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Efficacy of Novel Histone Deacetylase Inhibitor, AR42, in a Mouse Model of Human T-lymphotropic Virus Type 1 Adult T cell Lymphoma

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Abstract

Human T-lymphotropic virus type 1 (HTLV-1) causes a variety of forms of adult T-cell leukemia/lymphoma (ATL), a refractory CD4+/CD25+ T-cell malignancy. Novel approaches to treat ATL patients are required due to the resistance of ATL to conventional chemotherapies. Histone deacetylase inhibitors (HDACi), which induce histone hyperacetylation leading to chromatin remodeling and reactivation of transcriptionally repressed genes have shown efficacy against a variety of cancers. Herein, we tested if valproic acid and the novel orally bioavailable HDACi, AR-42 reduced the proliferation of ATL cell lines by promoting apoptosis and histone hyperacetylation. Both compounds were cytotoxic and elicited a dose dependent increase in cytochrome C and cleaved Poly (ADP-ribose) polymerase (PARP) indicating the induction of cell death by apoptosis and promoted acetylation of histone H3 in both MT-2 and C8166 cell lines. We then evaluated the effects of AR-42, for survival in an ATL NOD/SCID mouse model. A dietary formulation of AR-42 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 AR-42 against HTLV-1-associated lymphoid malignancies.

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Authors' contributions M.L., B.Z., C.S.C. and A.S. provided conception and design of study; K.L., B.Z., and S.F. provided acquisition of data; B.Z., A.S., S.F. and M.L. provided analysis and interpretation of data and all authors made substantial contributions by drafting the article, revising it critically for important intellectual content and gave final approval of manuscript submitted.

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Keywords

Human T-lymphotropic virus type 1 (HTLV-1); adult T-cell leukemia/lymphoma; histone deacetylase inhibitors (HDACi); therapy; apoptosis

1. 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 infect 15 to 20 million people worldwide and is endemic in southern Japan, the Caribbean basin, central Africa, in some Amerindian populations in Central and South America, remote groups in the Melanesian Islands in the Pacific basin, and some aboriginal populations in Australia [reviewed in [1, 2]]. Approximately 3 to 5 % of HTLV-1-infected individuals develop some form of ATL typically late in life (40 years and older) and present with malaise, fever, lymphadenopathy, hepatosplenomegaly, jaundice, weight loss, and various opportunistic infections, such as *Pneumocystis carinii* [reviewed in [2]]. Four stage classifications have been used to categorize HTLV-1-associated cancer: acute, smoldering, chronic and lymphoma stages [reviewed in [3]]. Acute ATL is highly refractory to standard chemotherapeutic approaches and patients exhibit hypercalcemia of malignancy, lytic bone lesions, elevated lactate dehydrogenase (LDH), and soluble interleukin-2 (IL-2) receptor in their serum [reviewed in [4]].

Due to the low penetrance and prolonged latency period (up to 70 years) between infection and clinical disease, it is postulated that genetic and epigenetic alterations precede the development of ATL [5]. Transcriptional regulation at the chromosomal level can be modified by the acetylation of histones and non-histone proteins resulting in the relaxation of chromatin and the promotion of transcription. This process is controlled, in part, by histone deacetylases (HDACs) that promote the removal of the acetyl groups from lysines on histones, restoration of positive charges, and condensation of DNA around the histone core decreasing transcription of affected genes. Histone deacetylases may also functionally influence non-histone proteins such as hormone receptors, chaperones (heat shock proteins), viral proteins, and cytoskeletal proteins [6].

In contrast, the inhibitors of HDACs (HDACi) promote the acetylated state of histone proteins and relaxed chromatin structure. The role of HDACi in cancer therapy has been reviewed [7, 8]. The HDACi 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 particular HDAC isoenzymes [9].

Recently, HDACi have been used as targeted therapies in cancer research [reviewed in [6, 7]]. Depsipeptide (FR901228) has been used in the treatment of peripheral and cutaneous T-cell lymphoma [10] and several other formulations of HDACi are in phase I and phase II clinical trials [11]. The HDACi anti-cancer properties are based on their ability to induce apoptosis, promote cell cycle arrest, disrupt Hsp90 and the aggresome, inhibit angiogenesis, trigger mitotic and autophagic cell death and promote senescence. The mechanism of HDACi induced cell death may vary depending on downstream targets of the HDAC in a particular type of cancer. For example, we have reported the cytotoxic effect of a novel HDACi, AR42, in malignant melanoma cell lines due to induction of apoptosis due to

caspase activation, in contrast to suberoylanilide hydroxamic (SAHA), which appears to be calpain-dependent [12].

