBMCs). At the conclusion of the study (week 8), rabbits were humanely euthanized and necropsied.

Compound and VPA treatment

Valproic Acid was obtained from Sigma (St. Louis, MO) and diluted in 0.9% sodium chloride (normal saline). Rabbits were treated with 70 mg/kg valproic acid subcutaneously three times per week. Rabbit serum levels of valproic acid were measured using Beckman Coulter SYNCHRON LX® Systems
particle enhanced turbidimetric immunoassay method in the clinical laboratories at the Ohio State University.

**Hematology**

White blood cell differential cell counts were obtained by counting 100 cells from blood films in a blinded manner by two independent examiners. Morphologic examination was used to classify blood leukocytes as normal lymphocytes, atypical lymphocytes (large lymphocytes with at least two nuclear indentations), heterophils, monocytes, basophils and eosinophils.

**Serological response to HTLV-1 infection**

Reactivity to specific viral antigenic determinants was detected using a commercial HTLV-1 western immunoblot assay (GeneLabs Diagnostics, Singapore) adapted for rabbit plasma by use of alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (1:1,000 dilution; BioMerieux, Inc.). Plasma (diluted 1:2000) showing reactivity to HTLV-1 Gag (p24 or p19) and Env (p21 or gp46) antigens was classified as positive for HTLV-1 seroreactivity.

**Detection of p19 (MA) matrix antigen from cultured rabbit PBMC**

To test for active HTLV-1 infection in rabbit PBMCs p19 matrix antigen (MA) enzyme-linked immunosorbent assays (ZeptoMetrix, Buffalo, NY) were
performed on supernatants from one day PBMC cultures (1.0 x 10^6 per well) as described (18).

**Detection of proviral copy number by PCR**

To quantify the proviral copy number genomic DNA was isolated from rabbit PBMCs using the manufacturer’s protocol (Qiagen). DNA concentration and quality was determined (NanoDrop Technologies). HTLV-1 polymerase gene (pol) primers were used at a final concentration of 200 nM of both forward and reverse primers. The forward pol primer 5’-CCC TAC AAT CCA ACC AGC TCA G-3’ and the reverse primer 3’-TTT TGG GCT ACC GTC GAA GTG GTG-5’ were used to amplify genomic DNA. Standard curves were generated using DNA from a molecular clone of HTLV-1, the ACH plasmid and assayed concurrently with test samples. Forward rabbit GAPDH (rGAPDH) primers 5’-TGC CCA GTG TCA AGT CTG TT-3’ and reverse primers 3’-TCT TCG TCG TGT GTC GTG TC-5’ were used at a final concentration of 250 nM to amplify genomic DNA. The standard curves for rGAPDH were generated by subcloning a 780 base pair fragment from the rGAPDH gene into the pCR®4-Topo vector (Invitrogen) and run concurrently with test samples. For each run, a standard curve was generated from triplicate samples of log 10 dilutions of plasmid DNA in DNAse/RNAse free water. The HTLV-1 proviral load was expressed as infected cells per 10,000 PBMCs and was calculated using the following formula: (HTLV-1 pol copy number) / (rabbit GAPDH copy number / 2) x 10000.
4.4 RESULTS

Establishment of therapeutic ranges of VPA blood levels in rabbits

To establish that our treatment protocol was effectively meeting therapeutic target ranges, we administered 70 mg/kg valproic acid subcutaneously three times per week to each rabbit. Group 1 rabbits were treated from week zero through week four of infection. Group 2 rabbits were treated during week four through week eight of infection. The concentration of VPA in the blood was determined by measuring levels in the whole blood by Beckman Coulter SYNCHRON LX® Systems particle enhanced turbidimetric immunoassay method in the clinical laboratories at the Ohio State University. Mean VPA serum concentrations were 96 $\mu$g/ml +/- 52 among four rabbits. This was within the therapeutic range for human epilepsy patients (50-120 $\mu$g/ml), provided by the Department of Clinical Laboratories. The blood concentration was determined two weeks into the experiment and immediately prior to the rabbits receiving their first treatment of week three.

