의학박사 학위논문

The role of histone deacetylase inhibitor romidepsin in EBV-positive diffuse large B-cell lymphoma

엡스타인-바 바이러스 양성 미만성 B대세포 림프종에서 히스톤 탈아세틸화 효소 억제제인 로미뎁신의 역할

2015년 8월

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The role of histone deacetylase inhibitor romidepsin in EBV-positive diffuse large B-cell lymphoma

By

Dong-Yeop Shin

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for the degree of Doctor of Philosophy in Medical Sciences
at Seoul National University, Seoul, Korea

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Approved by Thesis Committee:

Professor _____ **Chairman**

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Abstract

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Purpose: Epstein-Barr virus (EBV)-positive diffuse large B-cell lymphoma (DLBCL) is an aggressive subset of non-Hodgkin lymphoma which had been newly identified and incorporated into the revised 2008 international tumor classification system of the World Health Organization classification. We investigated the role of the histone deacetylase inhibitor, romidepsin, in EBV-positive diffuse large B-cell.

Materials and methods: We used EBV-positive and EBV-negative DLBCL cell lines and generated two EBV-transfected cell lines, LY7/EBV and U2932/EBV. For in vivo experiments, skin xenograft model of U2932 and U2932/EBV were generated using NOD/SCID mice. Selective knockdown of LMP1 and c-myc was performed using small inhibitory RNAs and co-localization of apoptosis and EBV lytic activation was checked using immunofluorescent antibodies for cleaved caspase-3 and zebra.

Results: Romidepsin was cytotoxic to cultured EBV-positive cells via the activation of the caspase cascade. Moreover, *in vivo* mice xenograft models demonstrated the cytotoxicity of romidepsin to EBV-positive DLBCL cells. The suppressed expression of LMP1 and c-myc after the romidepsin treatment was observed in Farage and U2932/EBV cells. Inhibiting either LMP1 or c-myc using small inhibitory RNAs caused partial cytotoxicity in EBV-positive DLBCL cell lines (Farage and U2932/EBV). The dual inhibition of LMP1 and c-myc showed a synergistic cytotoxic effect in EBV-positive cells similar in magnitude to that of romidepsin alone. In addition, either double blockade of LMP1 and c-myc activity or romidepsin single treatment activated EBV lytic cycle in EBV-positive cells.

Conclusions: In conclusion, romidepsin exerts strong anti-tumor activity in EBV-positive DLBCL via the inhibition of both LMP1 and c-myc. Our findings indicate that romidepsin might be a promising treatment for EBV-positive DLBCL.

Key words: c-myc, diffuse large B-cell lymphoma, Epstein-Barr virus, latent membrane protein-1, romidepsin

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List of Abbreviations and Symbols

ATCC: American Type Culture Collection

DLBCL: diffuse large B-cell lymphoma

DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen

EBER: Epstein-Barr virus-encoded small RNAs

EBNA: Epstein-Barr virus nuclear antigen

EBV: Epstein-Barr virus

FASL: Fas ligand

FISH: fluorescence in situ hybridization

GCB: germinal center B-cell

HDAC: histone deacetylase

H3: histone 3

LMP1: latent membrane protein-1

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

NK/T: natural killer/T

PARP: poly (ADP-ribose) polymerase

RT-PCR: reverse transcription polymerase chain reaction

siRNA: small inhibitory RNA

Introduction

The Epstein-Barr virus (EBV) is known to be prevalent, which more than 90% of people in the world have history of past infection. Epidemiologic studies showed that primary infection of EBV usually happened in childhood in underdeveloped countries, while it occurred in adolescence or adulthood in developed countries.[1] EBV exhibits tropism for specific cell types in human hosts, including B-cells and epithelial cells.[2] The primary EBV infection occurs through epithelial cells and then enters and promotes the transformation of B lymphocytes via unique EBV latency programs.[3] The EBV is associated with various malignancies involving epithelial cells and B lymphocytes, including nasopharyngeal cancer,[4] gastric cancer [5] and Burkitt's lymphoma.[6] The latency types of EBV-associated malignancies were classified according to the expression of EBV encoded viral genome like EBV nuclear antigen (EBNA), EBV-encoded small RNAs (EBER), and latent membrane protein (LMP). Burkitt lymphoma which don't express latent membrane protein-1 (LMP1) but express EBNA and EBER has a type I latency program, natural killer/T (NK/T) cell lymphoma which express LMP1 as well as EBNA 1 is classified as type II latency. Such lymphomas as post-transplantation lymphoproliferative disorder or human immunodeficiency virus-associated lymphoma are known to be classified as type III latency which shows additional expression of EBNA 2 to LMP1 and EBER.

Diffuse large B-cell lymphoma (DLBCL) is one of the most common hematologic malignancies worldwide.[7] Although CD20-targeted chimeric monoclonal antibody, rituximab remarkably improved treatment outcomes of DLBCL, about one third of patients still have experienced relapse or refractoriness. Since a previous study demonstrated the clinical dissection of DLBCL according to the different gene expression profiles,[8] following studies have proposed classification systems for determining of germinal center B-cell (GCB) type and non-GCB one using various combinations of immunohistochemical staining to several markers in tumor tissue.[9,10] However, there have been no other robust prognostic marker to identify a subset with poor outcomes except the molecular classification of GCB and non-GCB so far.

A recently identified DLBCL subset that is associated with EBV infection was incorporated into the revised 2008 international tumor classification system of the World Health Organization.[11] According to subsequent clinical studies, EBV-positive DLBCL has a lower cure rate and higher relapse rate than EBV-negative DLBCL, the latter of which responds better to standard chemotherapy and consolidative radiotherapy as frontline treatments.[12,13] Thus, novel treatment approaches that focus on EBV-associated carcinogenesis are needed.

Latent membrane protein-1 (LMP1) is an oncoprotein with a known role in EBV-induced hematopoietic cell immortalization.[14] LMP1 regulates various intracellular oncogenic pathways including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and bcl-2 in EBV-associated lymphoid malignancies,[15,16] and is thought to be one of the causes of cancer cell chemo-resistance.[17] This protein also has been postulated to be a key mediator of the aggressive features of various EBV-associated cancers such as nasopharyngeal carcinoma,[18] and extranodal NK/T cell lymphoma.[19] LMP1 is also associated with poor prognosis in patients with DLBCL.[20] LMP1 expression is also implicated in c-myc upregulation, which is one of the master modulators in EBV-associated malignancies.[21]

Romidepsin, a histone deacetylase (HDAC) inhibitor, may be a promising novel agent for treating EBV-associated DLBCL because it has proven to be clinically effective against hematopoietic malignancies such as peripheral T-cell lymphoma.[22,23] The anti-tumor activity of romidepsin involves a broad spectrum of mechanisms that inhibit multiple intracellular signaling pathways, such as the ERK/AKT pathway.[24-26] In addition to its activity on histones, romidepsin may also have acetylating activity on non-histone transcription factors such as c-myc.[27]

Although, several targeted agents have been tested in patients with relapsed or refractory DLBCL,[28,29] effective strategies to overcome the poor treatment

outcomes manifested by EBV-positive DLBCL have not yet been developed. Because c-myc interacts with LMP1 through intracellular signaling pathways,[15] we hypothesized an HDAC inhibitor might be effective in regulating the growth of LMP1-expressing EBV-positive DLBCL. Our study therefore explored the effect and mechanism of romidepsin in regulating EBV-positive DLBCL growth with a focus on the involvement of LMP1 and c-myc.

Materials and methods

DLBCL cell lines and reagents

Three human EBV-negative DLBCL cell lines (SU-DHL4, U2932 and OCI-LY7) were purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). An EBV-positive DLBCL cell line (Farage) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). OCI-LY7 cells were cultured in IMDM media with 10% fetal bovine serum, and the other cells were cultured in RPMI-1640 media with 10% serum. To generate EBV-transfected cells, we continuously cultured LY7 and U2932 cells in conditioned media from an EBV-positive cell line (Daudi; Burkitt lymphoma cells) for 3 months. The resulting stably transfected EBV-positive cell lines were designated LY7/EBV and U2932/EBV, respectively. For confirmation of EBV genome of transfected cell lines, EBER in situ hybridization was performed **(Figure 1)**, and fluorescent in situ hybridization of sex chromosome was also applied to rule out the possibility of contamination of Daudi cell lines in case of U2932/EBV **(Figure 2)**. Rituximab was purchased from Roche Pharmaceuticals (Hertfordshire, UK). Romidepsin was purchased from LC Laboratories (Woburn, MA, USA).

Figure 1. EBER in situ hybridization technique revealed that U2932/EBV and LY7/EBV cells were EBV-positive ones, while U2932 was EBV-negative.

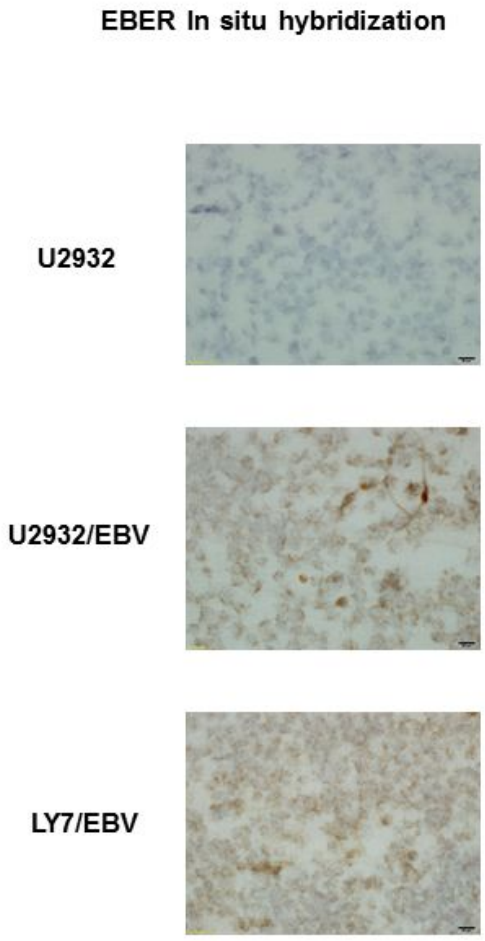
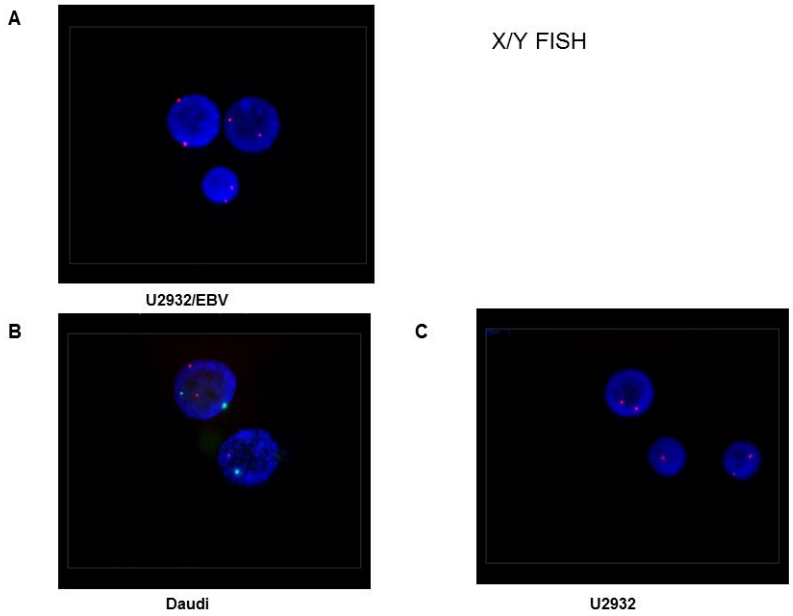