Herein, we evaluated the histone deacetylase inhibitors valproic acid (VPA) and the novel agent AR-42 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, caused dose dependent increases in cytochrome C, cleaved Poly (ADP-ribose) polymerase (PARP) and promoted acetylation of histone H3 in both MT-2 and C8166 cell lines. We then tested the efficacy of AR-42 in an established NOD/SCID mouse ATL model. Our data demonstrate that a dietary formulation of AR-42 prolonged the survival of mice engrafted with ATL cells. Our findings support further development of AR-42 against HTLV-1-associated lymphoid malignancies and demonstrate the efficacy of this novel oral bioavailable HDACi in this refractory T-cell lymphoma.

2. Materials and Methods

2.1. Cell lines

The HTLV-1 infected cell lines C8166-45[13], MT-2[14], 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 [15]. 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 2×10^7 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.

2.2. 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 $20 \pm 2^\circ\text{C}$ 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 2×10^7 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-2R α 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 AR-42 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 2×10^7 MET-1 cells. After the engraftment period of 21 days, the mice were fed an AIN-76A rodent diet with or without 208 ppm AR-42. Dietary intake was monitored by weight of diet consumed [16].

To ascertain differences in survival between treatment groups, mice were continually monitored until 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).

2.3. Compounds

AR-42 (Arno Therapeutics, Parsippany, NJ) was obtained from 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, AR-42 was prepared as a suspension in 0.5% methylcellulose and 0.1% Tween 80 in sterile water. The AR-42 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.

2.4. Growth Inhibition Assays

Forty thousand cells were plated in each well of a 96 well plate and incubated with media only, 1, 2.5, or 5 mM VPA or 0.5, 1, or 2.5 μ M OSU-HDAC 42. Concentrations of AR-42 and VPA were chosen based on the previous literature [16-18]. After 24, 48, and 72 hours, 20 μ l of MTS reagent (Cell Titer 96® AQueous, 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.

2.5. Western blot assays

Cells (2×10^6) were incubated with media only, 1, 2.5, or 5 mM valproic acid or 0.5, 1, or 2.5 μ M OSU-HDAC 42 for 24 hours. Cells were washed once in PBS and lysed. Aliquots of protein (20 μ g) 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 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, Tax (AbCam #26997), 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). Western blot analysis was used to evaluate the expression of Tax in MT-2 and C8166 cell lines as described [19].

2.6. 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.

2.7. Serum IL-2R α

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-2R α), 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.

2.8. 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. Time and also the interaction time by concentration were included in the models to study the change over time and whether this change was different depending on the concentration. The numbers of multiple comparisons were adjusted by Tukey's method. Kaplan-Meier curves were compared using the log-rank test for survival analyses. Cox regression models were performed to compare the effects of histone deacetylases on survival time.

3. Results

3.1. AR-42 and valproic acid significantly inhibit growth of ATL cell lines

The histone deacetylase inhibitors AR-42 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 a standard control to represent a CD4+ transformed cell line, but HTLV-1-negative.

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. 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 ($p < 0.0001$).

AR-42 inhibited growth in all three cell lines tested. In the C8166 cell line, there was a statistically significant decrease in growth with 0.5 μ M concentration and 48 hours duration ($p = 0.0011$). In MT-2 cells, a statistically significant decrease in growth was observed with 0.5 μ M AR-42 ($p < 0.0001$). Jurkat T-cells were significantly inhibited in growth at the 2.5 μ M AR-42 concentration ($p = 0.0001$) (Fig. 1B). Both drugs did not increase HTLV-1 Tax expression in MT-2 and C8166 cells, which maximally express the viral protein (Fig. 1C). Decreased Tax expression observed in C8166 cells is likely due to the cytotoxic effects of the drugs.

3.2. AR-42 and valproic acid induces apoptosis and hyperacetylation of histones

Whole cell lysates of C8166 and MT-2 cells treated with VPA and AR-42 for 24 hours were tested for hyperacetylation of histones and HDACi affects on non-histone target proteins by western immunoblot assay (Fig. 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. 2A and B). Collectively our western immunoblot data indicated that both VPA and AR-42 targeted the appropriate pro-apoptotic pathways to limit cell proliferation and justified in vivo studies with our NOD/SCID ATL mouse model.

3.3. AR-42 prolongs survival in the ATL NOD/SCID mouse model with continued drug administration

We utilized a previously established NOD/SCID ATL mouse model [15] to test the efficacy of AR-42 in vivo (Fig 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 AR-42 or vehicle alone respectively three times per week for four weeks. Serum was obtained every 14 days to measure the serum biomarker for tumor progression (serum IL-2R α). After one month, AR-42 was discontinued and mice were monitored for development of removal criteria. Mice treated with AR-42 tended to have lower serum IL-2R α levels, but did not differ in time to removal (data not shown).