Hematology evaluation and serologic responses following HTLV-1 infection

To evaluate the hematologic response to valproic acid treatment, differential cell counts were performed on whole blood samples from each rabbit at week 0, 1, 4, 6, and 8. One hundred white blood cells were categorized as heterophils, eosinophils, basophils, monocytes, lymphocytes, and atypical.
lymphocytes. Atypical lymphocytes, defined as large lymphocytes with at least two nuclear indentation of 1/3 nuclear width (Fig 4.2), were visualized at each time point. A decrease in lymphocytes was observed at week 4 in Group 1. This decrease was not observed in either Group 2 or Group 3 (Fig. 4.3A). In both Group 2 and Group 3 there was an increase in the atypical lymphocyte count at week 1, which was not present in the VPA treated Group 1 (Fig 4.3B).

At each time point, serum was analyzed by western blot for antibody response to HTLV-1. Western blot strips were evaluated for reactivity to four major viral proteins p19, p24, p21, and gp46 at defined intervals throughout the infection. Responses were rated 0-3 based on band intensity (Fig 4.4). The negative control rabbits remained negative throughout the study. At week one, antibodies to p19 were present in the serum at detectable levels in all infected rabbits. By week eight, the intensity of most bands had equilibrated (Fig 4.5). Valproic acid treatment did not appear to change the antibody response during the first eight weeks of infection.

HTLV-1 p19 production from ex vivo cultured PBMC

To monitor active virus replication in rabbits, ex vivo cultures of PBMC samples were obtained and supernatants tested for HTLV-1 p19 MA. In all three groups, there is a decrease in p19 production from week 1 to week 2. At week four and week six, Group 1 p19 levels increased while Group 2 and Group 3
remained static. Valproic acid treatment for one month resulted in increased p19 production (Fig. 4.6).

**Proviral copy numbers**

To measure proviral copy number in the PBMCs from each rabbit, genomic DNA was isolated from each sample of rabbit PBMCs. Fifty nanograms of DNA was probed for integrated provirus and normalized to rabbit GAPDH concentration for each sample. Proviral copy numbers of saline treated animals varied minimally during the eight week study. Group 1 rabbits proviral copy numbers were increased at week 1, 2, and 4, but decreased at week 6. The proviral load levels in this group rebounded at week 8 (one month removed from treatment). The proviral load set point in group 2 was greater than group 1 or group 3, but remained static after week 1.

### 4.5 DISCUSSION

Rabbits are the model used most often to study early HTLV-1 infection events, test viral transmission routes, and evaluate host immune responses against the virus infection. The rabbit model was used to confirm the modes of transmission in humans, as well as early studies of the immune response against the infection or to develop vaccines against HTLV-1 (19-24). Early studies utilizing the rabbit model of HTLV-1 helped confirm routes of viral transmission (21) and effective means to prevent the transmission of the virus (21;25-28).
Herein, we utilized the rabbit model to test the influence of VPA, a known modulator of histone acetylation, to test its influence on HTLV-1 expression in vivo. HTLV-1 expression is controlled by both methylation and histone acetylation (3-7). Both methylation and histone acetylation cause transcriptional silencing of HTLV-1 at the 5'LTR (8). In vitro, Mosley et al. estimated acetylation of histones controlled approximately 9% of the HTLV-1 proviral load (9). In addition, histone deacetylases such as HDAC1 decreased Tax mediated gene expression (10). Our data using the rabbit model of HTLV-1 infection indicate that VPA treatment at 70 mg/kg for four weeks caused a decrease in proviral load when administered during the first four weeks of infection. We also noted an increase in atypical lymphocytes in both group 2 and group 3 rabbits at one week after virus exposure. This increase was not observed in the VPA treated rabbits. In HTLV-1 infection, the presence of atypical lymphocytes is usually indicative of transformed T cells and is one of the diagnostic criteria for ATL (29). These cells have marked chromosomal abnormalities and aneuploidy linked to the expression of Tax (30). Atypical lymphocytes may also be present in the cerebrospinal fluid (CSF) of HAM/TSP patients (31). While the presence of atypical lymphocytes in rabbit blood smears has not been documented with HTLV-1 infection before, it can be inferred that the increase in atypical lymphocytes with HTLV-1 infection is expected due to increased Tax expression and viral replication. The static nature of the atypical lymphocytes in group 1 may correlate to the subsequent decrease in proviral load observed at week 6.
Our findings parallel and support previous studies of bovine leukemia virus infection in sheep (32). In BLV-infected sheep administered 80 mg/kg VPA three times per week for one month, all sheep seroconverted at similar times and had similar hematologic values. The authors did recognize a slight reduction in proviral load, although the sample size was too small to draw a statistical comparison (32).

