Histone deacetylase inhibitors are potent inducers of
gene expression in latent EBV and sensitize lymphoma cells to
nucleoside anti-viral agents

Sajal K. Ghosh, PhD¹,
Susan P. Perrine, MD¹,
Robert M. Williams, PhD²,
Douglas V. Faller, PhD MD¹*

¹Cancer Center, Boston University School of Medicine, Boston;
²Department of Chemistry, Colorado State University.

Address for Correspondence:
Douglas V. Faller, PhD. MD., Cancer Center, Boston University School of Medicine,
72 East Concord Street, K 701; Boston, MA 02118
Ph: 617-638-4173, E-mail: dfaller@bu.edu

Running Title: HDAC inhibitors as therapeutics for EBV-lymphoma
Abstract:

Induction of EBV lytic-phase gene expression, combined with exposure to an anti-herpesviral drug, represents a promising targeted therapeutic approach to EBV-associated lymphomas. Previously, short-chain fatty acids (SCFA) or certain chemotherapeutics have been used to induce EBV lytic-phase gene expression in cultured cells and mouse models, but these studies generally have not translated into clinical application. The recent success of a clinical trial with the pan-histone deacetylase (HDAC) inhibitor arginine butyrate and the anti-herpesviral drug ganciclovir (GCV) in the treatment of EBV lymphomas prompted us to investigate the potential of several HDAC inhibitors, including some new, highly-potent compounds, to sensitize EBV-positive human lymphoma cells to anti-viral agents in vitro. Our study included SCFAs (sodium butyrate and valproic acid); hydroxamic acids [oxamflatin, Scriptaid, SAHA, panobinostat (LBH589) and belinostat (PXD101)]; the benzamide MS275; the cyclic tetrapeptide apicidin, and the recently-discovered HDACi largazole. With the exception of SAHA and PXD101, all of the other HDAC inhibitors effectively sensitized EBV+ lymphoma cells to GCV. LBH589, MS275, and largazole were effective at nanomolar concentrations and were 10^4 to 10^5-times more potent than butyrate. The effectiveness and potency of these HDAC inhibitors make them potentially applicable as sensitizers to anti-virals for the treatment of EBV-associated lymphomas.
Introduction:

Latent infection with Epstein-Barr Virus (EBV), a γ-herpesvirus, is ubiquitous among human populations worldwide. Acute EBV infection results in the self-limiting illness infectious mononucleosis, although it can lead to severe and sometimes fatal disease in immunocompromised patients.¹ Latent EBV infection has also been associated with number of human malignancies such as Burkitt lymphoma (BL),² nasopharyngeal carcinoma (NPC),³ post transplantation lymphoproliferative disease (PTLD),⁴ Hodgkin lymphoma (HL),⁵ non-Hodgkin lymphoma,⁶ and sporadic cancers of the gastrointestinal tract and breast.⁷,⁸ Commonly used anti-herpesvirus drugs, such as the nucleoside analogs ganciclovir (GCV) or acyclovir (ACV), are inefficient at eliminating EBV from chronically-infected hosts, as EBV maintains a latent state of infection in these tumors and lytic-phase proteins are required to convert these pro-drugs to active anti-viral drugs.

In recent years, several laboratories have explored the concept that induction of EBV lytic replication, with or without the addition of anti-herpes virus drugs, could be therapeutically beneficial for EBV-associated tumors.⁹⁻¹¹ This approach would have high tumor-specificity, as only EBV-containing cells would be targeted by this treatment, while neighboring EBV-negative cells would remain unaffected. A number of disparate agents have been used to induce lytic-phase EBV-gene expression in tumor cells, including butyrate, valproic acid, rituximab, bortezomib, cisplatinum, gemcitabine, 5-azacytidine, and gamma-radiation.¹²⁻¹⁷ Although the specific mechanisms by which these agents induce EBV lytic-phase gene expression differ, they all modulate EBV gene transcription in infected cells. Butyrate and valproic acid, in particular, are inhibitors of histone deacetylases. Arginine butyrate in combination with GCV was used in a recent phase I/II multi-institutional clinical trial in patients with highly-refractory EBV-positive diverse lymphoid malignancies.¹⁸ Ten out of fifteen patients showed significant tumor responses, including complete clinical and pathological responses, in this study.