Figure 2. Daudi was established from a male patient (XY), while U2932 was originated from a female patient (XX). Fluorescence in situ hybridization (FISH) using probe detecting X and Y chromosome (probe: CEP X SpectrumOrange/Y SpectrumGreen Direct Labeled Fluorescent DNA Probe, Abott, US)



Cell viability assays

Cells were seeded at a density of 3×10^3 cells per well in 96-well culture plates and treated for 72 h with various doses of drugs, as indicated. Cells were incubated with the MTT reagent for 3 h at 37°C. The resulting formazan crystals then were solubilized with sodium dodecyl sulfate overnight. The absorbance of the formazan solution was measured at 595 nm using a microplate analyzer.

Soft agar assay

For each treatment condition, triplicate 60-mm dishes were coated with 0.9% agarose (SeaPlaque™; FMC Corporation, Rockland, ME). LY7 (5×10^4), U2392 (5×10^4), Farage (5×10^4), and U2932/EBV (2.5×10^4) cells were seeded onto the base agar layer in culture medium containing 0.45% agarose. Cells in culture dishes were allowed to settle at room temperature for 30 min, and then were moved to tissue culture incubators (37°C) after applying a 3ml upper layer of culture medium. After incubation for 14 days, colonies containing more than 50 cells were counted using an inverted microscope.

Flowcytometry analysis

For the apoptosis assays, DLBCL cells were seeded in 60-mm dishes (3×10^5 cells) and either treated with romidepsin (5 nM), or transfected with LMP1 small inhibitory RNA (siRNA), myc siRNA or both. For the cell cycle analysis, romidepsin-treated cells were harvested and fixed overnight in 70% ethanol. Fixed cells were washed and incubated with RNase followed by propidium iodide staining. Cells were harvested, washed, and resuspended in phosphate-buffered saline. Cells then were stained with annexin V and propidium iodide, and cell cycle progression and apoptotic cell death were evaluated using a FACSCanto™ flow cytometer (BD Biosciences, San Jose, CA, USA).

Western blotting

Western blot analysis was performed as described previously.[30] The primary antibodies were reactive against c-myc, Fas ligand (FASL), caspase-3, -8, and -9 (Cell Signaling Technology Inc., Danvers, MA, USA); p50, p65, Poly (ADP-ribose) polymerase (PARP), Acetyl-H3, H3, ZEBRA, EaD, β-actin (Santa Cruz Biotechnology, Santa Cruz, CA); p-myc, LMP1, and Bam H1Z (Abcam plc., Cambridge, MA, USA).

Reverse transcription polymerase chain reaction

Total RNA was extracted from the DLBCL cells using Trizol (Invitrogen, Carlsbad, CA, USA). Primer design for reverse transcription polymerase chain reaction (RT-PCR) were as follows: EBER1: 5'-AGGACCTACGCTGCCCTAGA-3' and 5'-CCCTAGAAATGGTGCCAATG-3', EBER2: 5'-AGGACAGCCGTTGCCCTAGTGGTTTCG-3' and 5'-AAAAACAGCGGACAAGCCGAATACC-3', EBNA1: 5'-GATGAGCGTTTGGGAGAGCTGATTCTGCA-3' and 5'-TCCTCGTCCATGGTTATCAC-3', EBNA2: 5'-GCTGCTACGCATTAGAGACC-3' and 5'-TCCTGGTAGGGATTCGAGGG-3', and LMP1: 5'-TCCTCCTCTTGGCGCTACTG-3' and 5'-TCATCACTGTGTCGTTGTCC-3'. For RT-PCR, samples were then subjected to 35 cycles of amplification by a Thermal Cycler 480 (Perkin-Elmer, Foster City, CA, USA), each cycle consisting of 94°C for 1 min (denaturation), 45°C for 2 min (annealing) and 72°C for 3 min (DNA extension). The extension time was prolonged to 6 min in the last cycle.

Mouse xenograft model

Four-week-old female NOD/SCID mice were obtained from Harlan Laboratories (San Pietro al Natisone, Italy). The mice were acclimated for at least 7 days before handling. To assess the anti-tumor effects of romidepsin, U2932 (1×10^7) and U2932/EBV (0.5×10^6) cells were harvested and resuspended in 100 μl of a 1:1 mixture of phosphate-buffered saline and

Matrigel® (BD Biosciences). Each mouse was subcutaneously injected in the flank using a 23-gauge needle. One week after implantation, mice were randomly divided into two groups. Romidepsin (2.4mg/kg) or vehicle was intraperitoneally injected into mice two times per week (for a total of 4 weeks). Tumor sizes in mice were measured every 4 days with a caliper, and tumor volumes were calculated using the formula: (length×width^2) ×0.5. The Korea Institute of Radiological and Medical Science approved the animal studies.

Small interfering RNA transfection

Farage (3×10^5) and U2932/EBV cells (2×10^5) were plated in 60-mm dishes in RPMI-1640 media. Cells were transfected with 50 nM NF-κB, LMP1 and myc siRNA (GE Healthcare Dharmacon Inc., Lafayette, CO, USA) or control non-targeting siRNA using Lipofectamine™ 2000 (Thermo Fisher Scientific/Invitrogen Corporation, Carlsbad, CA, USA). After 24 h, cells were treated with 5 nM romidepsin for a further 24 h. Apoptosis then was analyzed using flow cytometry. Whole cell lysates were subjected to western blotting analysis.

Immunofluorescence staining

Samples were prepared and stained as described previously.[31] Cells were treated with 5 nM romidepsin for 24 h. The primary antibodies used were specific for ZEBRA (Santa Cruz Biotechnology) and cleaved caspase-3 (Cell Signaling Technology Inc.). For nuclear staining, cells were incubated with 2 μg/ml of DAPI. The images of the immunostained slides were observed using a confocal laser scanning microscope (Leica, Bannock Burn, IL, USA).

Results

EBV-positive DLBCL cells express high levels of both LMP1 and c-myc

To confirm the EBV status of the DLBCL cell lines, we analyzed the expression of the EBV-associated viral genes EBER1, EBER2, EBNA1, EBNA2, and LMP1 using RT-PCR. EBV-positive cell lines (Farage, LY7/EBV, and U2932/EBV) expressed EBV-associated genes, while EBV-negative cell lines (SU-DHL4, OCI-LY7, and U2932) did not express these genes **(Figure 3)**. EBV LMP1 is an oncoprotein that increases cell survival and growth pathway activities, including the activities of NF-κB, PI3K, and c-myc. Because NF-κB is a major component of LMP1 signal transduction,[32,33] we examined nuclear NF-κB levels in DLBCL cells **(Figure 4A)**, and showed that nuclear NF-κB was elevated in EBV-positive DLBCL cells. However, the knockdown of NF-κB by siRNA did not induce cytotoxicity in EBV-positive DLBCL cells **(Figure 4B)**. These results imply that EBV LMP1-induced NF-κB activation may not be associated with EBV DLBCL survival. Western blot analysis revealed the expression of LMP1, c-myc, MEK, and Erk in EBV-positive cells, whereas weak or no expression of these molecules was observed in EBV-negative cells **(Figure 5)**.

Romidepsin differentially affects DLBCL cells according to their EBV

status

We first determined whether romidepsin induces different responses in DLBCL cells according to their EBV status. Figure 6 shows that romidepsin significantly induced cytotoxicity in EBV-positive cells compared with EBV-negative cells. Flow-cytometry analysis using annexin-V/propidium iodide revealed that romidepsin treatment preferentially increased apoptosis in EBV-positive versus EBV-negative cells **(Figure 7A and B)**. We also screened cytotoxicities according to EBV status in another subset of non-Hodgkin lymphoma cell lines. PI/annexin-V staining analysis confirmed selective cytotoxicity of romidepsin in NK/T cell lymphoma cell lines. Romidepsin induced more effective cytotoxicity in EBV-positive NK/T cell lymphoma cell line, HANK-1 than in EBV-negative NK-92 cells **(Figure 8)**. However, neither EBV-positive Burkitt lymphoma cell line, Raji nor EBV-negative Ramos respond to romidepsin **(Figure 9)**. Because the cytotoxicity of romidepsin is linked to the induction of histone 3 (H3) acetylation [34], we examined the effect of romidepsin treatment on acetyl-H3 status in DLBCL cells. H3 acetylation was increased in both EBV-positive and EBV-negative cells following romidepsin treatment, which implies that H3 acetylation does not affect the sensitivity of DLBCL to romidepsin. Conversely, romidepsin induced caspase and PARP cleavage in EBV-positive cells only **(Figure 10)**. Vinodhkumar *et al.* showed that romidepsin inhibits cell growth by regulating

the expression levels of cyclin B1 and p21.[35] We also confirmed that romidepsin induced G2/M phase arrest and selectively increased the sub-G1 phase (apoptotic cells) in EBV-positive, but not EBV-negative DLBCL cells **(Figure 11)**. These findings suggest that the mechanism of romidepsin-induced cell death in EBV-positive DLBCL cells might involve a pro-apoptotic pathway that precedes the caspase cascade rather than the mediation of H3 acetylation.