In parallel to human clinical trials, our data indicated an early increase of proviral copy number in PBMC in response to VPA followed by reductions. Patients administered VPA demonstrated a transient increase in proviral load, which lasted approximately 45 days prior to reductions (16). In addition, proviral loads of patients in this study that were treated with VPA fluctuated more than untreated controls. Consistent with this study, our group 1 rabbit’s proviral load fluctuated more than either group 2 or group 3.

HTLV-1 expression is controlled by both methylation and histone acetylation (3-7). Both methylation and histone acetylation cause transcriptional silencing of HTLV-1 at the 5’LTR (8). Acetylation of histones has been estimated to control approximately 9 % of the HTLV-1 proviral load in vitro (9). In addition, histone deacetylases such as HDAC1 decreased Tax mediated gene expression (10).

Controlling viral expression is critical to the outcome of HTLV-1 infection. Increased viral expression and corresponding proviral copy numbers in circulating lymphocytes has been associated with lymphocyte-mediated disease
syndromes associated with HTLV-1 infection. The goal of VPA therapy in early
HTLV-1 infection and in HAM/TSP is to force the virus out of latency and expose
it to the immune response. Expression of viral proteins causes an increase in
immune surveillance and destruction of HTLV-1 infected cells by cytotoxic T-
cells. During two months of treatment with VPA, HAM/TSP patients exhibited a
transient increase in proviral load followed by decreased proviral load (16).

Histone deacetylase inhibitors are not specific and have widespread
acellular effects. Concurrent immune response to HTLV-1 infected cells
complicate interpretation of the effects of HDACi on proviral copy numbers in
patients and in our infected animals. In vitro, Mosley et al., (9) demonstrated that
HDACi decreased the efficiency of CTL killing of HTLV-1 infected cells, possibly
by inhibition of HDAC-6 in mediating cell-to-cell contact. Because of the effect on
the CTL, they concluded that HDACi might not be efficient in controlling proviral
load and a poor therapy.

Our experiments provide data to assist in the design of future studies to
test the effects of HDACi on HTLV-1 early infection. Future studies to optimize
the duration of treatment in the rabbit model, as well as the response to removal
of VPA are warranted. Our data indicate early and prolonged intervention with
HDACi may be needed to control proviral load.
4.6 REFERENCES


**Figure 4.1 Experimental design for rabbit inoculation and treatment with VPA.** Twenty New Zealand white rabbits were inoculated with either R49 or Jurkat cells. At 0, 1, 2, 4, 6, 8 weeks, whole blood was collected from the central auricular artery and processed for hematology, VPA levels, humoral response, viral load, and viral production.
Figure 4.2 Linear timeline of valproic acid administration. All rabbits were inoculated with R49 or control cells at week 0. Group 1 received VPA starting at week zero, three times per week ending at week four. Group 2 received VPA beginning at week four of infection through week eight. Group 3 received saline beginning at week zero through week four.
Figure 4.3 Morphology of atypical lymphocytes. Atypical lymphocytes are larger than normal and had at least two nuclear indentations that were greater than 1/3 of the nuclear diameter. Panel A is a normal small lymphocyte. Panel B represents a reactive lymphocyte. Panel C is a representative atypical lymphocyte.
**Figure 4.4** Hematologic values by differential cell count.  

A: Percent of white blood cells that are lymphocytes. There is a marked variation in the percent lymphocytes in group 1. This fluctuation is not observed in group 2 or group 3. Grey bars denote duration of VPA treatment.  