Chromatin structure and gene transcription are tightly regulated by the acetylation state of the histone molecules in the nucleosome. Histone acetyl transferases (HATs) and histone
deacetylases (HDACs) play a major role in this epigenetic control of cellular gene transcription.\textsuperscript{19} Whereas HATs acetylate conserved lysine residues in histone tails and associate with transcriptional co-activators and other HATs to facilitate gene transcription, HDACs typically associate with a different set of co-repressor proteins such as SMRT, N-Cor, NURD and others to remove acetyl group from the acetylated lysines of histone tail, compact chromatin and induce transcriptional repression.\textsuperscript{20,21} Certain small molecules with anti-proliferative and pro-apoptotic activities in tumor cells were later identified as inhibitors of HDACs. Consequently, substantial effort has been made in the development of new HDAC inhibitors with potential therapeutic utility.\textsuperscript{22} Many of the HDAC inhibitors developed to date have been found to have strong anti-tumor activity in laboratory models. Several HDAC inhibitors have already been clinically evaluated in multiple types of malignancies.\textsuperscript{23} Some of them have demonstrated efficacy in hematological malignancies, such as cutaneous T-cell leukemia (CTCL), peripheral T-cell leukemia (PTCL), acute myeloid leukemia (AML) and HL.\textsuperscript{24} Two HDAC inhibitors, suberoyl anilide hydroxamic acid (SAHA or Vorinostat)\textsuperscript{25} and FK-228 (Romidepsin)\textsuperscript{26} have been approved for the treatment of CTCL.

Our previous studies demonstrated that butyrate, a general HDAC inhibitor, acts as inducer of EBV lytic-phase gene expression, and together with GCV, efficiently kills EBV-infected cells. In this study we evaluated efficacy of several newer and more potent inhibitors of multiple HDAC subclasses to induce EBV lytic-phase gene expression and GCV-dependent killing of infected cells. We report here that HDAC inhibitors MS275 (benzamide class), LBH589 (hydroxamic acid class) and largazole (cyclic depsipeptide class) efficiently killed EBV-infected Burkitt’s lymphoma cell P3HR1 in the presence of GCV. We further demonstrate that some of these inhibitors also have potent activity in other EBV-infected Burkitt lymphoma (BL) cells and lymphoblastoid cell lines (LCLs).
Materials and Methods:

Cells

Two Burkitt lymphoma cell lines P3HR1 (EBV-producer cell line originally obtained from the Jijoye cell line)\textsuperscript{27} and Daudi (EBV-positive but non-producer line)\textsuperscript{28} were used in this study. The EBV transformed lymphoblastoid cell line JY, also used in this study, was originally generated from a homozygous Indiana Amish population.\textsuperscript{29} Two EBV-negative B-cell lines, BJAB and Toledo, originally generated from a Burkitt lymphoma\textsuperscript{30} and a non-Hodgkin’s lymphoma,\textsuperscript{31} respectively, were also used in this study. Except for BJAB, all cells were maintained in RPMI 1640 with 10% fetal bovine serum (FBS) containing 100 U of penicillin and 100 µg of streptomycin per ml. BJAB cells were maintained in DMEM with 20% FBS and antibiotics.

HDAC inhibitors

A complete list of HDAC inhibitors used in this study is listed in Table 1. Their structures are shown in Figure 1. Sodium Butyrate, and Suberoyl anilide hydroxamic acid (SAHA) were purchased from Sigma Chemical Company (St. Louis). Valproic acid, Scriptaid, apicidin, and oxamflatin were purchased from Calbiochem. MS275, LBH589, PXD101 and various largazole derivatives\textsuperscript{32} were synthesized at the Department of Chemistry, Colorado State University. Stock solutions of sodium butyrate was prepared fresh in sterile water. Valproic acid stock was prepared in water pH adjusted to 7.5 with 1M NaOH. Stock solutions of all other HDAC inhibitors were prepared in DMSO and stored in aliquots at –20°C.

Analysis of lytic gene expression

To determine EBV lytic-phase gene expression, levels of TK and BGLF4 gene transcripts were analyzed by reverse transcription and real-time PCR. Total RNA from P3HR1, Daudi or JY cells, either untreated or treated with various HDAC inhibitors, was isolated by Trizol (Invitrogen, Carlsbad, CA) exaction following the manufacturer’s protocol. Genomic or episomal DNA contamination from the RNA preparations were removed by RNase-free DNase treatment at a
concentration of 0.1 U/µl. Five µg of total RNA was reverse transcribed by Superscript III (Invitrogen) using random hexamer primers. The TK, BGLF4 or β-actin cDNA from these preparations were then amplified using SYBR green PCR amplification technology in an ABI PRISM 7500 sequence detection system (Applied Biosystems, Foster City, CA). Primers used in the real-time PCR amplification are listed in Table 2. Relative quantification of gene expression was determined by the comparative threshold method (ΔCT), as described previously. Expression of the β-actin mRNA in each individual sample was used to normalize the dataset.