Romidepsin has anti-tumor effects in vivo

To confirm that EBV infection affects DLBCL sensitivity to romidepsin, we performed *in vitro* assays and *in vivo* studies in a mouse model. First, we performed an *in vitro* clonogenic assay in two EBV-negative cell lines (U2932 and OCI-LY7) and two EBV-positive cell lines (Farage and U2932/EBV). Romidepsin (5 nM) significantly suppressed the formation of colonies by >80% in Farage and U2932/EBV cells, whereas colony growth was unaffected by romidepsin in LY7 and U2932 cells **(Figure 12)**.

We further conducted *in vivo* animal studies using naïve U2932 or U2932/EBV-transfected tumor xenografts in NOD/SCID mice. Animals were intraperitoneally injected with romidepsin (2.4 mg/kg) twice per week. After 4 weeks, romidepsin significantly reduced tumor volume in mice with

U2932/EBV-bearing tumors, whereas romidepsin did not suppress tumor growth in naïve U2932-xenografted mice **(Figure 13)**. These *in vitro* and *in vivo* results indicate that romidepsin differentially inhibits tumor growth according to EBV status in DLBCL.

Romidepsin enhances DLBCL apoptosis by activating the EBV lytic cycle

Based on a 2012 study in which HDAC inhibition induced the EBV lytic cycle and cancer cell apoptosis in nasopharyngeal carcinoma, an EBV-associated malignancy,[36] we examined whether the HDAC inhibitor romidepsin could activate the EBV lytic cycle in EBV-positive DLBCL cells. THe immunoblot assay revealed that romidepsin induced the expression of early EBV lytic proteins, including ZEBRA, EaD, and Bam HIZ, in EBV-positive DLBCL cells (U2932/EBV and Farage) in a concentration and time-dependent manners **(Figure 14)**. In contrast to romidepsin, rituximab caused no difference in cell viability between EBV-positive Farage cells and EBV-negative U2932 cells **(Figure 15)**. Moreover, rituximab did not induce EBV lytic proteins in EBV-positive Farage cells **(Figure 16)**. Immunofluorescence staining revealed the co-localization of ZEBRA and cleaved caspase-3 in the nuclei of EBV-positive DLBCL cells in response to romidepsin treatment **(Figure 17)**. Together, these results suggest that

romidepsin induces apoptosis in EBV-positive DLBCL cells by activating the EBV lytic cycle.

Romidepsin induces DLBCL cytotoxicity by down-regulating LMP1 and c-myc

Based on our findings that the baseline expression of LMP1 and c-myc in DLBCL cells differs according to their EBV status **(Figure 5)**, we examined the change in LMP1 and c-myc in these cells in response to romidepsin. Western blot analysis revealed decreased LMP1 and c-myc expression in EBV-positive, but not EBV-negative cells, after romidepsin treatment **(Figure 18)**. Knockdown of either LMP1 or c-myc individually using siRNAs did not induce sufficient cytotoxicity in Farage and U2932/EBV. These data show that one-sided inhibition of the LMP1/c-myc interactive pathway does not fully suppress cell survival.

Thus, we next performed a dual inhibition of these molecules using scrambled LMP1 and c-myc siRNAs transfected into EBV-positive cells, which demonstrated synergistic anti-tumor activity similar to that of romidepsin **(Figure 19)**. To further elucidate whether the cytotoxic effect of knockdown of LMP1 is through NF-κB or not, we performed an additional experiment using different combinational pairs of siRNAs, which suggests the mechanism

of action of LMP1 knockdown might be through a pathway rather than NF-κB **(Figure 20)**. Western blot analysis revealed that LMP1 siRNA alone weakly suppressed the LMP1-mediated downstream pathway activation of p-MEK and p-Erk as well as c-myc in EBV-positive DLBCL cells (Farage and U2932/EBV). However, the additional inhibition of c-myc activation by LMP1 siRNA synergistically induced a profound suppression of those signaling molecules **(Figure 21A)**. In addition, the dual suppression of LMP1 and c-myc also induced early EBV lytic cycle activation **(Figure 21B)**. Furthermore, cytotoxic effect of romidepsin was not different irrespective of pretreatment with siRNAs for LMP1 or c-myc **(Figure 22)**. This suggested that LMP1 and c-myc play major roles in romidepsin-induced cytotoxicity in EBV-positive DLBCL cells. Immunofluorescence staining against cleaved caspase-3 and ZEBRA proved the synergistic effect of the dual inhibition of LMP1 and c-myc on EBV lytic cycle activation in caspase-activated nuclei **(Figure 23)**. Taken together, LMP1 and c-myc synergistically mediate romidepsin-induced tumor cell lysis in EBV-positive DLBCL.

Figure 3. The results of RT-PCR using EBV-encoded RNA probes revealed that LMP1, EBER, and EBNA were detected only in EBV-positive DLBCL cell lines (i.e., Farage, LY7/EBV, and U2932/EBV).

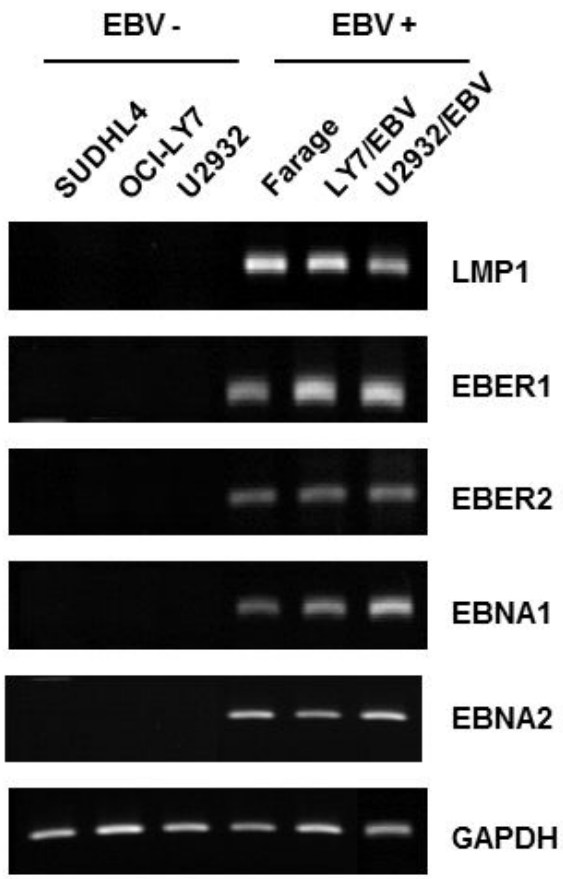


Figure 4. A) Western blot analysis on nuclear fractions showed an increased expression of NF-κB in EBV-positive versus EBV-negative DLBCL cells. B) The anti-tumor effect from inhibiting NF-κB using siRNA was not significantly different between EBV-positive and EBV-negative cells.

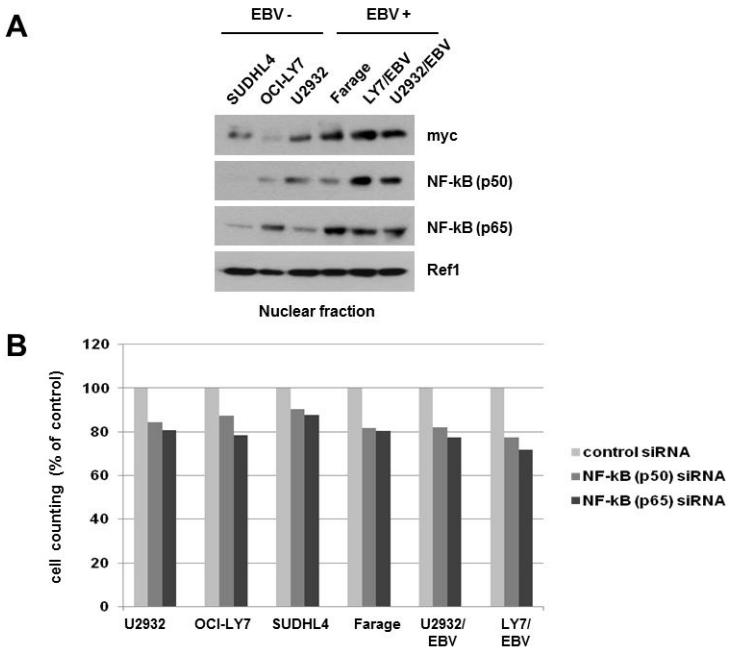


Figure 5. EBV-positive DLBCL cells harbor increased basal expression levels of LMP1, c-myc, p-MEK, and p-Erk compared with EBV- negative cells

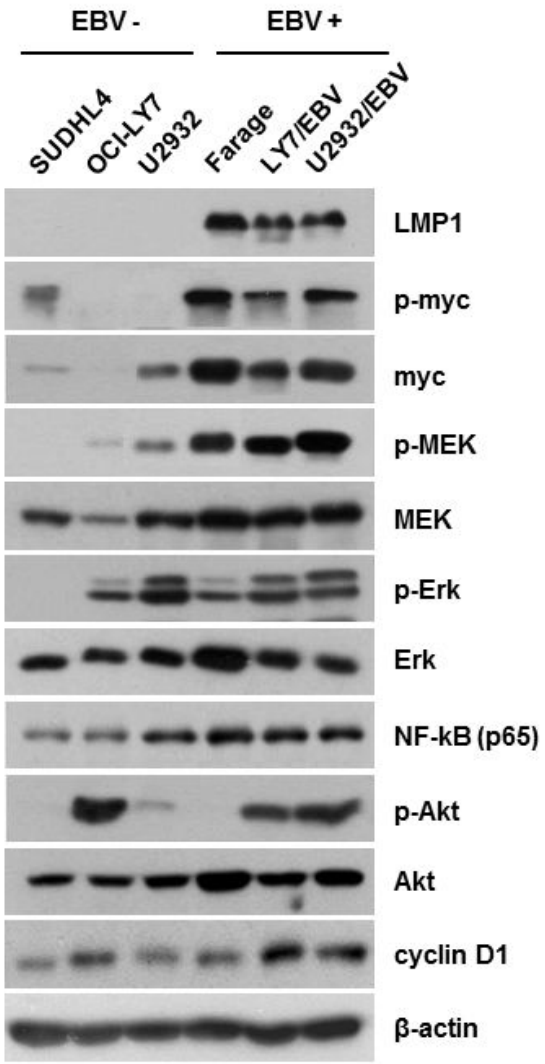


Figure 6. The MTT assay demonstrated a greater cytotoxic effect of romidepsin on EBV-positive DLBCL cell lines (Farage, LY7/EBV, and U2932/EBV) versus EBV-negative cell lines.

