Depsipeptide (FR901228) Induces Histone Acetylation and Inhibition of Histone Deacetylase in Chronic Lymphocytic Leukemia Cells Concurrent with Activation of Caspase-8 Mediated Apoptosis and Down Regulation of c-FLIP Protein

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Abstract

Depsipeptide is in clinical trials for chronic lymphocytic leukemia (CLL) based upon earlier observations demonstrating selective \textit{in vitro} activity in this disease. We sought to determine the relationship of histone H3 and H4 acetylation, inhibition of histone deacetylase, and apoptosis observed in CLL cells to justify a pharmacodynamic endpoint in these clinical trials. Herein we demonstrate that \textit{in vitro} depsipeptide induces histone H3 and H4 acetylation and histone deacetylase enzyme inhibition at concentrations corresponding to the LC$_{50}$ (concentration producing 50% cell death) for cultured CLL cells (0.038 µM depsipeptide). The changes in histone acetylation are lysine-specific, involving H4 K5, H4 K12, and H3 K9, and to a lesser extent H4 K8, but not H4 K16 or H3 K14. Depsipeptide-induced apoptosis is caspase-dependent, selectively involving the TNF-receptor (extrinsic pathway) initiating caspase 8 and effector caspase 3. Activation of caspase 8 was accompanied by the down-regulation of FLICE-inhibitory protein (c-FLIP, I-FLICE) without evidence of Fas (CD95) up-regulation. Change in other apoptotic proteins including Bcl-2, Bax, Mcl-1, and XIAP was not observed. Collectively, our results demonstrate a relationship between target enzyme inhibition of histone deacetylase, histone H3 and H4 acetylation and apoptosis involving the TNF-receptor pathway of apoptosis that is not utilized by other therapeutic agents in CLL. These data provide further support for clinical trials of depsipeptide in CLL and suggest usage of histone H3 and H4 acetylation, inhibition of histone deacetylase, and down-regulation of FLIP as pharmacodynamic endpoints for evaluation of this drug in patients \textit{in vivo}. 
Introduction

Chronic lymphocytic leukemia (CLL) is one of the most common types of leukemia diagnosed in the Western Hemisphere, with 7,000 projected new cases in the United States in the year 2002 and a nationwide prevalence of approximately 50,000 persons (1). CLL is characterized by disrupted apoptosis, as opposed to proliferation, leading to a gradual accumulation of leukemia cells eventually producing symptoms related to cytopenias or organomegaly (2,3). A small proportion of CLL patients have indolent disease, but the majority of patients either present with advanced stage disease or progress to the state of requiring treatment. The traditional therapeutic approach to CLL has been to utilize chlorambucil or fludarabine as initial therapy, although recently completed studies favor the latter with improved response rate and progression free survival over alkylator-based regimens (4,5).

Unfortunately, the majority of patients either fail to gain a complete response to fludarabine therapy or eventually relapse. This emphasizes the importance of focusing upon identification of new and specific therapies for CLL. Ideally, such treatments would work through activation of an apoptotic pathway different from standard regimens and would involve a specific drug target for which the minimally effective pharmacologic dose can be determined in vivo, thus avoiding unnecessary toxicity. Current treatments for CLL (e.g., fludarabine, cladribine, and alkylator-based therapies) induce apoptosis via a mitochondria-dependent pathway involving activation of the protease caspase 9 (6-9). An alternative pathway for apoptosis involves cell death protease caspase 8 (FLICE) and is triggered by Fas (CD95) and other tumor necrosis factor (TNF) receptor family members. This cytokine-mediated pathway is generally not functional in B-cell CLL (9-11). Attempts to increase expression of the Fas receptor on CLL cells with CD40 ligand (CD40L)
or bryostatin have been successful (12), but these leukemic cells remain resistant to Fas-mediated ligation, implying a post receptor block to apoptosis. Since defects in the mitochondrial pathway of apoptosis are likely to exist in chemotherapy refractory CLL, identification of agents that exert their cytotoxic effect via the FAS/TNF receptor pathway of apoptosis would represent a major therapeutic advance for the treatment of patients with CLL and present a new opportunity for combination therapies.