**PCR assay for the presence of EBV**

The presence of EBV genome in the cell lines used in the study was determined by PCR analysis of EBV EBER1 sequences in the genomic and extrachromosomal DNA extract from these cells. One microgram of DNA was subjected to PCR amplification with β-actin- or EBER1-specific primer sets (Table 2) for 35 cycles and products were analyzed on 2% agarose gel.

**Cell viability assay**

Viability of EBV positive lymphoma cells following treatment with various HDAC inhibitors in presence or absence of GCV was enumerated by the trypan blue dye-exclusion method using a Countess automated cell counter (Invitrogen).

**Immunoblotting**

Expression of EBV early antigen-diffuse or BMRF1 proteins following treatment of P3HR1 cells with individual HDAC inhibitors was determined by immunoblotting, essentially as described previously.33
Results:

**Induction of EBV lytic-phase gene expression with HDAC inhibitors**

To investigate the efficiency of the several newer HDAC inhibitors for induction of EBV lytic-phase gene expression, we measured their effect on the expression of the gene encoding the viral TK enzyme. The EBV-TK enzyme is normally expressed only during the lytic-replication stage of the virus life cycle. We initially studied the EBV-positive BL cell line P3HR1, which typically maintains a type I latency, wherein only the EBV latency genes EBNA1, EBER1 and EBER2 are expressed. The cells were exposed to individual HDAC inhibitors (shown in Figure 1) for 48 hrs, followed by total RNA extraction. TK transcript expression in these preparations was then analyzed by reverse transcription and real-time PCR analysis. Exposure to most of the HDAC inhibitors produced increases in TK expression in a dose-dependent manner compared to the level of expression in vehicle-treated control cells (Figure 2). The highest level of increase in TK expression was produced by hydroxamic acids LBH589 (panobinostat) and PXD101 (belinostat) (over 250- and 26-fold, respectively). Butyrate, MS275, oxamflatin, apicidin and largazole induced TK expression to a moderately high level (17-, 11-, 6-, 6.5- and 4.5-fold, respectively). Scriptaid, SAHA and valproic acid induced TK expression only marginally above untreated cells. LBH589, PXD101 and apicidin were extremely toxic to the cells at the highest concentrations tested, which limited our ability to assess their potency in this assay. These results thus demonstrated that most of the structurally-diverse HDAC inhibitors used in this study are inducers of EBV TK gene expression and therefore inducers of EBV lytic-phase gene expression.

**Cell growth analysis with HDAC inhibitors in combination with GCV**

Since most of the HDAC inhibitors efficiently induced TK expression in P3HR1 cells, we next analyzed whether presence of anti-herpesvirus drug GCV during this induction of lytic-phase gene expression would facilitate killing of the EBV-infected cells. We exposed the cells to the individual HDAC inhibitor, GCV, or the combination for 72 hr. The HDAC inhibitors were then removed and the cells were maintained in fresh media containing GCV only for a further 72 hr. The short-chain
fatty acid class HDAC inhibitor valproic acid (VA) showed significant growth inhibition in combination with GCV, as only 29% cells survived when treated with VA and GCV together compared to 84% when treated with GCV alone (Figure 3A). A relatively high concentration of VA, however (up to 1.0 mM), was necessary to achieve this effect. VA was therefore similar to butyrate, another short-chain fatty acid, in its potency. Higher concentrations of VA were cytostatic for the P3HR1 cells. The cyclic tetrapeptide apicidin also was efficient in cell killing but the effective dose range was very limited. At 100 nM, 34% of the cells survived with the combination treatment whereas 59% survived with GCV treatment alone. Higher concentrations of apicidin were toxic to the cells. The benzamide HDAC inhibitor MS275, in contrast, was relatively non-toxic to the cells at the concentration ranges effective to induce sensitivity to GCV. In our assays, MS275 at 500 nM was found to have optimal cell killing activity when used together with GCV (39% survival in combination treatment versus 63% with GCV alone). We also tested three cyclic depsipeptide HDAC inhibitors of the largazole class. Largazole was originally isolated from a marine cyanobacterium and showed strong cytotoxic activity particularly against tumor cells. The three compounds largazole, largazole-A and largazole-B were prepared by total synthesis, as described earlier. Chemical structures of the largazole and its synthetic analogs as well as all other HDAC inhibitors used in the study are shown in Figure 1. Of these three largazole derivatives, the parent natural product (largazole itself) showed the most significant cell killing effect at a low concentration (at 100 nM, 22% survival in combination treatment versus 60% with GCV alone). Largazole analog B also showed cytotoxic activity in combination, albeit at a lower level. At least ten other largazole derivatives were tested for the ability to sensitize EBV-infected tumor cells to GCV but only one other largazole analog had activity comparable to parent largazole (data not shown). Four additional HDAC inhibitors of the hydroxamic acid class (oxamflatin, SAHA, LBH589 and scriptaid) were tested in the same assays. Of these four inhibitors, SAHA had little effect on cell killing in presence of GCV. Scriptaid produced strong cytotoxic activity, requiring concentrations in the range of 1 µM or higher. Oxamflatin showed efficient cytotoxic activity at a 200 nM concentration (24% survival in combination treatment versus 59% with GCV alone).
LBH589 was highly effective in the range of 10 to 50 nM concentration and was the most potent HDAC inhibitor found to sensitize the tumor cells in this study (44% survival in combination treatment versus 69% with GCV alone at a 50 nM concentration). Our study clearly demonstrates that many of the HDAC inhibitors have strong cytotoxic activity in presence of the anti-herpesvirus drug GCV.