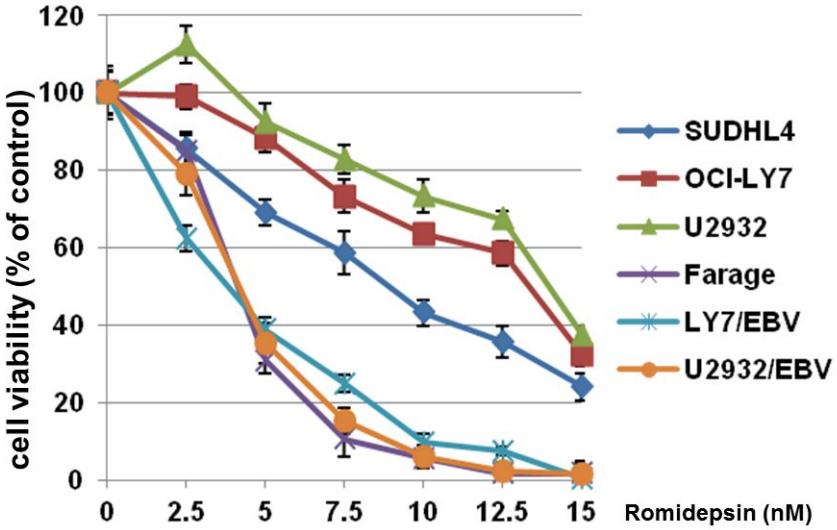


Figure 7. A) and B) Flow cytometry analysis demonstrated increased annexin V staining, reflecting apoptosis, in romidepsin-treated EBV-positive cells (Farage, U2932/EBV, and LY7/EBV) versus EBV negative cells.

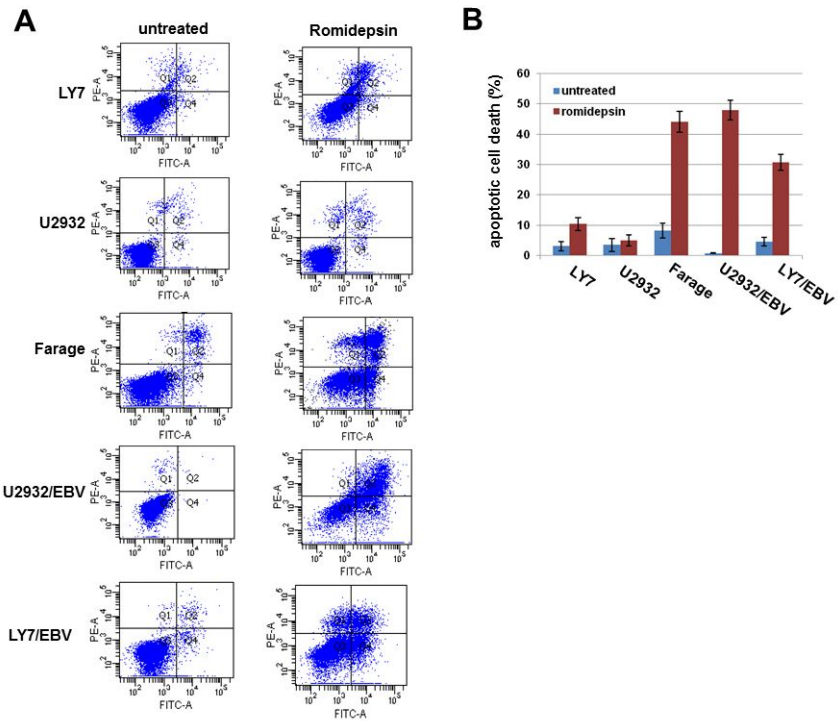


Figure 8. Flowcytometric analysis using PI/Annexin-V antibody demonstrated that romidepsin induced more effective cytotoxicity in EBV-positive NK/T cell lymphoma cell line, HANK-1 than in EBV-negative NK-92 cell line.

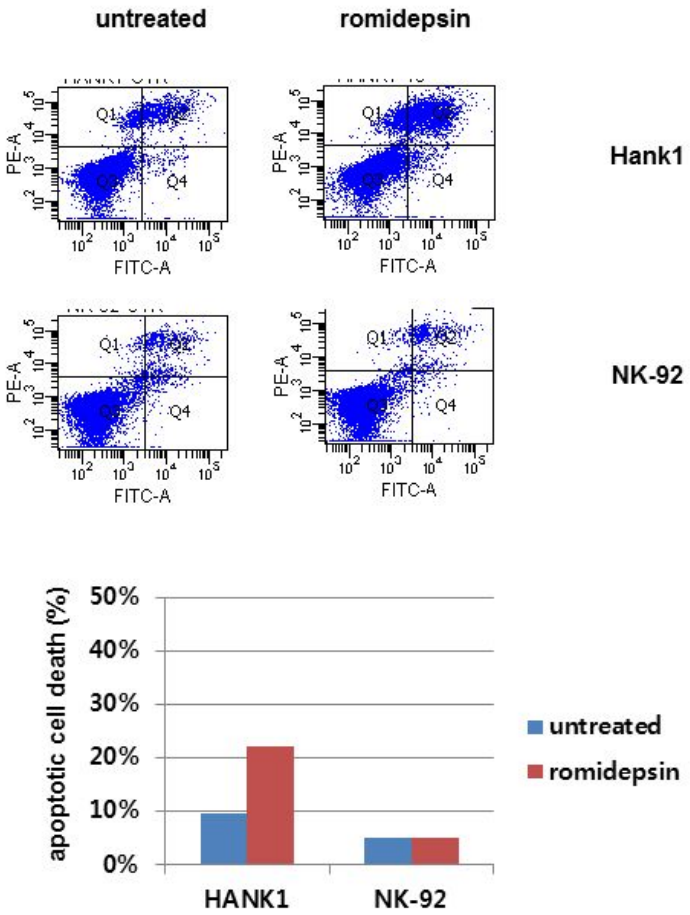


Figure 9. No difference of cytotoxicity according to EBV status was observed in burkitt lymphoma cell lines treated with romidepsin (Raji: EBV-positive, Ramos: EBV-negative).

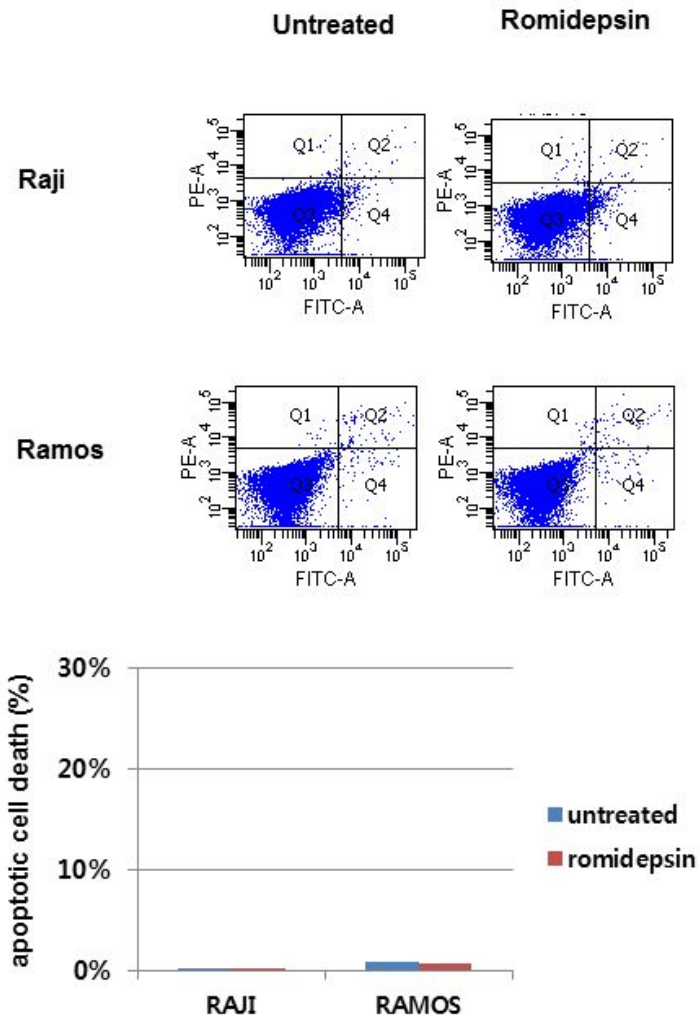


Figure 10. Romidepsin activated caspase-3, caspase-8, and caspase-9 in EBV-positive DLBCL cells but not EBV-negative cells. In contrast, romidepsin-induced histone 3 (H3) acetylation was similar in EBV-positive and EBV-negative DLBCL cells. Each experiment was triplicated.

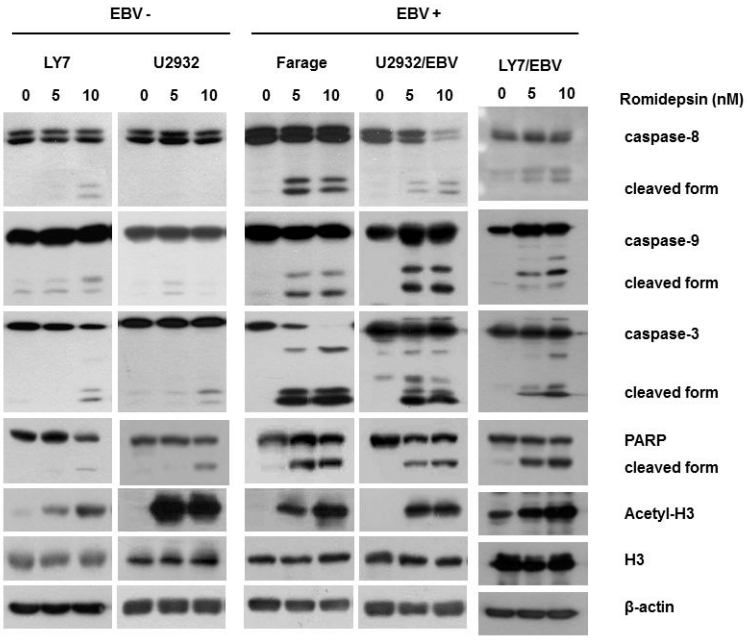


Figure 11. Cell cycle analysis indicated a sub-G1 peak and G2/M arrest in romidepsin-treated EBV-positive DLBCL cells (Farage and U2932/EBV).