Depsipeptide, a bicyclic depsipeptide currently in phase I clinical trials (13, 14), is one such unique compound that has promise for CLL patient therapy. Our group recently reported (15) that this agent exhibits selective cytotoxicity toward CLL B-cells as compared to both normal mononuclear cells and bone marrow progenitor cells, with maximal cytotoxicity observed following a 4-hour drug exposure. Others have demonstrated that depsipeptide has selective cytotoxicity toward drug-resistant P388 leukemia cell lines as compared to non-resistant P388 cells, causing down-regulation of c-myc and morphologic normalization of Ras-transformed cells (16-19). In such dividing cell lines, it appears that depsipeptide mediates its effects through inhibition of the enzyme histone deacetylase (20). Indeed, histone acetylation and DNA methylation are two of the primary mechanisms that control gene transcription (21-25). Inhibition of histone deacetylase (HDAC) in tumor cell lines potentially activates differentiation-related or tumor suppressor genes by removing transcriptional repression (21, 22).

The relationship between alteration of histone acetylation and apoptosis by depsipeptide observed in non-dividing CLL cells is presently uncertain. We therefore studied histone acetylation and HDAC inhibition at depsipeptide concentrations that result in increased apoptosis
in cultured CLL cells. We also further dissected the pathway of apoptosis utilized by depsipeptide and examined its treatment effect on levels of select apoptotic regulatory proteins.

**Materials and Methods**

**Patients, Cell Separation, and Culture Conditions**

Approval for patient blood collection was obtained from The Ohio State University Institutional Review Board for these studies. Informed consent was provided according to the Declaration of Helsinki. Cells were procured from patients previously diagnosed with CLL as defined by the modified NCI criteria (26). All of the CLL patients had been without prior therapy for a minimum of two months. Mononuclear cells were isolated from the peripheral blood utilizing density gradient centrifugation (Ficoll-Paque Plus, Pharmacia Biotech, Piscataway, NJ). Isolation of mononuclear cells in this manner provides >90% positive co-expressing CD19 and CD5 clonal B-lymphocytes. HeLa (CCL-2) and K562 (CCL-243) cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin-G, 100 µg/ml streptomycin, and 2 mM L-glutamine (Life Technologies, Grand Island, NY). The broad caspase inhibitor Z-VAD-fmk was obtained from Kamiya Biochemical, Seattle, WA. Depsipeptide (FR901228 or NSC649890) and 2-F-ara-A were obtained from the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute.

**Apoptosis and Flow Cytometric Studies**

Apoptosis was assessed in permeabilized cells using a phycoerythrin (PE)-labeled anti-active Caspase-3 polyclonal antibody (BD Pharmingen, San Diego, CA) that is specific for the
active form of this enzyme. After drug treatment, the cells were washed twice in cold PBS. Cells were fixed for 20 minutes with cold Cytofix/Cytoperm solution (BD Pharmingen, San Diego, CA). Cells were then washed twice with Perm/Wash buffer (BD Pharmingen, San Diego, CA) and then stained with the PE-labeled anti-active Caspase-3 antibody for 30 minutes at room temperature. After a final wash, the cells were analyzed by flow cytometry.

Cationic dyes such as rhodamine-123 readily accumulate in actively respiring mitochondria to a degree dependent of the mitochondrial membrane potential. Thus, rhodamine-123 can be used to monitor the integrity of mitochondria following depsipeptide treatment. Media and depsipeptide treated cells were washed once in RPMI 1640 media and then incubated in RPMI 1640 media containing 50 ng/ml rhodamine-123 (Molecular Probes, Eugene, OR) for 30 minutes at 37°C. Stained cells were washed once in RPMI 1640 media, placed on ice, and then quickly analyzed by flow cytometry.

The surface expression of CD95 on CLL cells was assessed with anti-CD95 PE and anti-CD19 FITC antibodies with appropriate isotype controls (Becton Dickinson). Cells were washed with PBS and then analyzed by flow cytometry.

Histone Extraction

For acetylated histone studies, approximately 5 x 10^7 human CLL cells were used. Nuclei were isolated after 10 min incubation with NIB + TritonX (10mM Tris pH 7.5, 1.5mM MgCl₂, 1.0mM CaCl₂, 2.0mM ZnSO₄, 0.25M sucrose, 0.2mM PMSF, 0.5% Triton–X 100). Cells were transferred to a teflon homogenizer and broken with 20 strokes. Nuclei were pelleted by centrifugation at 2000 x g for 5 min. Nuclear pellets were washed twice in NIB. The washed nuclear pellets were resuspended in 1 ml of 0.4N sulfuric acid and incubated on ice 30 min.
followed by a 20 min. centrifugation at maximum speed in a microfuge. 100% TCA was added to
the supernatant to a final concentration of 20% TCA, and this was incubated at least 30 min. on
ice. After centrifuging for 10 min. at maximum speed in a microfuge, cold acetone washes with
shaking were used to remove salts from the pellet. Histones were then removed from the pellet
by shaking using 0.5ml distilled water and were stored at –20C.