During phase II of our study, we used a dietary formulation of AR-42 and compared it to an isocaloric diet lacking drug. Mice were inoculated intraperitoneally with 2×10^7 MET-1 cells and allowed three weeks to engraft. To ascertain differences in survival between treatment groups, mice were continually monitored until euthanasia criteria were met. Mice were euthanized 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). At necropsy, tumor engraftment was confirmed in all mice by histologic examination. Neoplastic round cells are present in the brain (meninges), stomach, heart, lung, kidney, and liver (Fig. 4). At three weeks post inoculation, mice were given free choice access to either the control diet or the AR-42 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. 5). Serum levels of IL-2R α did not differ significantly between the groups (Fig. 6A), however mice treated with AR-42 overall survived longer than the vehicle only treated group (Fig. 6B). Collectively our data support the efficacy of this novel oral bioavailable HDACi in this refractory T-cell lymphoma.

4. Discussion

Our preclinical data indicate that HDACi may be an effective treatment for ATL. In vitro, both VPA and AR-42 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 AR-42 induced apoptosis. This was indicated by accumulation of cytochrome C and increased PARP cleavage. We are the first to demonstrate that AR-42 was safe and efficacious in the ATL NOD/SCID mouse model. In addition, our feed formulation design demonstrated that oral dosing of AR-42 was more effective compared to oral gavage in improving survival of the engrafted mice. In our study, we demonstrated that the novel and oral bioavailable drug, AR-42, prolonged survival when provided as a continuous feed supplement. We postulate that the addition of continued drug treatment (versus intermittent dosing) prolonged survival and prevented tumor cells from regaining growth capacity. These findings support the further development of AR-42 against HTLV-1-associated lymphoid malignancies.

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 [20]. New therapeutic approaches have focused on targeted therapies [reviewed in [4]]. In vitro and in vivo studies with targeted therapies have included monoclonal antibodies (alemtuzumab [21], Anti-Tac [22]), NF- κ B

inhibitors [23, 24], an Aurora A kinase inhibitor, [25] and HDACi [26, 27]. The p53 status of the cell lines used in our study has been published [28]. Our cell lines while expressing p53 have functionally impaired p53 activity. This has been attributed to Tax expression in MT-2 and C8166 cell lines. Interestingly, a recent study indicates that small molecule drugs that increase p53 activity in these cell lines is correlative to apoptotic activity further supporting the use of HDAC inhibitors in ATL [28].

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 [6]]. In ATL cell lines, the histone deacetylase inhibitor FR901228 induced apoptosis and reduced tumor size in mice [26]. In our study, we compared two HDACi (VPA and AR-42) 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 [29]. Recently, it has been shown to have efficacy in the treatment of cancer through the hyperacetylation of histones 3 and 4 [30]. VPA inhibits prostate cancer cell growth [31] medulloblastoma [32], and multiple myeloma [33] in vitro. AR-42 is a phenylbutyrate derived HDACi included in the Rapid Access to Intervention Development (RAID) program through the National Cancer Institute [17], which has potent activity in vitro and in vivo in multiple tumor cell lines. In a prostate cancer model, AR-42 was shown to decrease cell viability, decreased phospho-Akt, Bcl-xL, and Survivin [16]. In vivo, a prostate cancer xenograft mouse model had decreased tumor burden [16] and decreased tumor levels of phospho-Akt and Bcl-xL in response to the drug [17].

Our results have important implications for the design of human trials using AR-42 as adjunct therapy to standardized protocols and support further investigations to establish more effective therapeutic dosing regimens and drug cycles of AR-42 in both animal and human clinical trials against T-cell lymphomas.

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Figure 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 AR-42. At 24, 48, and 72 hours, growth was measured by the addition of MTS reagent and absorbance at 490 nm. C. Immunoblot analysis of HTLV-1 Tax expression in MT-2 and C8166 cells exposed to VPA and AR-42.

Figure 2. Western blot assay indicating induction of cell death by apoptosis and promoted acetylation of histone H3 in ATL cell lines

MT-2 cells were treated with media only, 1, 2.5, 5 mM valproic acid or 0.5, 1, 2.5 μ M AR-42 and tested at 24 hours following treatment. 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 μ M AR-42. Cells showed a dose dependent increase in cleaved PARP, cytochrome C, and acetylated histone H3.

Figure 3. Diagram of dosing schedule

In phase I, mice were treated by gavage with 50mg/kg AR-42 or 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 AR-42 or vehicle in diet at day 21.

Figure 4. Engraftment of MET-1 cells in multiple organs

Examples of NOD/SCID mice inoculated intraperitoneally with 2×10^7 MET-1 cells. Neoplastic round cells in the brain (meninges), stomach, heart, lung, kidney, and liver. All tissues magnified 200X, bar denotes 50 µm.

Figure 5. Daily feed 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. In both instances, the mice were allowed to survive until removal criteria were achieved.

Figure 6. Dietary AR-42 enhanced survival in the NOD/SCID ATL mouse model

A.) Serum IL-2R α levels of mice during the Phase II study. Solid squares indicate values for mice administered vehicle only. Open circles indicate values for mice administered AR-42.

B.) Kaplan Meier curve for Phase II in vivo experiments. The solid line represents control mice. Dotted line represents prolonged survival AR-42 treated group.