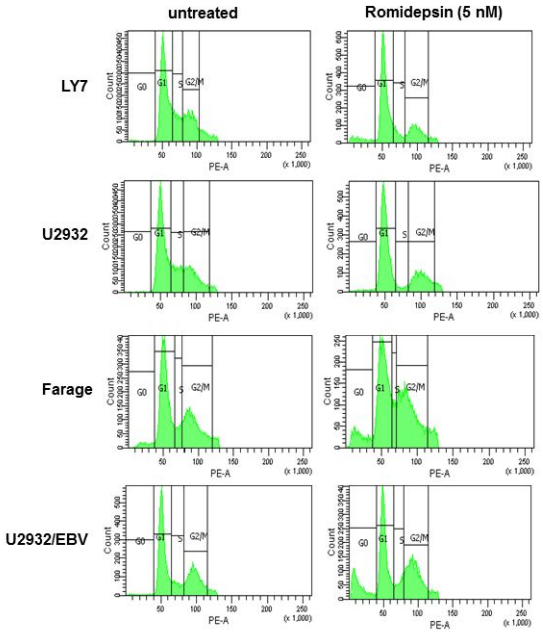


Figure 12. A, B and C) Romidepsin (5 nM) suppressed colony formation by >50% in Farage, U2932/EBV, and LY7/EBV cells, whereas colony formation in OCI-LY7 and U2932 cells was unaffected by this inhibitor at this concentration in clonogenic assays. All experiments were repeated three times.

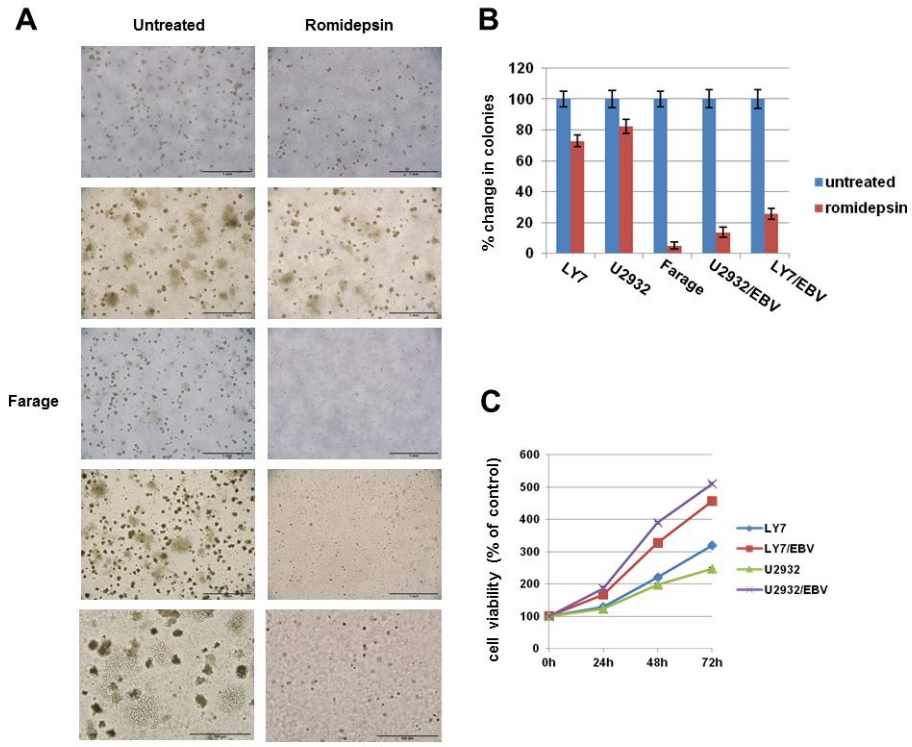


Figure 13. Systemic romidepsin administration inhibited the growth of EBV-positive DLBCL tumor xenografts (U2932/EBV) in NOD/SCID mice, but did not affect the growth of EBV-negative tumor grafts. Tumor volumes were calculated using the formula: (length×width^2) ×0.5 (mm).

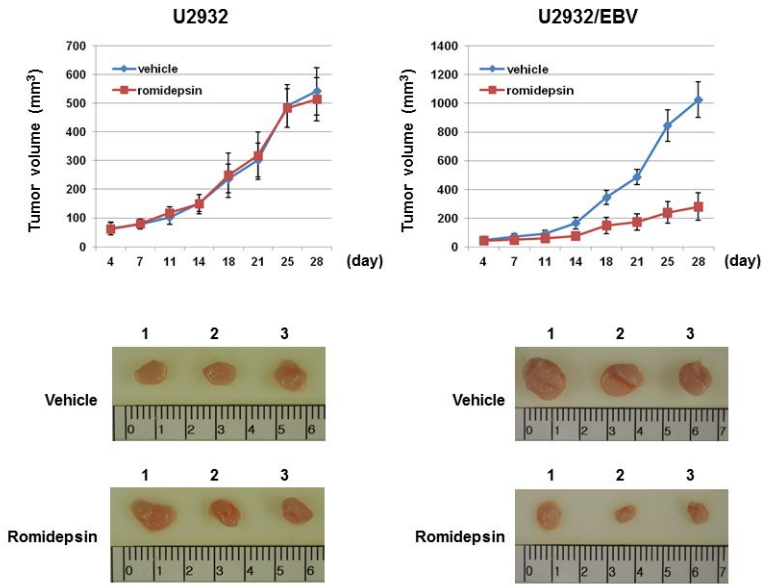


Figure 14. Romidepsin induced early EBV lytic cycle activation in a (A) concentration- and (B) time-dependent manner in EBV-positive DLBCL cells.

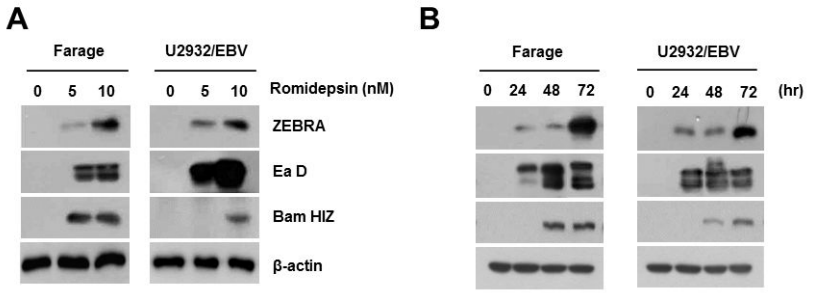
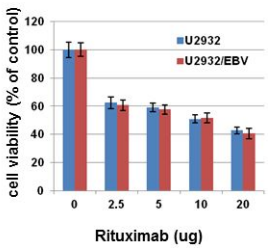


Figure 15. A) Romidepsin had different effects on EBV-positive cells (U2932/EBV) and EBV-negative cells (U2932), whereas rituximab did not have different effects on EBV-positive and EBV-negative cells. B) Rituximab did not induce EBV lytic cycle activation in EBV-positive cells (Farage).



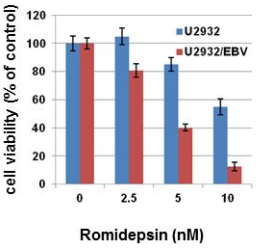


Figure 16. Rituximab did not induce EBV lytic cycle activation in EBV-positive cells (Farage)

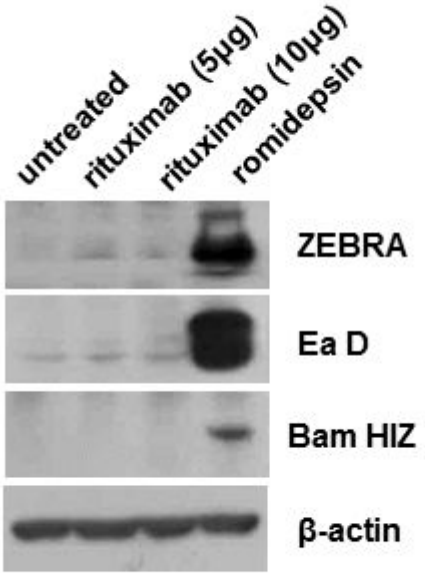


Figure 17. ZEBRA and cleaved caspase-3 co-localized in EBV-positive DLBCL cell nuclei (U2932/EBV).

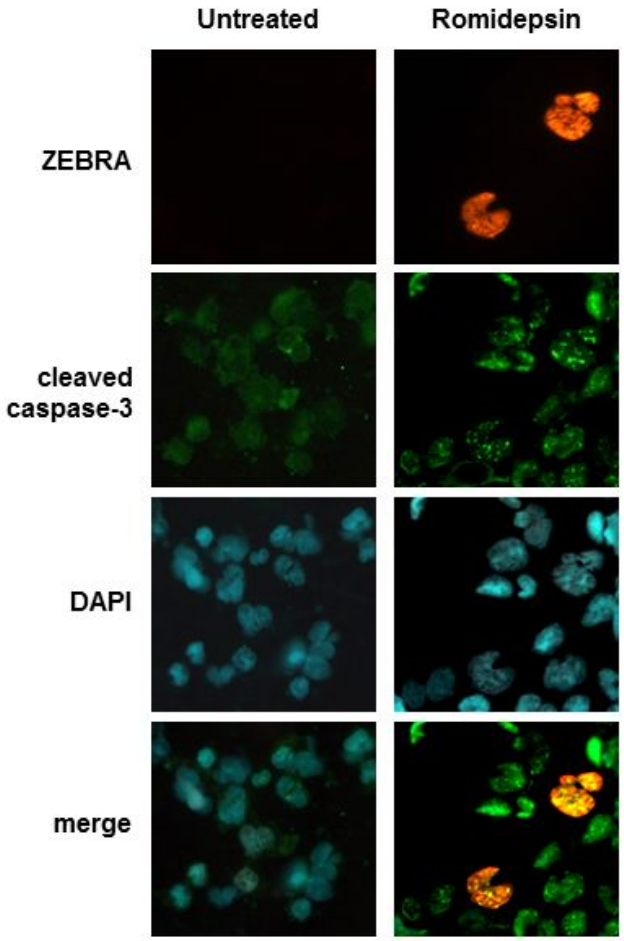


Figure 18. Romidepsin inhibited LMP1 and c-myc in EBV-positive (Farage and U2932/EBV), but not EBV-negative DLBCL cells (U2932).