**Western Blot Analysis**

Whole cellular lysates were prepared as previously described (28). Total protein in each
sample was quantified by the BCA method (Pierce, Rockford, IL). Lysates or extracted histones
were analyzed by SDS-PAGE/immunoblotting with antibodies recognizing acetylated or
methylated histones (Upstate, Lake Placid, NY) which are specific for various histone acetylation
or histone methylation sites (Serotec, Raleigh NC), pro-caspase-3 (Santa Cruz Biotechnology,
Santa Cruz, CA), caspase-8 (PharMingen, San Diego, CA), caspase-9 (Oncogene Research
Products, San Diego, CA), XIAP (Transduction Laboratories), bcl-2 (Santa Cruz Biotechnology),
bax (Santa Cruz Biotechnology), BID (Cell Signaling Technology, Beverly MA), PARP
(Oncogene Research Products) and c-FLIP (a kind gift from Dr. Marcus Peter, University of
Chicago). Protein samples were separated along with molecular weight markers (Bio-Rad,
Hercules, CA) in 10-14% polyacrylamide gels. Gels were transferred onto 0.45 µm nitrocellulose
membranes (Schleicher and Schuell, Keene, NH). Gel loading equivalence was confirmed by
Coomassie blue stain (Sigma, St. Louis, MO) of membranes or by probing with antibodies for
GAPDH (Chemicon International, Temecula, CA) or actin (I-19, Santa Cruz Biotechnology).
Species-specific IgG-HRP secondary antibodies were purchased from Bio-Rad. Blots were
developed with chemiluminescent substrate (Pierce Super-Signal, Pierce) and autoradiography
was performed using X-OMAT film (Kodak, Rochester, NY). Protein bands were quantified by computer densitometry (ImageQuant, Amersham Biosciences, Sunnyvale, CA).

**Immunocytochemistry Studies of Acetylation on Histone H3 and Histone H4 Proteins**

Slides were prepared by depositing $1 \times 10^5$ cells isolated from CLL patient blood onto glass slides using a Cytospin 3 centrifuge (Thermo Shandon, Pittsburgh, PA). Slides were Wright-Giemsa stained or fixed for one minute at room temperature in a solution of 95% ethanol and 5% glacial acetic acid, and then washed two times in PBS. Cells were permeabilized for 10 minutes at room temperature with 0.2% Triton X-100, and then blocked in 10% normal donkey serum (NDS) in PBS for 1 h at room temperature before incubating overnight at 4 °C with anti-acetylated histone H3 (Upstate Biotechnology, Lake Placid, NY) diluted 1:150 or with anti-acetylated histone H4 (Upstate Biotechnology) diluted 1:100 in 2% NDS in PBS. After washing three times in PBS, the slides were stained with donkey anti-rabbit Cy3-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1.5 h, and mounted with Cytoseal 60 (Electron Microscopy Science, Fort Washington, PA). In a parallel control experiment, it was observed that omission of either primary antibody eliminated staining. Slides were analyzed using an Olympus BX51 microscope equipped with an Olympus PM30 camera (Olympus Inc., Melville, NY).

**Caspase-3, Caspase-8 and Caspase-9 Enzyme Activity**

A colorimetric reaction assay kit (R&D Systems, Minneapolis, MN) was used to determine the enzymatic activities of caspases 3, 8, and 9 according to the manufacturer's instructions. The colorimetric reaction products were measured using an Anthos 2001 microplate reader at 405-nm wavelength light.
Histone Deacetylase Assay

Assays were performed using cell lysates containing 50 ug of protein, 40 ul 10mM Tris-HCl pH 7.0 plus 1mM benzamidine, Sigma protease inhibitor cocktail (1:100 dilution), 1 mM PMSF, and 2 mM NaVO₄, ³H acetate labeled histones extracted from K562 cells (see below) at approximately 1000 counts per minute (CPM), and distilled water to a total volume of 200ul. Assay mixtures were centrifuged briefly to collect components in bottom of tube. Incubations were performed in an Eppendorf Thermomixer at 37°C at 750 rpm for 3 hours. Each reaction was stopped using 50 ul of a Quenching Solution (3M HCl + 0.6M glacial acetic acid) and tubes were vortexed. Ethyl acetate (0.6ml) was added to each reaction, tubes were vortexed vigorously for 1 minute, and samples were centrifuged to separate the phases. Radioactivity was determined in two 200 ul aliquots of the ethyl acetate phase using a beta-counter (MF615). Counts per minute (CPM) were corrected for extraction efficiency (50%) and reported as a percentage of the total CPM of ³H histone added per assay. To provide a positive and negative control, HeLa cell lysates incubated with and without 250mM sodium butyrate were included in all experiments. Samples were assayed in duplicate.