To verify that the combinatory effect of GCV and HDAC inhibitors we observed on P3HR1 cells was indeed due to the presence of EBV, we also tested the combination treatment on two EBV-negative B-lymphoma lines, BJAB and Toledo. As shown in Figure 3B, exposure to the HDAC inhibitors butyrate, MS275 or LBH589 did not produce additional cytotoxic activity in the presence of GCV in either BJAB or Toledo cells. When used as a single agent, most of the HDAC inhibitors studied generated higher cytotoxicity in Toledo cells compared to the other cell lines utilized in this study. This prompted us to employ lower concentrations of HDAC inhibitors, at which Toledo cells remained healthy. We confirmed by PCR analysis that BJAB and Toledo cells were indeed EBV-negative, whereas, P3HR1 was EBV-positive (right panel of Figure 3B).

**Induction of viral BGLF4 and BMRF-1 expression by HDAC inhibitors**

In order for GCV to act as an anti-viral or cytotoxic drug, it requires initial mono-phosphorylation by an EBV-encoded kinase, with subsequently conversion to a tri-phosphate by a cellular kinase. The resulting GCV-TP then gets incorporated into an actively-replicating DNA strand and causes premature termination of DNA synthesis, evoking apoptosis. A few studies have reported that GCV is a better substrate for the EBV serine-threonine protein kinase BGLF4 than for the viral TK, both of which are exclusively expressed during lytic-phase replication of EBV. We analyzed BGLF4 expression in P3HR1 cells following exposure to certain HDAC inhibitors in comparison with the TK expression profiles. Butyrate, largazole, MS275, PXD101 and LBH589 were tested, as these HDAC inhibitors were found to be the most efficient at generating cytotoxicity in combination with GCV, or at inducing TK expression. As shown in Figure 4A, for each of the HDAC inhibitors tested, the pattern of change of BGLF4 expression paralleled TK expression. Exposure to
progressively higher concentrations of HDAC inhibitors (butyrate or largazole), which led to increases in TK induction, also increased BGLF4 expression. We have previously reported that exposure of P3HR1 cells to butyrate induces EBV early antigen-diffuse protein (EA-D or BMRF1), a major protein of the EBV lytic replication cycle.\textsuperscript{33} We evaluated EA-D protein expression in P3HR1 cells that were exposed to certain of the individual HDAC inhibitors used in this study. As shown in Figure 4B, LBH589 strongly induced EA-D protein, whereas PXD101 did so very weakly and the other HDAC inhibitors did not. Our results therefore demonstrated that although HDAC inhibitors induce TK and BGLF4 expression in a similar manner, induction of the EA-D protein may require co-ordinate expression of other factors which different HDAC inhibitors regulate differentially.