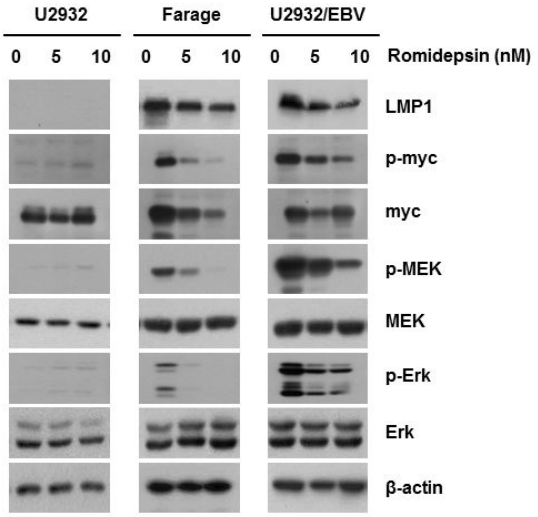


Figure 19. A and B) The dual inhibition of LMP1 and c-myc using siRNAs synergistically increased apoptosis.

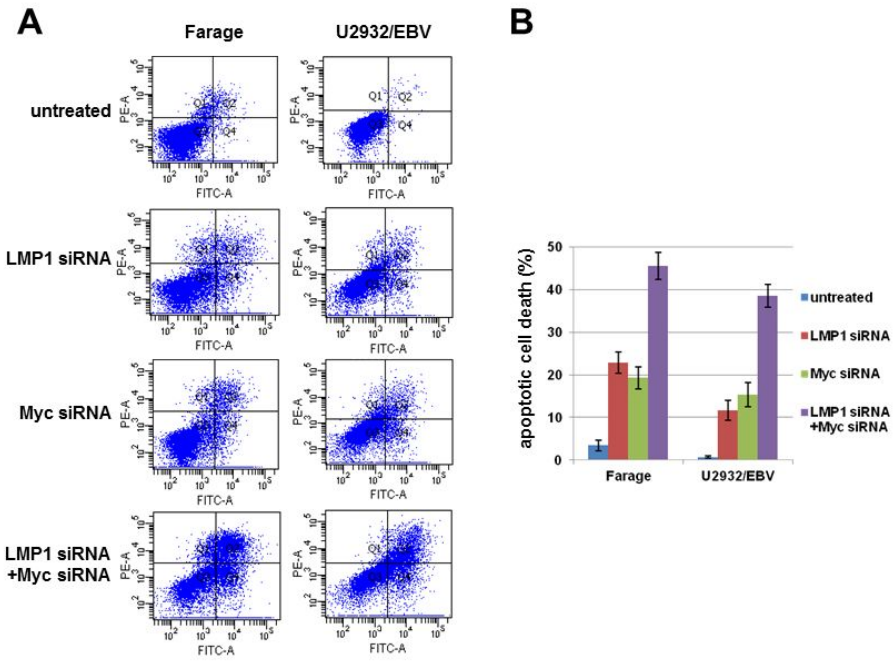


Figure 20. NF-κB knockdown showed minimal cytotoxicity in EBV-positive cell. However, additional knockout by either LMP1 siRNA or c-myc siRNA to NF-κB siRNA did not demonstrate synergistic cytotoxicity.

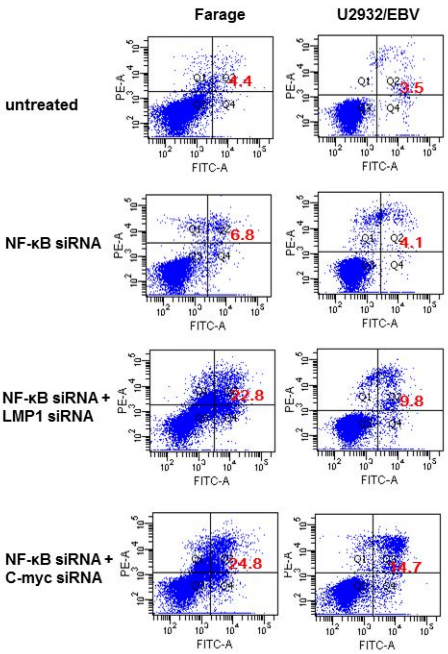


Figure 21. A) The dual inhibition of LMP1 and c-myc using siRNAs decreased the expression of LMP1-driven intra-cellular signalling pathways involving c-myc, p-MEK, and p-Erk in EBV-positive cells (Farage and U2932/EBV).

B) The dual inhibition of LMP1 and c-myc also induced EBV lytic cycle activation.

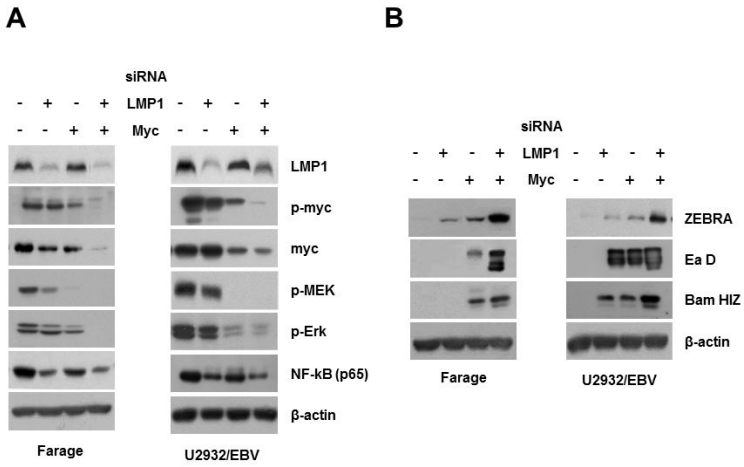


Figure 22. Cytotoxic effect of romidepsin was similar irrespective of pretreatment with siRNAs, which indirectly suggested that LMP1 and c-myc play major roles in romidepsin-induced cytotoxicity

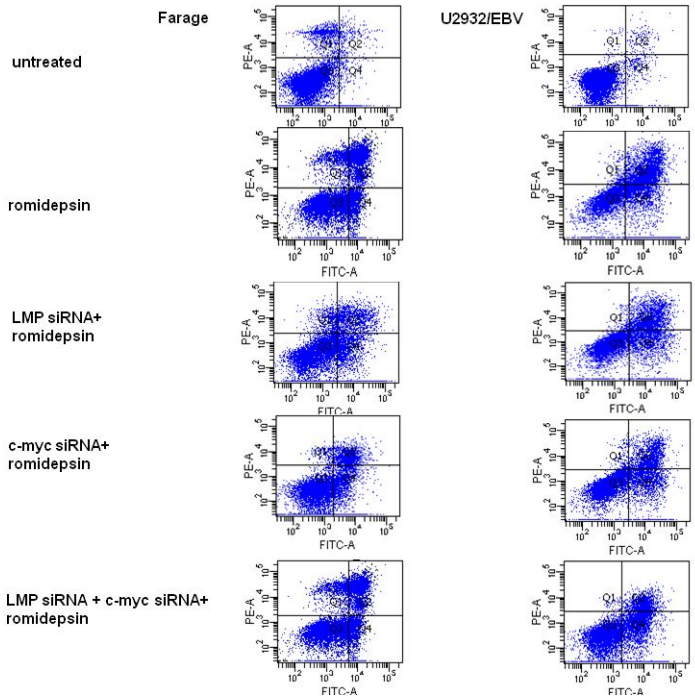
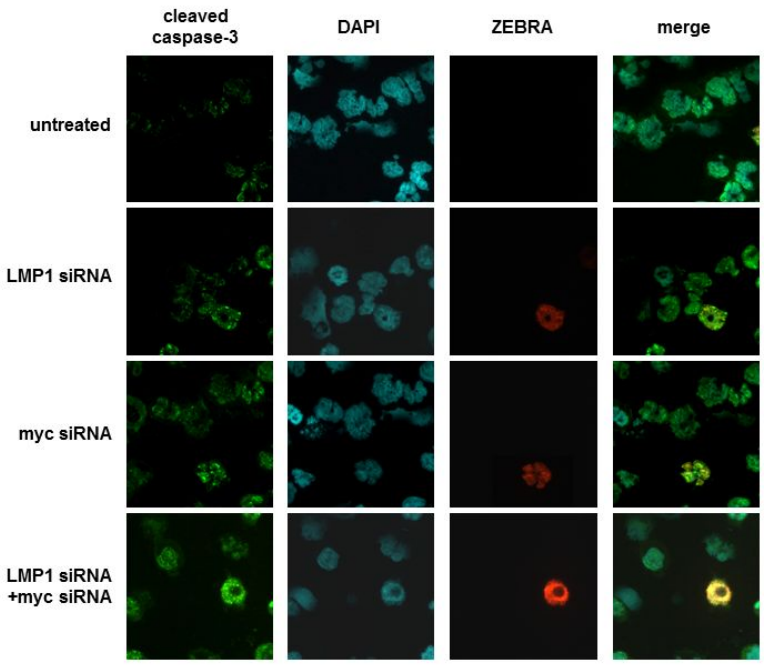


Figure 23. The synergistic overexpression of ZEBRA induced by LMP1 and c-myc blockade happened in the same locus.



Discussion

EBV-positive DLBCL has a poor prognosis because it responds poorly to conventional chemoimmunotherapy and frequently relapses.[12,13] Our experiments using U2932/EBV-positive cells demonstrated more invasive growth features than naïve EBV-negative U2932 cells in clonogenic assays. In addition, the growth rate of xenografted tumors in EBV-positive DLBCL-bearing mice was faster than in EBV-negative DLBCL-bearing mice. These results firmly support previous clinical observations of poorer outcomes in EBV-positive DLBCL.

Our study found that romidepsin, a clinically promising HDAC inhibitor, activates apoptosis in EBV-positive DLBCL cells. Romidepsin inhibited EBV-driven LMP1 and c-myc overexpression. The selective inhibition of both LMP1 and c-myc by siRNAs mimicked the effects of romidepsin. The caspase cascade activated by romidepsin was correlated with EBV lytic cycle activation, which was reproduced through the synchronous dual blockade of LMP1 and c-myc by siRNAs.