B. Percent Atypical lymphocytes. Atypical lymphocyte percentages increase at week 1 in both group 2 and group 3. This is not observed in group 1.
Figure 4.5 Rabbit serum response to HTLV-1. The antibody response to HTLV-1 infection in the rabbits at eight weeks is similar among the groups. Rabbits 4 and 8 were inoculated with Jurkats and remained uninfected throughout the study.
Figure 4.6 Grading of western blot strip intensity. Four serum antibody responses were measured at each timepoint in each infected rabbit. Responses were rated 0-3 based on intensity. Solid lines represent group 1. Dotted lines represent group 2. Dashed lines represent group 3.
Figure 4.7: Proviral load varies with time. Proviral load in group 1 fluctuates more than proviral load in group 2 or group 3.
Figure 4.8 p19 production in the rabbits. Ex vivo viral production, as measured by p19 ELISA. There is a general decrease in p19 production with time in all groups.
CHAPTER 5
SYNOPSIS AND FUTURE DIRECTIONS

5.1 Animal Models of ATL

Adult T-cell leukemia/lymphoma (ATL) is a refractory CD4/CD25+ CD8- T-cell malignancy caused by human T-lymphotrophic virus type 1 (HTLV-1) (1). Persistent HTLV-1 infections are established following transmission from infected mothers to their children, through breast feeding, and horizontally, through transfer of contaminated whole blood products. In approximately 1 to 5% of persistently infected individuals, clinical disease may manifest as ATL or other lymphocyte mediated disorders including the neurologic disease tropical spastic paraparesis (HAM/TSP) (2).

The mechanism of carcinogenesis induced by HTLV-1 infection is not completely understood, but it is thought to occur following viral induced cell dysregulation and accumulation of other transformation events. ATL is classified into four primary clinical subtypes. The two most aggressive forms are the acute and lymphomatous (3). These forms are associated with humoral hypercalcemia of malignancy (HHM), bone lysis, lymphadenopathy, visceral and skin
involvement. The lymphomatous form typically is associated with a normal peripheral blood leukocyte cell count, whereas in the acute form, ATL cells are found in high numbers in the peripheral blood. The more indolent clinical subtypes are the smoldering and chronic forms.

In this thesis we have investigated the use of targeted therapeutics in a preclinical, NOD/SCID ATL mouse model and in a rabbit model of HTLV-1 infection. The number of available mouse models of ATL is limited, in part, due to the lack of ATL cell lines obtained directly from patients. HTLV-1 cell lines transformed in cell culture systems often fail to engraft in immunodeficient mice (4-7). The mouse model chosen (MET-1 cells in NOD/SCID mice, (8) has as aggressive but consistent phenotype with all mice succumbing to ATL-like disease within 9 -12 weeks. A distinct advantage of this animal model is the use of a biomarker to noninvasively measure tumor burden by measuring the human specific serum IL-2Rα levels. Unfortunately, the MET-1 cell line does not grow in vitro so compounds cannot be tested in this cell line prior to administration in the mouse. An ideal mouse model would be one in which we could test compounds in vitro and explore mechanisms of action in vitro prior to the use of the same cell line in the immunodeficient mouse. This would help predict behavior and would also confirm in vitro that the in vivo effects are due to the expected targeting. Generation of new mouse models of ATL is dependent on procuring leukemic cells from infected patients and inoculating them into immunodeficient mice. In our experience, not every patient sample will grow and engraft in the
immunodeficient mouse. If the ATL cells do engraft in the immunodeficient mouse, they may not engraft on subsequent passage through additional mice and they may not grow in vitro. This is why the development of additional ATL cell lines is paramount.

5.2 Targeted Therapies Against ATL

Adult T-cell leukemia/lymphoma is a refractory malignancy with a uniformly poor prognosis regardless of therapy in its acute form (9-13). The acute form of ATL is a refractory malignancy with a median survival time of less than one year regardless of therapy (14). Adult T-cell leukemia/lymphoma-derived cells have multiple ways of surviving and persisting in presence of chemotherapy. While, HTLV-1 is clearly linked to the development of ATL, active virus replication is not required to maintain tumor growth. The outgrowth of ATL cells is believed to be initiated by key viral proteins such as Tax, but is more dependent upon genetic or epigenetic changes of the host cell for eventual selection of clonally expanded malignant lymphocytes. Over expression of the multidrug resistance protein has been reported in ATL (15;16) further complicating treatment options.