**Prolonged exposure to HDAC inhibitors is not essential for efficient sensitization to anti-viral agents**

We rationalized that the potential clinical efficacy of the combination of an HDAC inhibitor and anti-viral agent would be improved if we could determine the minimal time of exposure to HDAC inhibitor required to sensitize the tumor cells to anti-viral agents. To determine this, P3HR1 cells were exposed to HDAC inhibitors for 24 hr or 48 hr in the combination treatment protocols and the results were compared with those using 72 hr of exposure to HDAC inhibitors (as in Figure 2). As shown in Figure 5A, using a 20 nM concentration of LBH589 in P3HR1 cells together with GCV, the relative tumor cell cytotoxicity induced by 48 hrs of exposure to the HDAC inhibitor was similar to cytotoxicity observed in cells which had been exposed for 72 hrs (45% survival with 48 hr exposure; 48% with 72 hr exposure). The cytotoxicity induced by the HDAC inhibitor alone, however, also increased with each longer interval of exposure. When MS275 was used as the HDAC inhibitor at 1 \( \mu \)M, total cytotoxicity with 48 hr of exposure was equivalent to that seen with 72 hr exposure, and the relative cytotoxicity conferred by addition of GCV (measured as fraction of surviving cells) was equivalent for all three intervals of HDAC inhibitor exposure. In this experiment, a higher concentration of the inhibitor (as identified in Figure 2) was also studied, to determine if
shorter exposure to a higher concentration of the HDAC inhibitor would effectively sensitize cells to GCV. We found that a 24 hr exposure to 5 µM MS275 plus GCV was more cytotoxic than 48 hr or 72 hr exposure to either 0.5 µM or 1µM MS275 plus GCV (Figure 5B). However, exposure to 5 µM MS275 as a single agent produced substantial cytotoxicity within the 24 hr treatment period.

**HDAC inhibitors in combination with an anti-viral induce efficient cell killing of other EBV-positive lymphoma cells**

We have carried out the experiments described above in the Burkitt lymphoma (BL) cell line P3HR1, which maintains an EBV type 1 latency. To determine whether the combination of HDAC inhibitor and GCV would be effective against other EBV-positive lymphoma cells, we tested butyrate and one of the other most effective HDAC inhibitors, LBH589, in combination with GCV in cytotoxicity assays. We employed another BL line, Daudi, and a LCL line, JY, for this purpose. EBV replication in LCLs is of type 3 latency, wherein all eleven latency gene products are expressed. EBV replication in LCLs resembles that found in the clonal or multiclonal B-cell populations in patients with post-transplantation lymphoproliferative disease (PTLD). The EBV expression and latency status of these lines was confirmed by RT-PCR analysis of the expression of EBER1, Qp, Cp and Wp-specific transcripts. As shown in Figure 6A, all three lines abundantly expressed EBER1 RNA. As expected, transcripts from Qp promoter were observed in both of the BL cell lines, P3HR1 and Daudi. The Qp transcript was also detected in JY cells. Although it is not common, some type 3 latency LCLs do express the Qp transcript.\(^{37}\) Although wide variations of Wp expression among different latency types have been noted previously, expression of the Cp transcript is specific to cells with type 3 EBV latency.\(^{37}\) The JY cells, but neither the P3HR1 or Daudi cells, expressed Cp-specific transcripts. We analyzed ability of butyrate and LBH589 to sensitize both Daudi and JY cells to an anti-viral agent. Both HDAC inhibitors, as single agents, significantly reduced the number of the Daudi cells at both concentrations tested, but had less of a cytotoxic effect on the JY cells (Figure 6B). LBH589 in combination with GCV had a modest cytotoxic effect on Daudi cells (61% relative survival with combination treatment versus 84% with
GCV alone) but a more significant effect on JY cells (48% relative survival in combination treatment versus 74% with GCV alone). Butyrate in combination with GCV produced a strong cytotoxic effect on both Daudi cells (34% relative survival in combination treatment versus 66% with GCV alone) and JY cells (44% relative survival in combination treatment versus 70% with GCV alone). These results clearly demonstrate that combination of an HDAC inhibitor and GCV is effective in killing EBV-positive lymphoma cells of diverse origins.
Discussion:

In this study we demonstrate that HDAC inhibitors of disparate classes and structures induce EBV lytic-phase gene expression and sensitize EBV+ tumor cells to cytotoxicity in presence of the anti-herpesvirus drug GCV. Effective sensitizing concentrations for certain of the HDAC inhibitors, such as LBH589, apicidin, MS275 and largazole, were in the range of 20 nM to 500 nM. Our previous in vitro and preclinical studies demonstrated that the HDAC inhibitor sodium or arginine butyrate strongly induced viral TK expression in a patient-derived EBV lymphoma cell line, as well as in the patient himself, and suggested the therapeutic potential of the approach. A phase I/II clinical trial later showed that this treatment strategy indeed significantly reduced the tumor burden in two-thirds of patients with very refractory EBV-associated lymphomas. The lymphomas which responded included post-transplant lymphoproliferative diseases, T/NK lymphomas, EBV+ large B cell lymphomas, and EBV+ cutaneous T cell lymphomas. However, because of poor oral bioavailability and its short half-life in vivo, this regimen required that butyrate be infused continuously for many days, and at a high dose, for therapeutic activity. In contrast, some of the HDAC inhibitors we have studied in this report are 10^4- to 10^5-fold more potent than butyrate, and have superior pharmacokinetics. Interestingly, a few of these novel HDAC inhibitors are already in clinical trials.