EBV-positive tumor cells express LMP1 in many cases, and LMP1 is associated with the maintenance and proliferation of EBV-infected cells.[37] LMP1 activates various down-stream signaling pathways such as NF-κB, stat3, PI3K, and c-myc which enhance tumor growth and survival in many

malignancies.[15,16,38] Our data showed that EBV-positive cells express higher levels of LMP1 and its down-stream proteins than EBV-negative cells **(Figure 1B)**. Previous studies suggested that NF-κB is a critical pathway activated by LMP1 in EBV-positive DLBCL;[15,39] therefore, we tested the effect of NF-κB siRNA treatment in EBV-positive and EBV-negative DLBCL cells. However, the inhibition of NF-κB by siRNA resulted in only a slight difference in cytotoxicity according to the EBV status of the cells **(Figure 4)**. The NF-κB blockade by siRNA was not synergistic even with LMP1 siRNA or c-myc siRNA. In addition, the inhibition of NF-κB by siRNA did not result in the inhibition of LMP1 or c-myc **(Figure 20)**.

Next, we focused on the role of c-myc in EBV-positive DLBCL cells. LMP1 and c-myc are interconnected through intracellular signaling pathways,[40,41] and NF-κB activation by LMP1 induces c-myc expression.[15] In contrast to the minimal effect of NF-κB with inhibition on cytotoxicity, inhibiting c-myc using siRNA caused measurable but incomplete cytotoxic activity in EBV-positive DLBCL cells **(Figure 19)**. Inhibiting LMP1 by siRNA also resulted in partial cytotoxicity and the partial suppression of c-myc **(Figure 19)**, which indirectly supports the importance of the interaction between LMP1 and c-myc. A previous study[38] demonstrated the overexpression of c-myc in LMP1-positive human nasopharyngeal carcinoma tissue samples, which is consistent with our finding that LMP1 and c-myc co-operatively contribute to cell

survival in EBV-positive DLBCL. Because the co-treatment of EBV-positive DLBCL cells with LMP1 and c-myc siRNAs resulted in synergistic cytotoxicity, we inferred that LMP1-dependent and LMP1-independent c-myc activities are cooperatively engaged in the pathogenesis of EBV-positive DLBCL. To strengthen our hypothesis, we also checked the cytotoxic effect of romidepsin in EBV-positive DLBCL cells pretreated with siRNAs for LMP1 or c-myc **(Figure 22)**. The results strengthened our hypothetic mechanism of romidepsin, that is, romidepsin not only inhibits c-myc but also LMP1, which in turn suppressed LMP1-driven intracellular pathways as well as LMP1-dependent c-myc activity. As a result romidepsin induced EBV lytic cycle activation and apoptosis coupled with the activation of caspase cascade **(Figure 24)**.

HDAC inhibitors that were thought to act through histone acetylation have been revealed to also be associated with the acetylation of various non-histone transcription factors and co-regulators.[27,42] Because c-myc has the unique ability to bind to more than 10% of all human genes,[43] and recruits specific co-activators or co-repressors such as HDACs,[44] a HDAC inhibitor might also act as a c-myc inhibitor by interfering in the direct interaction between HDACs and c-myc. Our data showed that romidepsin suppressed c-myc expression in EBV-positive DLBCL cells **(Figure 18)**, which is consistent with the results of a previous study in which HDAC inhibition decreased c-

myc transcript and protein levels in lymphoma cells.[45] However, previous studies have not fully explained the differential cytotoxicity of romidepsin according to EBV status in DLBCL cells. Our study revealed that the underlying mechanism mediating the greater induction of cytotoxicity in EBV-positive versus EBV-negative DLBCL by romidepsin involves the dual inhibition of LMP1 and c-myc. To our knowledge, our study is the first to elucidate the selective cytotoxic effect of romidepsin on EBV-positive DLBCL, acting via dual LMP1 and c-myc inhibition.

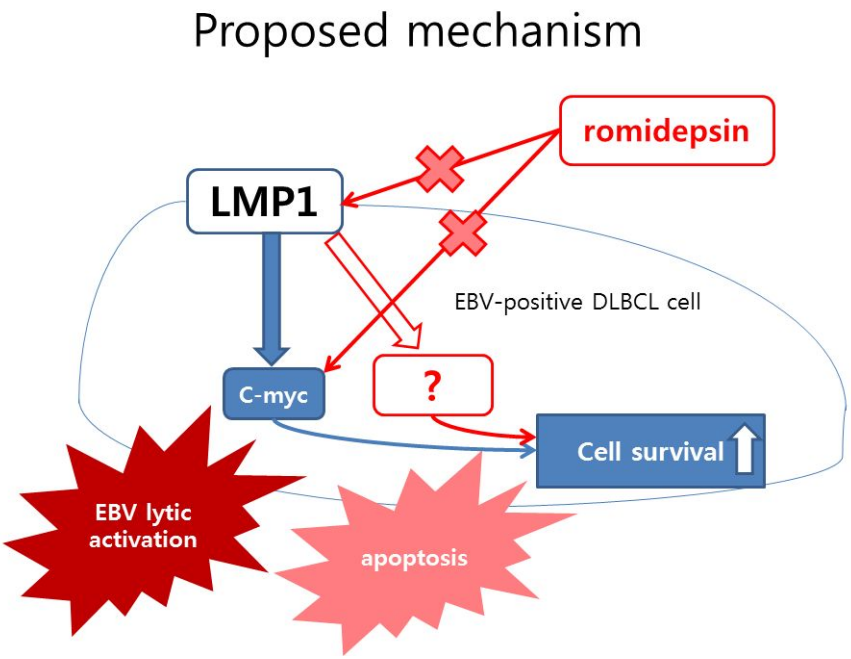
Clinical trials using romidepsin have focused mainly on patients with indolent T-cell non-Hodgkin lymphoma. The early clinical trials of romidepsin as a monotherapy for patients with cutaneous T-cell lymphoma, a slow-growing subtype of non-Hodgkin lymphoma, showed promising results.[46] A subsequent European phase II study published in 2011/2012 also demonstrated the favorable efficacy of romidepsin in patients with relapsed or refractory peripheral T-cell lymphoma.[22,23] Based on our preclinical results, future clinical studies using romidepsin to treat EBV-positive DLBCL are warranted.

Our study had several limitations. First, we did not use a large variety of EBV-positive DLBCL cell lines or tumor samples from patients to expand and consolidate our findings. However, this limitation is a reflection of the

difficulties in studying EBV-positive DLBCL due to the paucity of patients with EBV-positive DLBCL and the availability of additional appropriate cell lines. Although romidepsin showed selective cytotoxicity according to EBV status in NK/T lymphoma cell lines **(Figure 8)** as well as DLBCL cell lines, romidepsin had no effect on Burkitt lymphoma cell lines irrespective of EBV status **(Figure 9)**. Further studies will be needed to elucidate this difference which might be from the hidden mechanisms regulating complex signalling pathways unique to specific lymphoma cell line subsets. Second, we could not confirm molecular changes in patient tumors following *in vivo* treatment with romidepsin, because this drug has not yet been approved for DLBCL patients. Third, we did not assess the titre of EBV in the supernatant of original Daudi cell culture media used for EBV transfection. This means that there is a possibility that U2932/EBV or LY7/EBV is just a partial EBV viral gene-incorporated cell line rather than EBV whole genome-transfected one. If the whole EBV genome was not transfected, romidepsin might inducec EBV early lytic protein expression rather than the induction of EBV virus lytic cycle through the true viral proliferation. Despite these limitations, our study produced novel results that elucidated the specific cytotoxic effect of romidepsin on EBV-positive DLBCL cells via dual inhibition of LMP1 and c-myc. This study is also the first to demonstrate that romidepsin induces the EBV lytic cycle and apoptosis via the caspase cascade.

In conclusion, romidepsin has promising anti-tumor activities especially against EBV-positive DLBCL. Our findings are noteworthy in view of the urgent need for new clinical strategies to treat patients with relapsed or refractory EBV-positive DLBCL, a rare disease with a poor clinical prognosis.

Figure 24. Proposed mechanism of romidepsin-induced cytotoxic effect in EBV-positive DLBCL.



References

1. Thompson MP, Kurzrock R. Epstein-Barr virus and cancer. Clin ical cancer research : an official journal of the American Association fo r Cancer Research 2004;10:803-21.

2. Murata T, Sato Y, Kimura H. Modes of infection and oncogene sis by the Epstein-Barr virus. Reviews in medical virology 2014.

3. Hong GK, Gulley ML, Feng WH, Delecluse HJ, Holley-Guthrie E, Kenney SC. Epstein-Barr virus lytic infection contributes to lympho proliferative disease in a SCID mouse model. Journal of virology 2005; 79:13993-4003.

4. Mounier N, Canals C, Gisselbrecht C, et al. High-dose therapy and autologous stem cell transplantation in first relapse for diffuse larg e B cell lymphoma in the rituximab era: an analysis based on data fro m the European Blood and Marrow Transplantation Registry. Biology of blood and marrow transplantation : journal of the American Society fo r Blood and Marrow Transplantation 2012;18:788-93.

5. Fitoussi O, Belhadj K, Mounier N, et al. Survival impact of rit uximab combined with ACVBP and upfront consolidation autotransplanta tion in high-risk diffuse large B-cell lymphoma for GELA. Haematologi ca 2011;96:1136-43.

6. Schwartz EJ, Molina-Kirsch H, Zhao S, Marinelli RJ, Warnke

RA, Natkunam Y. Immunohistochemical characterization of nasal-type ex tranodal NK/T-cell lymphoma using a tissue microarray: an analysis of 84 cases. American journal of clinical pathology 2008;130:343-51.

7. Chihara D, Ito H, Matsuda T, et al. Differences in incidence a nd trends of haematological malignancies in Japan and the United State s. British journal of haematology 2014;164:536-45.

8. Rosenwald A, Wright G, Chan WC, et al. The use of molecula r profiling to predict survival after chemotherapy for diffuse large-B-cell lymphoma. The New England journal of medicine 2002;346:1937-47.

9. Hans CP, Weisenburger DD, Greiner TC, et al. Confirmation of the molecular classification of diffuse large B-cell lymphoma by immu nohistochemistry using a tissue microarray. Blood 2004;103:275-82.

10. Muris JJ, Ylstra B, Cillessen SA, et al. Profiling of apoptosis genes allows for clinical stratification of primary nodal diffuse large B-cell lymphomas. British journal of haematology 2007;136:38-47.

11. Sabattini E, Bacci F, Sagramoso C, Pileri SA. WHO classificati on of tumours of haematopoietic and lymphoid tissues in 2008: an over view. Pathologica 2010;102:83-7.

12. Park S, Lee J, Ko YH, et al. The impact of Epstein-Barr virus status on clinical outcome in diffuse large B-cell lymphoma. Blood 20 07;110:972-8.