Histone Protein Substrate Preparation

Approximately 1 x 10⁸ K562 cells were grown in RPMI media with additions as described above. Cells were pre-incubated in media with 200 ug/ml cycloheximide and 10 mM sodium butyrate 60 min at 37°C in a 75-ml Corning tissue culture flask. Cells were then incubated in 10 ml of the above media with 300 uCi ³H acetate (DuPont NEN, Boston MA) in a 50-ml centrifuge tube at 37°C. Cells were washed three times in 10 ml PBS + 10mM sodium butyrate. The histones were then isolated as described above.
Results

Deipsipeptide Induces a Dose-Dependent, Lysine-Specific Increase in Histone Acetylation

The majority of studies with HDAC inhibitors such as deipsipeptide have been performed in dividing tumor cell lines rather than non-dividing patient cells. Using non-proliferating cells from CLL patients (n=5), we sought to determine if a short exposure (4 hours) to concentrations (0.04-0.4 μM) of deipsipeptide, similar to those attained in phase I studies, could promote histone acetylation. Figure 1 shows the in vitro treatment of three representative CLL samples with deipsipeptide. These studies show that deipsipeptide induces a dose-dependent increase in acetylation of total histone H4. Baseline acetylation of histone H3 is greater than histone H4, but also increases modestly with deipsipeptide treatment. No change in acetylation was noted at concentrations lower than those reported herein (data not shown). By comparison, incubation with the active metabolite of fludarabine, 2-F-ara-A (1 μM), did not promote histone acetylation (data not shown).

Acetylation of specific lysine residues on histone H3 and histone H4 has been associated with transcriptional activation, differentiation, and deposition of synthesized histones onto newly replicated DNA. We analyzed the histone acetylation patterns of three separate CLL patient samples treated with deipsipeptide to quantify increases in global histone acetylation and to determine the lysine specificity of the acetylation. The pattern of lysine residue acetylation demonstrated increases in H4 K5, H4 K12 and H3 K9 acetylation in all patient specimens examined. A small change in H4 K8 was observed, and no changes in H4 K16 acetylation, H3 K14 acetylation, or H3 K9 methylation were observed. These findings suggest that inhibition of HDAC by deipsipeptide induces acetylation of specific lysine residues on H3 and H4.
Figure 1: Depsipeptide Induces Lysine-Specific Changes in Histone Acetylation.

Analysis of changes in histone acetylation by immunoblot does not distinguish whether modifications are occurring in large increments in a small proportion of tumor cells, versus equivalent increments among the majority of tumor cells. We therefore examined changes in global H4 and H3 acetylation by immunohistochemistry where individual cells can be evaluated.
These experiments revealed changes in H4 (Figure 2) and H3 (data not shown) acetylation in virtually all the CLL cells examined.

Figure 2. Depsipeptide Induces Histone H4 Acetylation in CLL Cells

Depsipeptide-induced Histone Acetylation in CLL Cells is Promoted By Inhibition of Histone Deacetylase

Several compounds, including arsenic trioxide (29), have been noted to induce histone acetylation even in the absence of enzyme inhibition of HDAC. To determine the specificity of the change in histone acetylation with inhibition of the target enzyme HDAC, we studied the kinetics of inhibition of depsipeptide following a 4-hour treatment with this agent in three separate patients. Figure 3 demonstrates that significant (p<0.001) inhibition of HDAC activity in primary CLL tumor cells occurs following 4-hour exposure to 0.038 μM and 0.38 μM...
concentration of depsipeptide as compared to cells incubated in media alone. Specifically, 76% inhibition of global HDAC activity was observed at the depsipeptide concentration of 0.038 µM, which represents the LC_{50} concentration at 4 days identified in our previous study. No HDAC inhibition was observed following incubation of cells with 2-Fara-A (data not shown). These data demonstrate that depsipeptide-mediated histone acetylation in non-proliferating cells occurs through direct inhibition of the HDAC enzyme.