Most of the HDAC inhibitors used in our study induced EBV TK expression, and their relative activity in TK induction correlated with their ability to generate tumor cytotoxicity in presence of GCV. This correlation was not universal with all the HDAC inhibitors studied, however. For example, valproic acid did demonstrate cytotoxicity in combination with GCV, although its TK-inducing ability was modest in comparison to the newer HDAC inhibitors studied here. Conversely, SAHA produced up to 3-fold inductions of TK but did not augment cytotoxicity in the presence of GCV. This occasional lack of correlation with TK induction suggested that kinases other than TK might also be involved in the conversion of the pro-drug GCV. Some studies have reported that the EBV protein kinase coded by the BGLF4 gene is a more efficient kinase for the phosphorylation of GCV than TK. In our studies, the induction of BGLF4 always paralleled the induction TK by all
of the HDAC inhibitors tested. It is therefore possible that HDAC inhibitor-mediated induction of BGLF4 and subsequent expression of EBV-PK may also have contributed to the phosphorylation of GCV. However, the differential induction of these two potential GCV-kinases does not explain the lack correlation of TK-induction and sensitization to GCV by SAHA or valproic acid. Irrespective of the mechanism of HDAC inhibitor-mediated sensitization, however, the control experiments with EBV-negative lymphoma cell lines demonstrated that the presence of EBV is critical for the combination of agents to generate efficient cytotoxicity.

As noted above, although butyrate in combination with GCV was quite effective in killing EBV positive tumor cells in vitro and in clinical studies, the pharmacokinetic limitations of butyrate in clinical application mandated infusion at high doses to patients continuously over a number of days. We demonstrated that a 2-day exposure to selected HDAC inhibitors was as effective in sensitizing tumors to GCV as a 3-day exposure. With MS275, even a 24 hr exposure produced cytotoxic activity in combination with GCV comparable to 2- or 3-day exposure. These findings suggest that prolonged exposure to HDAC inhibitors might not be necessary in the clinical setting, potentially limiting secondary toxicities. Indeed, we have previously reported that shorter durations of exposure to butyrate also efficiently killed EBV+ lymphoma cells in the presence of GCV. Based on this data, one patient with refractory EBV+ lymphoma was treated in a protocol employing butyrate for 5 days, and administration of GCV or valganciclovir for 21 days. The lymphoma burden was dramatically reduced within one cycle. Furthermore, previously high EBV viral loads, as well as the viral loads of two other herpesviruses (CMV and HHV6), became undetectable.

The reported pharmacokinetics and pharmacodynamics for MS275 and LBH589 as single agents appear superior to butyrate. A Phase I clinical trial with MS275 administered orally demonstrated that the area under the plasma concentration versus time curve (AUC) easily reached 59-268 ng·h/ml for doses of 2-8 mg/m² and was sustained for a minimum of 34 ± 26 h across all dose levels. Administration of MS275 induced acetylation of histone H3 and H4 in circulating peripheral blood mononuclear cells in these studies, and in variety of tumor cell lines.
including that of the prostate, pancreas and breast at these concentrations in vitro.\textsuperscript{41} LBH589 also displayed rapid absorption when administered orally in a phase I clinical trial, with a serum half-life of about 14.6 h and an AUC of 134 ng.h/ml for a single 20 mg dose.\textsuperscript{42}