Conventional chemotherapies against ATL have produced inconsistent or limited results in treatment outcome and prolongation of mean survival times (reviewed in (1;13;17). A combination of Cyclophosphamide, Adriamycin, vincristine, and prednisolone is typically the first line of therapy, but is of limited
efficacy. The use of nucleoside analogs, interferon, zidovudine, topoisomerase inhibitors, and monoclonal antibodies has produced more encouraging results; however the prognosis for ATL patients remains poor. Bone marrow transplant has been reported to “cure” ATL (18). This is complicated by patient age, condition, and likelihood of immunologic match and therefore is of limited use.

With the development of the ATL mouse model, preclinical efficacy studies in the mouse have provided insight into the use of targeted chemotherapy. These compounds take advantage of the genetic or epigenetic alterations that are consistently found in the neoplastic cells but not the normal cells of the body. This provides specificity for the neoplastic population that is not found with the standard cytotoxic chemotherapies.

5.3 The Need for Prolonged Dosing to Achieve Efficacy in ATL

In the previous chapters, we demonstrated a marked in vitro efficacy of preclinical compounds. The compounds tested in vitro were tested in the NOD/SCID mouse model of ATL with varied success. In Chapter 2, we showed that the combination of PS-341 and 17-AAG decreased tumor burden in the mice when measured immediately after treatments. Phase II studies in which PS-341 and 17-AAG were withdrawn and mice were allowed to survive until removal criteria were met. Extending the study resulted in a dampening of the benefits of combination therapy.
In chapter 3, we tested the novel histone deacetylase inhibitor, OSU-HDAC42, in vitro in ATL cell lines and in the NOD/SCID ATL mouse model. In the in vitro experiments, cell death and histone hyperacetylation was evident. Translating this to the in vivo model was less clear. In phase I of this study, mice dosed by gavage and removed from therapy did not have a significantly different change in survival when compared to the vehicle only treated mice. In phase II, we used daily in feed dosing to provide a more consistent and longer term dosing of the mice. While the change in survival was not statistically significant, this was likely due to sample size. One might infer from the Kaplan Meier curve of the in feed study that with an expanded sample size there would be a significant effect on survival.

Based on the information provided by the previous chapters, investigation of long term therapy for ATL is warranted. Our model indicates that removal of compounds diminishes the benefits of the targeted therapy.

Several other neoplastic diseases require long term adjuvant therapy to prolong survival. As early at 1974, long term chemotherapy was suggested in breast cancer (19) and is now one of the mainstays of estrogen receptor positive breast cancer (20).

5.4 Evaluation of Combinational Therapy Against ATL

In chapter 2 of this thesis we present data to indicate that the combination therapy of PS-341 and 17-AAG when tested both in vitro and in an established
ATL mouse model is effective to block the growth of ATL cell lines and reduce tumor burdens mice. Using HTLV-1 transformed cell lines, we found that the addition of 17-AAG to PS-341 induced apoptosis. Our data is the first to demonstrate that treatment with PS-341 in combination with 17-AAG reduced the tumor burden of established ATL cells in a mouse model. Of significance, our survival studies in ATL engrafted mice indicated that the differences observed between the treatment groups were minimized upon removal of drug therapy. Thus, while this combination of targeted therapy offers promise to decrease tumor burden, our results indicate that additive effects of 17-AAG are transient and require continued presence of the drug.

The effect of PS-341 on the growth of tumors in a Tax transgenic mouse model revealed no consistent inhibition of NF-κB activation in vivo, however transplanted Tax tumors in Rag-1 mice showed consistent inhibition of tumor growth and prolonged survival (21). In a separate study, PS-341 decreased tumor burden in ATL xenograft mice (22). In the MET-1 ATL mouse model, PS-341 was not beneficial alone, however enhanced efficacy was noted in combination with humanized anti-Tac (CD25) monoclonal antibody(23). Our studies reveal that PS341 and 17-AAG, while effective at reducing established tumor burdens in short term studies, our survival study indicated a need for continuous drug pressure to maintain the additive benefits of both drugs. Our design allowed us to test the effects of the continued pressure of the combination therapy. Our mouse model, while providing an excellent preclinical model in
which to assay the effects of the compounds on the ATL cells, does not assay the effects of prolonged therapy in context to ATL patients or in the presence of a functional immune system. Despite the limitations of the model system, our results have implications for the design of human trials using this combination of drugs or when considering 17-AAG or derivatives as adjunct therapy to standardized protocols. Our data support further investigations to test various dosing regimen and drug cycles of PS-341 and 17-AAG in both animal and human clinical trials. The website http://www.clinicaltrials.gov shows, current clinical trials using this combination include phase I dose escalation studies in leukemia, lymphoma, and small intestinal cancer.