Figure 3: Depsipeptide inhibits histone deacetylase activity in CLL cells

Depsipeptide Induces Caspase-Dependent Apoptosis

We previously documented that a 4-hour incubation of CLL cells with depsipeptide induces apoptosis in a dose-dependent fashion (15) and have demonstrated (Figure 3) that greatest inhibition of HDAC activity occurs at concentrations corresponding to where apoptosis is noted. Apoptosis can occur through caspase-dependent and independent pathways. In the caspase-
dependent pathway, caspase 3 serves as an effector molecule by cleaving cellular proteins, including Poly (ADP-Ribose) polymerase (PARP), that are key for cell survival. We detected caspase 3 activation in depsipeptide-treated cells, as assessed using flow cytometric analysis with a PE-directed antibody specific for the active cleavage product (Figure 4a). In addition, we demonstrated cleavage of both caspase 3 with appearance of the 17 kD active protease (data not shown) along with the downstream substrate PARP by immunoblotting (Figure 4b). Furthermore, addition of the pan-caspase inhibitor Z-VAD-fmk inhibits this process (data not shown).

Figure 4a: Depsipeptide Treatment Results in Activation of Caspase-3 as assessed by flow cytometry using a PE-directed antibody specific for the active cleavage product of caspase 3.

Figure 4b: Depsipeptide Treatment Results in Cleavage of PARP
Depsipeptide Induced Apoptosis Occurs via the Tumor Necrosis Factor Receptor Pathway

Caspase-dependent apoptosis can be initiated through a variety of signal cascades, including the tumor necrosis factor (TNF) receptor (caspase 8) and mitochondria (caspase 9) pathway of apoptosis. These initiator caspases subsequently activate the effector caspase 3. To further characterize the apoptotic pathway utilized by depsipeptide to activate caspase 3, we examined whether the activity levels of either caspase 8 or caspase 9 increased following treatment, an indication of proteolytic processing and activation. Five patient samples were exposed to media or depsipeptide (0.38 μM) for four hours, and subsequently analyzed for caspases 8 and 9 activity. These data, depicted in Figure 5a, demonstrate that caspase 3 and caspase 8 activities increase over baseline at one day, but caspase 9 activity changes only minimally following treatment.

These data were confirmed by five separate in vitro experiments that showed no decrease in the non-cleaved caspase 9 but a dose-dependent decline in uncleaved caspase 8, generally corresponding to activation of this caspase following exposure to increasing concentrations of depsipeptide (Figure 5b). This suggests that depsipeptide utilizes the TNF-receptor pathway of apoptosis to activate caspase 8, which leads to recruitment of caspase 3 and subsequent cleavage of PARP. While caspase 8 can cross-activate caspase 9 through cleavage of BID in some systems, we were unable to demonstrate either baseline or induced expression of BID (data not shown) in CLL cells as one other group has also reported (30). Furthermore, we did not detect caspase 9 processing, as shown in Figure 5b.
Figure 5a: Depsipeptide induces activation of caspases 3 and 8 but not caspase 9

Figure 5b: Depsipeptide induces processing of the caspase 8 but not the caspase 9 pro-form
FLIP Expression Decreases without CD95 Induction Following Depsipeptide Exposure

Caspase 8 activation can occur through a variety of TNF-receptor pathways including signaling via Fas and Fas ligand. The FLICE-inhibitory protein (FLIP) acts downstream of Fas to inhibit TNF-receptor mediated apoptosis. FLIP can bind to the adaptor protein FADD and to caspase 8, interfering with caspase 8 activation. Given that Fas ligation promotes, and FLIP impedes, caspase 8 activation, we assessed expression of these proteins on days 1 and 2 following in vitro exposure to depsipeptide in cells from six patients with CLL. At baseline, no patient samples expressed Fas on greater than 10% of the CLL cells, and we detected neither an increased population of Fas-expressing CLL cells nor an up-regulation of cell surface Fas expression following depsipeptide exposure. In contrast, FLIP was noted to decrease as early as 4 hours in seven of the nine CLL patient samples treated with depsipeptide. As decreased levels of c-FLIP following depsipeptide could represent degradation occurring as an event associated with apoptosis, we performed a parallel assessment of apoptosis by annexin/PI, mitochondrial membrane potential, and c-FLIP expression analyses as demonstrated in Figure 6 in four separate patients. Similar results were noted for all four patients; representative data from one patient is shown in figure 6. In each case, at the time apoptosis was noted by annexin-V/PI staining (24 hours and beyond, Figure 6b), loss of mitochondrial membrane potential occurred. Notably, depsipeptide treatment resulted in a substantial decrease in c-FLIP-L expression prior to detectable induction of apoptosis as early as 4 hours. The splice variant FLIP-S was not detected in these samples. These findings suggest that depsipeptide induces apoptosis via a caspase 8 pathway that does not require Fas expression and ligation, but instead involves FLIP down-
modulation. Examination of other anti-apoptotic proteins including Bcl-2, Bax, Mcl-1 and XIAP at these same time points demonstrated no change with depsipeptide treatment (data not shown).