Our results demonstrate that the EBV latency type in the lymphoma is not crucial for the success of combination therapy approach with HDAC inhibitors and GCV. The P3HR1 cells, a line originally derived from Burkitt Lymphoma cell line Jijoye, produce virus particles that are transformation defective.\textsuperscript{43} Daudi cells were also isolated from a Burkitt lymphoma patient and are transformation-defective, but are a EBV non-producer line.\textsuperscript{44} Our data demonstrated that the inherent viral defects of the P3HR1 or Daudi cells do not interfere with HDAC inhibitor-mediated induction of lytic-phase gene expression and cytotoxicity in presence of an anti-herpesviral drug. JY cells, an EBV transformed LCL with a different latency pattern, responded equally well to the combination treatment approach, with either butyrate or LBH589 as the viral-inducing agent. These results in the JY cell line mirror the observed responsiveness of the PTLD patients to the combination of butyrate and GCV in the previous clinical trial.\textsuperscript{18} PTLD, commonly arising in immunosuppressed individuals, is caused by unchecked proliferation of EBV+ B cells in the absence of immune surveillance. In both LCL and PTLD, EBV maintains a type 3 latency, wherein all of the EBV latent gene products are expressed.

In summary, our study demonstrated that a number of structurally-distinct HDAC inhibitors are efficient agents for sensitizing EBV+ lymphoma cells to anti-herpesvirus drugs. Only nanomolar concentrations of the most potent of these agents are necessary for optimal effect. As our previous work has demonstrated that the HDAC inhibitor butyrate has impressive early-phase clinical activity in the treatment of patients with EBV+ lymphomas, data from this study suggests that there is also potential for the application of these new HDAC inhibitors in combination therapy with an anti-herpesvirus drugs for treating EBV+ lymphomas. In particular, certain of the new HDAC inhibitors with substantial clinical and safety data might provide more convenient treatment regimens, and even, in combination with oral anti-virals, a completely outpatient-based protocol.
Acknowledgement:

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Authorship Contributions:

The studies were conceived and planned by SKG, DVF and SPP. Certain of the compounds were synthesized by RMW. The studies were carried out by SKG. The data was analyzed by SKG, DVF and SPP. All authors contributed to the writing of the manuscript.

Conflict of Interest Disclosures:

SPP, DVF and RMW have had research or consulting agreements with HemaQuest Pharmaceuticals, Inc. in the past, and own equity in HemaQuest Pharmaceuticals, Inc.
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### Table 1. HDAC inhibitors used in the study

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<th>Name</th>
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N/A = Not available
### Table 2. Primers used in the study

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<td>TCCGGAGCCAGCTTCTCTCC CGTGATTGTGTAGCCGG</td>
<td>275 bp</td>
<td>131353 – 131334 (NC_007605) 131079 – 131098</td>
<td>Ghosh et. al.33</td>
</tr>
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<td>Thymidine Kinase-R</td>
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<td>This work</td>
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<td>6794 - 6774</td>
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<td>339 bp</td>
<td>62426 – 62452 (B95-8)</td>
<td>Komano et. al.48</td>
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<td>Qp-R</td>
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<td>Cp-F</td>
<td>CACTACAAAGACCTAGCCTCCATCCATACCCATC</td>
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<td>11425 – 11454 (B95-8)</td>
<td>Komano et. al.48</td>
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<td>Cp-R</td>
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<td>14832 – 14813, 17636-17626</td>
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<td>Komano et. al.48</td>
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<td>14832 – 14813, 17636-17626</td>
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Figure Legends

Figure 1
Chemical structures and chemical classes of the histone deacetylase inhibitors employed in these studies.

Figure 2
HDAC inhibitor-mediated induction of TK transcript in EBV-positive lymphoma cells. Three million P3HR1 cells in 3 ml RPMI 1640 media were exposed to individual HDAC inhibitors for 48 hr. HDAC inhibitors used include short chain fatty acids (butyrate and valproate), cyclic tetrapeptide (apicidin), cyclic depsipeptide (parent largazole and analogs A and B), benzamide (MS275), and hydroxamic acids (oxamflatin, LBH589, SAHA, PXD101 and Scriptaid). Inhibitor concentrations were empirically determined so that cytotoxicity remained minimal. Total RNA extraction, reverse transcription and real-time PCR analysis were performed as described in Materials and Methods. Real-time PCR was performed in triplicate on each HDAC inhibitor-treated sample for both TK mRNA and β-actin mRNA and these values were used to determine respective ΔCt and fold-induction. RNA from P3HR1 cells treated with 1.0 mM and 2.5 mM sodium butyrate were used as internal control in each experiment (not shown for each inhibitor). Each assay was repeated three times and error bars in each individual figure represents standard deviations.