### 5.5 HDACi in ATL Therapy

Our chapter 3 data presented in this thesis indicate that the use of HDACi is a potential effective treatment strategic against ATL. In vitro, both VPA and OSU-HDAC42 abate growth of ATL cell lines. Exposure of ATL cell lines to both VPA and OSU-HDAC42 resulted in hyperacetylation of histones and induction of apoptosis. Our data are the first to demonstrate that OSU-HDAC42 is safe and efficacious in the ATL NOD/SCID mouse model. In addition, our feed formulation design demonstrated that oral dosing of OSU-HDAC42 appeared to be more effective compared to oral gavage in improving survival of the engrafted mice.

Human T-lymphotropic virus type 1 is linked to the development of ATL; however, active virus replication is not required to maintain tumor growth. The
outgrowth ATL cells appear to be dependent on genetic and epigenetic changes of the host cell for selection of clonally expanded malignant lymphocytes. The refractory nature of ATL indicates that novel treatments are needed against the malignancy to more selectively target key cancer cell pathways to block tumor cell growth. Conventional chemotherapies against ATL have produced inconsistent or limited results against ATL (1). New therapeutic approaches have focused on targeted therapies.

Histone deacetylase inhibitors promote the acetylated state of histones and relax chromatin structure leading to induction of apoptosis, cell cycle arrest, disruption of Hsp90 and the aggresome, inhibition of angiogenesis, mitotic cell death, and senescence (reviewed in (24). In ATL cell lines, the histone deacetylase inhibitor FR901228 induced apoptosis and reduced tumor size in mice (25). In chapter 3 of this thesis, we compared two HDACi (VPA and OSU-HDAC42) for their ability to abate ATL cell growth and reduce tumor burdens in a mouse model. Recently, VPA has been shown to have efficacy in the treatment of cancer through the hyperacetylation of histones 3 and 4 (26). OSU-HDAC42 is a phenylbutyrate derived HDACi included in the Rapid Access to Intervention Development (RAID) program through the National Cancer Institute (27). In a prostate cancer model, OSU-HDAC42 was shown to decrease cell viability, decreased phospho-Akt, Bcl-xL, and Survivin and decreased tumor burden in a mouse model (28).
In our study, we demonstrated that OSU-HDAC42 while not improving survival in short term tumor engraftment studies did prolong survival when provided as a continuous feed supplement. Thus, OSU-HDAC42 was required on a more continuous basis to demonstrate antitumor activity. The addition of continued drug pressure for a prolonged time appeared to prolong survival and prevent tumor cells from regaining growth capacity. Expanding the numbers of mice to provide for statistically significant sample size for a second survival study is one logical continuation. It would also be valuable to design a study similar to the phase I study of chapter 2 in which the mice are euthanized at a single timepoint and the tumor burden is quantitated using histology and immunohistochemistry.

The mouse model, while providing an excellent preclinical model in which to assay the effects of the compounds on the ATL cell growth, does not assay the effects of prolonged therapy in context to ATL patients or in the presence of a functional immune system. Our results have implications for the design of human trials using OSU-HDAC42 as adjunct therapy to standardized protocols and support further investigations to establish more effective therapeutic dosing regimens and drug cycles of OSU-HDAC42 in both animal and human clinical trials. We have shown that shorter duration dosing may be more effective than every other day dosing.
5.6 Control of Proviral Load in HTLV-1 Infection