Figure 6
Discussion

Previous pre-clinical studies of CLL cells derived from patients with this disease have demonstrated depsipeptide selectively induces apoptosis of tumor cells, relative to normal mononuclear cells or bone marrow progenitor cells (15). In the study presented here, we have demonstrated that apoptosis induced by depsipeptide in CLL B-cells corresponds to increases in histone H3 and H4 acetylation that is restricted to specific lysine residues. This increase in histone acetylation is noted early following depsipeptide exposure and occurs as a consequence of inhibition of the enzyme histone deacetylase, confirming the results of one prior study of this agent in proliferating cell lines (20). We further demonstrate that apoptosis induced by depsipeptide involves a caspase-dependent pathway, utilizing the TNF-Receptor pathway of apoptosis (caspase 8) followed by activation of the effector caspase 3 to promote apoptosis in human CLL cells. Activation of the caspase 8 pathway does not appear to involve induction of expression of FAS (CD95) or of its ligand CD95L. However, we observed that c-FLIP, a protein that inhibits caspase 8 activation, is down-modulated by depsipeptide treatment at a time before apoptosis is noted. The observation that depsipeptide operates via a caspase 8-mediated process in human CLL cells is quite significant, as this pathway is not activated by any other therapeutic agents currently utilized in the treatment of this disease (6-9).

Post-translational modification of the histone proteins is a central component to timely activation and inhibition of genes important to cell growth and survival. Such modifications occur on the tails of histone proteins and include acetylation, phosphorylation, methylation, ubiquitination, and ADP ribosylation. In this study we have demonstrated that depsipeptide
induced inhibition of histone deacetylase and subsequent increased histone acetylation appears to be lysine specific, as illustrated by varied acetylation patterns of distinct lysine residues. The most notably acetylated lysine residues were those associated with chromatin formation and assembly (H4 K5 and K12, and H3 K9) (31). Hyperacetylation of the K9 residue on H3 may prevent the silencing of genes by preventing methylation of H3 K9, which contributes to gene repression (32,33). In addition, changes in histone acetylation and other modifications can influence post-translational modifications at other sites. To date, studies examining the direct consequences of lysine-specific modifications (acetylation, methylation, and phosphorylation) in neoplastic cells have been limited. The lysine residue pattern of histone acetylation induced by depsipeptide is similar to that resulting from mutation of the yeast histone deacetylase RPD3 (34, 35, 36). RPD3 is the prototype of the human class I histone deacetylase class (HDAC 1-3), suggesting that depsipeptide may target these enzymes (37-40). Understanding the role of specific histone deacetylase enzymes on these lysine residues and which of these are affected by specific histone deacetylase inhibitors will be important for eventual clinical exploitation of these agents in CLL and other diseases.

The mechanism by which depsipeptide and other histone deacetylase inhibitors induce cytotoxicity in CLL cells and other hematologic malignancies is still uncertain but may involve down-regulation of cytokines necessary for survival (41, 42) or differentiation (43-45), or induction of genes that promote apoptosis. Indeed, much research on histone deacetylase inhibitors has focused upon the ability of these agents to promote differentiation (43-45), presumably as a result of transcriptional activation of several genes. This increase in acetylation provides enhanced DNA access by transcription factors. Preliminary studies by our group have
not demonstrated expression changes in B-cell differentiation markers such as CD22 and CD25 induction as observed by others with the differentiating agent bryostatin (data not shown). Others have demonstrated that histone deacetylase inhibitors such as trichostatin A and sodium butyrate cause diminished IL-2-mediated gene expression prior to induction of apoptosis (41,42). Furthermore, in cytokine-dependent and independent hematopoietic cell lines, acetylation of histone proteins was always noted while apoptosis was only observed in cell lines dependent upon IL-2 for growth (41,42). Such pathways are currently under investigation in our laboratory.