Figure 3
Cytotoxic activity of HDAC inhibitors in the presence of an anti-herpesvirus drug. A. Three hundred thousand P3HR1 cells were exposed to either 40 μM GCV or vehicle, and the indicated concentrations of individual HDAC inhibitors, in a 1 ml volume in 24-well plates in triplicate. Three days later, 800 μl of the media was removed without disturbing the settled cells and 1 ml of fresh growth media containing GCV (40 μM) was added and the cells allowed to grow for another 3 days. HDAC inhibitors used include butyrate, valproate, apicidin, largazole and its analogs, MS275, oxamflatin, LBH589, SAHA and Scriptaid. The number above the HDAC+GCV bar represents the
percentage of cells surviving relative to the cultures exposed to that particular HDAC inhibitor alone (assigned a value of 100%). Error bars represent standard deviations in individual experiments. B. Cytotoxic activity of selected HDAC inhibitors (butyrate, MS275 and LBH589) in presence of GCV in the EBV-negative B-lymphoma lines BJAB and Toledo. Experiments were carried out essentially as in panel A. The right panel shows detection of EBER1- and β-actin-specific PCR products generated from cellular DNA of BJAB, P3HR1 and Toledo cells, analyzed in a 2% agarose gel. A vertical line has been inserted to indicate a repositioned gel lane.

Figure 4
HDAC inhibitor-mediated induction of EBV lytic-phase gene expression. (A) Comparison of TK and BGLF4 transcript expression in P3HR1 cells following exposure to different HDAC inhibitors. Cells were treated with individual HDAC inhibitors as indicated and total RNA was analyzed by reverse transcription and real-time PCR analysis as described in the legend to Figure 2. Expression of β-actin mRNA under similar treatment conditions was used to normalize the dataset. (B) Immunoblot analysis for EA-D protein. Thirty microgram of whole cell lysates from individual HDAC inhibitor-treated cells (for 48 hr) or untreated cells were separated in 10% SDS-PAGE, transferred to a nitrocellulose membrane and immunoblotted with a 1:2000 dilution of mouse anti-EBV EA-D antibody (Millipore Corporation). Equal loading of proteins was verified by immunoblotting with 1:15,000 dilution of mouse anti-β-actin antibody (Sigma Chemical Company). Each assay was repeated three times and error bars in each individual figure represents standard deviations.

Figure 5
Sensitization of EBV lymphoma cells to GCV-mediated killing by brief exposure to HDAC inhibitors. (A) Three hundred thousand P3HR1 cells in a 1 ml volume were treated with 20 nM LBH589 for indicated period of time in presence or absence of 40 µM GCV. Culture medium was completely removed after centrifugation at the end of incubation with LBH589 and replenished with fresh growth media, with or without GCV, as indicated. Media for all cells was replaced again at 72 hr.
Cells were counted at 144 hr (day 6). (B) Similar protocol as in panel A, but evaluated MS275 at three different concentrations. The overwhelming toxicity following exposure to MS275 at 5 µM for 48 or 72 hr precluded any meaningful cell count. Cells exposed to sodium butyrate (NaB) at 1 mM were used as an internal control. The number above the HDAC+GCV bar represents the percentage of cells surviving, relative to the cell count after exposure to the HDAC inhibitor alone (assigned a value of 100%). Experiments were repeated three times and error bars represent standard deviations in individual experiments.

Figure 6
Effect of HDAC inhibitor and GCV combination treatment on other EBV-positive lymphoma cells. (A) Analysis of promoter utilization by the three different EBV lymphoma cell lines used in the study. Reverse transcription and PCR analysis of total RNA was carried out using primers that specifically detected the Qp, Wp or Cp transcripts (Table 2). Products were analyzed on a 2% agarose gel with a 100 bp DNA ladder as marker. A vertical line has been inserted to indicate a repositioned gel lane. (B) Effect of combination treatment on the BL line Daudi. Four hundred thousand cells per ml per well were used along with 60 µM GCV in the appropriate wells. Assay parameters were as described in the legend of Figure 2. (C) Effect of combination treatment on the EBV-transformed lymphoblastoid cell line JY. In this case, two hundred thousand cells per ml per well were used along with 60 µM GCV, as appropriate. Experiments were repeated three times and error bars represent standard deviations in individual experiments.
Figure 1.
Figure 2.
Figure 3.
Figure 4.

A

![Graph showing fold induction for different treatments.](image)

B

![Western blot images for EA-D and β-Actin proteins.](image)
Figure 5.

A

B
Figure 6.

A

B

C

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Histone deacetylase inhibitors are potent inducers of gene expression in latent EBV and sensitize lymphoma cells to nucleoside anti-viral agents

Sajal K. Ghosh, Susan P. Perrine, Robert M. Williams and Douglas V. Faller