Rabbits are the model used most often to study early HTLV-1 infection events, test viral transmission routes, and evaluate host immune responses against the virus infection. The rabbit model was used to confirm the modes of transmission in humans, as well as early studies of the immune response against the infection or to develop vaccines against HTLV-1 (29-34). Early studies utilizing the rabbit model of HTLV-1 helped confirm routes of viral transmission (31) and effective means to prevent the transmission of the virus (31;35-38). Herein, we utilized the rabbit model to test the influence of VPA, a known modulator of histone acetylation, to test its influence on HTLV-1 expression in vivo. HTLV-1 expression is controlled by both methylation and histone acetylation (39-43). Both methylation and histone acetylation cause transcriptional silencing of HTLV-1 at the 5’LTR (44). Acetylation of histones has been estimated to control approximately 9 % of the HTLV-1 proviral load in vitro (45). In addition, histone deacetylases such as HDAC1 decreased Tax mediated gene expression (46). Our data using the rabbit model of HTLV-1 infection indicate that VPA treatment at 70 mg/kg for four weeks caused a decrease in proviral load when administered during the first four weeks of infection. We also noted an increase in atypical lymphocytes in both group 2 and
group 3 rabbits at one week after virus exposure. This increase was not observed in the VPA treated rabbits. In HTLV-1 infection, the presence of atypical lymphocytes is usually indicative of transformed T cells and is one of the diagnostic criteria for ATL (47). These cells have marked chromosomal abnormalities and aneuploidy linked to the expression of Tax (48). Atypical lymphocytes may also be present in the cerebrospinal fluid (CSF) of HAM/TSP patients (49). While the presence of atypical lymphocytes in rabbit blood smears has not been documented with HTLV-1 infection before, it can be inferred that the increase in atypical lymphocytes with HTLV-1 infection is expected due to increased Tax expression and viral replication. The static nature of the atypical lymphocytes in group 1 may correlate to the subsequent decrease in proviral load observed at week 6.

Our findings parallel and support previous studies of bovine leukemia virus infection in sheep (50). In BLV-infected sheep administered 80 mg/kg VPA three times per week for one month. All sheep seroconverted at similar times and had similar hematologic values. The authors did recognize a slight reduction in proviral load, although the sample size was too small to draw a statistical comparison (50).

In parallel to human clinical trials, our data indicated an early increase of proviral copy number in PBMC in response to VPA followed by reductions. Patients administered VPA demonstrated a transient increase in proviral load, which lasted approximately 45 days prior to reductions (51). In addition, proviral
loads of patients in this study that were treated with VPA fluctuated more than untreated controls. Consistent with this study, our group 1 rabbit’s proviral load fluctuated more than either group 2 or group 3.

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Controlling viral expression is critical to the outcome of HTLV-1 infection. Increased viral expression and corresponding proviral copy numbers in circulating lymphocytes has been associated with lymphocyte-mediated disease syndromes associated with HTLV-1 infection. The goal of VPA therapy in early HTLV-1 infection and in HAM/TSP is to force the virus out of latency and expose it to the immune response. Expression of viral proteins causes an increase in immune surveillance and destruction of HTLV-1 infected cells by cytotoxic T-cells. During two months of treatment with VPA, HAM/TSP patients exhibited a transient increase in proviral load followed by decreased proviral load (51).

Histone deacetylase inhibitors are not specific and have widespread cellular effects. Concurrent immune response to HTLV-1 infected cells complicate interpretation of the effects of HDACi on proviral copy numbers in patients and in our infected animals. In vitro, Mosley et al., (45) demonstrated
that HDACi decreased the efficiency of CTL killing of HTLV-1 infected cells, possibly by inhibition of HDAC-6 in mediating cell-to-cell contact. Because of the effect on the CTL, they concluded that HDACi might not be efficient in controlling proviral load and a poor therapy.

Our experiments provide data to assist in the design of future studies to test the effects of HDACi on HTLV-1 early infection. Future studies to optimize the duration of treatment in the rabbit model, as well as the response to removal of VPA are warranted. Our data indicate early and prolonged intervention with HDACi may be needed to control proviral load.

5.7 Summary and impact of work

In the preceding four chapters, we have studied the effects of targeted therapies on HTLV-1 infection and disease. Conventional chemotherapy has shown little improvement over no treatment in ATL and HAM/TSP. The development of targeted therapeutics is necessary to combat these diseases. Our work serves as a stepping stone to larger scale animal studies and supports the ongoing Phase I clinical trials for several therapeutic regimens.
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