Other histone deacetylase inhibitors including butyrate, suberoylanilide hydroxamic acid (SAHA), trichostatin, and apicidin have been investigated in either myeloid or lymphoblastic cell lines (45-51) with varied results suggesting extrinsic, intrinsic, and caspase-independent apoptosis as the relevant death pathway. Only one of these studies, by Amin and colleagues (51), included primary tumor cells. This study investigated the affect of trichostatin, SAHA, and sodium butyrate on acute promyelocytic leukemia (APL) cells and in several APL cell lines. In these experiments trichostatin, SAHA and butyrate were demonstrated to induce caspase-dependent apoptosis, but results of investigation of the relevant upstream initiator caspase were not conclusive, leaving the importance of caspase 8 or 9 activation in this process unknown. The relevance of these previous studies to our own are limited, as the experiments reported herein were carried out with a histone deacetylase inhibitor in primary non-proliferating human tumor cells, as opposed to actively proliferating transformed cell lines.

In this study, we have demonstrated that depsipeptide induces caspase-dependent apoptosis in human CLL cells that involves relatively selective activation of the same apoptosis pathway utilized by the TNF-family receptors. Our studies have shown that activation of this
process in CLL does not involve induction of FAS with subsequent activation of the FAS-FAS ligand signaling pathway, as observed in APL cell lines with apicidin (50). Alternative pathways of activating this pathway through DR4, DR5, and TNF-receptors were not examined that could explain this and these are currently under study in our laboratory. However, it is of interest that depsipeptide down-regulated c-FLIP concurrent with processing of caspase 8. C-FLIP blocks death receptor-mediated signaling by preventing caspase 8 activation at and/or release from the death-inducing signaling complex (52,53). The extrinsic pathway of apoptosis is generally not functional in patients with CLL, even when expression of CD95 occurs on the surface of CLL cells following treatment with bryostatin or CD40 ligand (12). Lack of apoptosis in this setting may occur as a consequence of CD40 ligand-induced up-regulation of c-FLIP, or bryostatin induced up-regulation of XIAP, which are effective inhibitors of caspase 8 and caspase 3, respectively (12). However, in this manuscript we have provided evidence that depsipeptide treatment results in a decrease in c-FLIP in the majority (7 of 9) patient samples assessed. Reasons for not observing a decrease in c-FLIP in these other two patients may reflect alternative regulatory mechanisms of c-FLIP in different genetic subtypes of CLL or possibly altered cellular uptake of depsipeptide due to varied presence of MDR expression/efflux (54). Effective activation of this apoptosis pathway in CLL may explain previous work by our group that demonstrated no difference in \textit{in vitro} CLL cell sensitivity to depsipeptide relative to previous treatment status (15). Exploitation of the ability of depsipeptide to activate the caspase-8 pathway in combination with other therapies such as bryostatin, CD40 ligand, IL-12, and CD154 adenovirus gene transfer, which increase CLL cell FAS expression, may offer a new treatment strategy for this incurable disease.
Virtually all agents used in the treatment of cancer are most effective when administered in combination with other treatments. Depsipeptide targets a specific class of enzymes whose activity can be followed to prevent unnecessary dose escalation that may enhance toxicity without additional therapeutic benefit. Herein, we have provided in vitro validation of using the early pharmacodynamic endpoint of change in histone acetylation and inhibition of histone deacetylase, with later reductions in c-FLIP in human CLL cells. This provides a strategy to proceed with the clinical development of depsipeptide, targeting the minimally effective pharmacologic dose in vivo in patients that promotes acetylation of H3 and H4 histone proteins in primary CLL tumor cells. This will be most relevant to studies with depsipeptide that seek to combine it with therapies that have demonstrated cytotoxic and differentiation synergy in other diseases, but also produce both medullary and extramedullary toxicity. Using the biologic endpoints described in this report to target the minimally effective pharmacologic dose in initial clinical trials with depsipeptide will avoid exceeding the favorable therapeutic index of depsipeptide with normal immune effector cells and bone marrow progenitor cells, and will facilitate effective combinations with other therapies. Based upon these data, such trials are currently underway in patients with CLL (55).
Bibliography


8. Kitada S, Pearson M, Flinn IW, Shinn CA, Reed JC, Byrd JC. The mechanism of tumor cell
clearance by rituximab in vivo in patients with B-cell chronic lymphocytic leukemia involves


10. King D, Pringle JH, Hutchinson M, Cohen GM. Processing/activation of caspases -3 and -7
and -8 but not caspase-2, in the induction of apoptosis in B-chronic lymphocytic leukemia

leukaemia cells: Role of type I versus type II cytokines and autologous fasL-expressing T

12. Kitada S, Zapata JM, Andreeff M, Reed JC. Bryostatin and CD40-ligand enhance apoptosis
resistance and induce expression of cell survival genes in B-cell chronic lymphocytic


1999;94:1401-1408.


Legends:

Figure 1. The amount of global and lysine-specific acetylated H3 and H4 in CLL cells increases in a dose-dependent manner following a 4-hour incubation with depsipeptide. Patients' mononuclear cells were isolated and cultured in media or depsipeptide (0.04 and 0.4 µM) for 4 hours. Histone extractions were performed at 4 hours and analyzed by SDS-PAGE/immunoblotting with anti-human antibodies for acetylated H3 and H4 and specific lysine residues on these histones. Equivalent loading was verified by staining with Coumassie blue.

Figure 2. Increasing histone H3 acetylation in majority of CLL cells following treatment with depsipeptide. CLL cells were incubated in media or depsipeptide (0.038 and 0.38 µM) for 4 hours. Acetylation of H4 was analyzed at 4 hours by immunofluorescence detection with appropriate negative control antibodies.

Figure 3. Depsipeptide inhibits histone acetylase at concentrations that promote histone acetylation in vitro in CLL cells. CLL cells were incubated in media or depsipeptide (0.00038, 0.0038, 0.038 and 0.38 µM) for 4 hours. Histone deacetylase activity was measured by conversion of a tritiated K562 histone substrate. Histone deacetylase activity in the media control is set at 100%, and the depsipeptide treatment data is expressed relative to this.
Figure 4a. **Depsipeptide in human CLL cells activates caspase 3.** CLL cells were treated with media or depsipeptide (0.038 and 0.38 µM) for 4 hours and subsequently incubated in media for 20 hours. Cells were washed, permeated, and stained with a PE-directed antibody specific for the active cleavage product of caspase 3.

Figure 4b. **Depsipeptide mediated apoptosis promotes processing of Poly (ADP-ribose) polymerase (PARP).** To determine if depsipeptide treatment caused alteration of a caspase 3 substrate, we examined both the unprocessed and processed forms of PARP in fresh human CLL cells at 24 hours following a 4 hour incubation of CLL cells with media, 0.038 µM depsipeptide, or 0.38 µM depsipeptide. Protein lysates were prepared and 50 micrograms protein per lane was separated on a 14% SDS-PAGE gel. Loading equivalence was confirmed by blotting with an antibody for constitutively expressed protein GAPDH. PARP and its cleaved product were detected utilizing an anti-PARP polyclonal antibody.

Figure 5a. **Depsipeptide incubation of human CLL cells results in increased activity of caspases 3 and 8.** Human CLL cells at were exposed to depsipeptide (0.38 µM) for 4 hours and compared to media control at 24 hours. Changes in caspase 3, caspase 8 and caspase 9 activities were determined by a colorimetric reaction assay.

Figure 5b. **Depsipeptide induces processing of caspase 8, but not caspase 9 in human CLL cells.** To confirm the findings of selective caspase 8 activation in CLL cells, protein lysates were prepared and 50 micrograms protein per lane was separated on a 14% SDS-PAGE gel. Loading
equivalence was confirmed by blotting with an antibody for constitutively expressed protein GAPDH. The unprocessed form of caspase 8 and caspase 9 was detected utilizing appropriate antibodies.

**Figure 6: c-FLIP Decreases in CLL Cells Following *In Vitro* Exposure to Depsipeptide.** CLL patient cells were incubated with or without depsipeptide (DDP) for 4, 24, and 48 hours. **A.** Protein lysates were prepared at each timepoint, separated on a 14% SDS-PAGE gel, and immunoblotted with polyclonal anti-c-FLIP. Gel loading equivalence was confirmed by blotting with an antibody for constitutively expressed protein beta-actin. FLIP expression in these samples was measured by laser densitometry, and is shown relative to the 4 hour untreated sample after equalizing to actin. The positive control (+) is lysate from CLL cells stimulated with CD40L for 12 hours. **B.** Prior to lysing, an aliquot of cells from each condition was assessed for early apoptosis by flow cytometry with annexin-V FITC and propidium iodide.
Depsipeptide (FR901228) induces histone acetylation and inhibition of histone deacetylase in chronic lymphocytic leukemia cells concurrent with activation of caspase-8-mediated apoptosis and downregulation of c-FLIP protein

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