13. Yoshino T, Nakamura S, Matsuno Y, et al. Epstein-Barr virus i nvolvement is a predictive factor for the resistance to chemoradiotherap y of gastric diffuse large B-cell lymphoma. Cancer science 2006;97:163-6.

14. Cahir McFarland ED, Izumi KM, Mosialos G. Epstein-barr viru s transformation: involvement of latent membrane protein 1-mediated act ivation of NF-kappaB. Oncogene 1999;18:6959-64.

15. Dirmeier U, Hoffmann R, Kilger E, et al. Latent membrane pr otein 1 of Epstein-Barr virus coordinately regulates proliferation with co ntrol of apoptosis. Oncogene 2005;24:1711-7.

16. Pratt ZL, Zhang J, Sugden B. The latent membrane protein 1 (LMP1) oncogene of Epstein-Barr virus can simultaneously induce and i nhibit apoptosis in B cells. Journal of virology 2012;86:4380-93.

17. Kim JH, Kim WS, Yun Y, Park C. Epstein-Barr virus latent m embrane protein 1 increases chemo-resistance of cancer cells via cytopla smic sequestration of Pim-1. Cellular signalling 2010;22:1858-63.

18. Shen Z, Zeng Y, Guo J, et al. Over-expression of the special AT rich sequence binding protein 1 (SATB1) promotes the progression of nasopharyngeal carcinoma: association with EBV LMP-1 expression. Journal of translational medicine 2013;11:217.

19. Mao Y, Zhang DW, Zhu H, et al. LMP1 and LMP2A are pote

ntial prognostic markers of extranodal NK/T-cell lymphoma, nasal type (ENKTL). Diagnostic pathology 2012;7:178.

20. Paydas S, Ergin M, Erdogan S, Seydaoglu G. Prognostic signifi cance of EBV-LMP1 and VEGF-A expressions in non-Hodgkin's lympho mas. Leukemia research 2008;32:1424-30.

21. Faumont N, Durand-Panteix S, Schlee M, et al. c-Myc and Rel /NF-kappaB are the two master transcriptional systems activated in the l atency III program of Epstein-Barr virus-immortalized B cells. Journal o f virology 2009;83:5014-27.

22. Coiffier B, Pro B, Prince HM, et al. Results from a pivotal, o pen-label, phase II study of romidepsin in relapsed or refractory periphe ral T-cell lymphoma after prior systemic therapy. Journal of clinical onc ology : official journal of the American Society of Clinical Oncology 2 012;30:631-6.

23. Piekarz RL, Frye R, Prince HM, et al. Phase 2 trial of romide psin in patients with peripheral T-cell lymphoma. Blood 2011;117:5827-34.

24. Fecteau KA, Mei J, Wang HC. Differential modulation of signa ling pathways and apoptosis of ras-transformed 10T1/2 cells by the dep sipeptide FR901228. The Journal of pharmacology and experimental ther apeutics 2002;300:890-9.

25. Yu X, Guo ZS, Marcu MG, et al. Modulation of p53, ErbB1, ErbB2, and Raf-1 expression in lung cancer cells by depsipeptide FR90 1228. Journal of the National Cancer Institute 2002;94:504-13.

26. Kobayashi Y, Ohtsuki M, Murakami T, et al. Histone deacetyla se inhibitor FK228 suppresses the Ras-MAP kinase signaling pathway b y upregulating Rap1 and induces apoptosis in malignant melanoma. Onc ogene 2006;25:512-24.

27. Yang XJ, Seto E. HATs and HDACs: from structure, function a nd regulation to novel strategies for therapy and prevention. Oncogene 2007;26:5310-8.

28. Hernandez-Ilizaliturri FJ, Deeb G, Zinzani PL, et al. Higher res ponse to lenalidomide in relapsed/refractory diffuse large B-cell lympho ma in nongerminal center B-cell-like than in germinal center B-cell-like phenotype. Cancer 2011;117:5058-66.

29. Morschhauser FA, Cartron G, Thieblemont C, et al. Obinutuzum ab (GA101) monotherapy in relapsed/refractory diffuse large b-cell lymp homa or mantle-cell lymphoma: results from the phase II GAUGUIN st udy. Journal of clinical oncology : official journal of the American Soci ety of Clinical Oncology 2013;31:2912-9.

30. Kim A, Park S, Lee JE, et al. The dual PI3K and mTOR inhi bitor NVP-BEZ235 exhibits anti-proliferative activity and overcomes bort

ezomib resistance in mantle cell lymphoma cells. Leukemia research 20 12;36:912-20.

31. Kim A, Lee JE, Jang WS, et al. A combination of methotrexat e and irradiation promotes cell death in NK/T-cell lymphoma cells via down-regulation of NF-kappaB signaling. Leukemia research 2012;36:350 -7.

32. Kieser A, Kaiser C, Hammerschmidt W. LMP1 signal transducti on differs substantially from TNF receptor 1 signaling in the molecular functions of TRADD and TRAF2. The EMBO journal 1999;18:2511-21.

33. Izumi KM, Kieff ED. The Epstein-Barr virus oncogene product latent membrane protein 1 engages the tumor necrosis factor receptor-a ssociated death domain protein to mediate B lymphocyte growth transfor mation and activate NF-kappaB. Proceedings of the National Academy of Sciences of the United States of America 1997;94:12592-7.

34. Schrump DS. Cytotoxicity mediated by histone deacetylase inhi bitors in cancer cells: mechanisms and potential clinical implications. Cl inical cancer research : an official journal of the American Association for Cancer Research 2009;15:3947-57.

35. Vinodhkumar R, Song YS, Devaki T. Romidepsin (depsipeptide) induced cell cycle arrest, apoptosis and histone hyperacetylation in lun g carcinoma cells (A549) are associated with increase in p21 and hypo

phosphorylated retinoblastoma proteins expression. Biomedicine & pharm acotherapy = Biomedecine & pharmacotherapie 2008;62:85-93.

36. Hui KF, Ho DN, Tsang CM, Middeldorp JM, Tsao GS, Chiang AK. Activation of lytic cycle of Epstein-Barr virus by suberoylanilide hydroxamic acid leads to apoptosis and tumor growth suppression of na sopharyngeal carcinoma. International journal of cancer Journal internatio nal du cancer 2012;131:1930-40.

37. Lam N, Sugden B. CD40 and its viral mimic, LMP1: similar means to different ends. Cellular signalling 2003;15:9-16.

38. Tudor CS, Dawson CW, Eckhardt J, et al. c-Myc and EBV-LM P1: two opposing regulators of the HLA class I antigen presentation ma chinery in epithelial cells. British journal of cancer 2012;106:1980-8.

39. Montes-Moreno S, Odqvist L, Diaz-Perez JA, et al. EBV-positiv e diffuse large B-cell lymphoma of the elderly is an aggressive post-ger minal center B-cell neoplasm characterized by prominent nuclear factor-kB activation. Modern pathology : an official journal of the United Stat es and Canadian Academy of Pathology, Inc 2012;25:968-82.

40. Saha A, Robertson ES. Epstein-Barr virus-associated B-cell lym phomas: pathogenesis and clinical outcomes. Clinical cancer research : a n official journal of the American Association for Cancer Research 201 1;17:3056-63.

41. Levens DL. Reconstructing MYC. Genes & development 2003;17:1071-7.

42. Drummond DC, Noble CO, Kirpotin DB, Guo Z, Scott GK, Benz CC. Clinical development of histone deacetylase inhibitors as anticancer agents. Annual review of pharmacology and toxicology 2005;45:495-528.

43. Li Z, Van Calcar S, Qu C, Cavenee WK, Zhang MQ, Ren B. A global transcriptional regulatory role for c-Myc in Burkitt's lymphoma cells. Proceedings of the National Academy of Sciences of the United States of America 2003;100:8164-9.

44. Kurland JF, Tansey WP. Myc-mediated transcriptional repression by recruitment of histone deacetylase. Cancer research 2008;68:3624-9.

45. Kretzner L, Scuto A, Dino PM, et al. Combining histone deacetylase inhibitor vorinostat with aurora kinase inhibitors enhances lymphoma cell killing with repression of c-Myc, hTERT, and microRNA levels. Cancer research 2011;71:3912-20.

46. Piekarz RL, Frye R, Turner M, et al. Phase II multi-institutional trial of the histone deacetylase inhibitor romidepsin as monotherapy for patients with cutaneous T-cell lymphoma. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 2009;27:5410-7.

국문초록

목적: 엡스타인-바 바이러스 (EBV) 양성 미만성 B 대세포 림프종 (DLBCL)은 공격적이고 불량한 예후를 보이는 비호지킨 림프종으로, 2008 년 개정된 세계보건기구(WHO) 국제종양분류체계에 새로 편입된 아형이다. 우리는 엡스타인-바 바이러스 양성 미만성 B 대세포 림프종에서 히스톤 탈아세틸화 효소인 로미뎁신의 역할을 연구하였다.

재료 및 방법: EBV 양성과 EBV 음성 DLBCL 세포주가 사용되었고, 전달감염을 통해 만들어진 2 가지의 EBV 양성 세포주 (LY7/EBV, U2932/EBV)가 사용되었다. 생체실험을 위해 U2932 와 U2932/EBV 의 피부이식모델을 NOD/SCID 마우스를 이용하여 제작하였다. LMP1 와 c-myc 의 선택적인 저해 실험은 소간섭 RNA 를 이용해 수행되었고, 분할 카스파제-3 와 제브라에 대한 형광항체를 이용하여 세포자멸사와 EBV 용해주기의 활성화가 같은 장소에서 일어나는지 여부를 확인하였다.

결과: 배양된 EBV 양성세포에 로미뎁신을 처리하여 세포독성은 확인하였고, 로미뎁신은 카스파제 연쇄반응을 통해 세포독성을 보였다. 더 나아가, 생체 마우스 이종이식 모델이 사용되었는데, 이 또한 EBV 양성 세포주에서 로미뎁신의 세포독성을 확인시켜 주었다.

로미뎁신 처치후 LMP1 과 c-myc 의 발현의 감소가 Farage 와 U2932/EBV 에서 관찰되었다. 소간섭 RNA 를 이용하여 LMP1 이나 c-myc 을 억제하면 EBV 양성 세포주인 Farage 와 U2932/EBV 세포주에서 부분적인 세포독성이 야기되었다. LMP1 과 c-myc 의 복합적인 억제는 EBV 양성 세포주에서 상승적인 세포독성을 보여주었는데, 이는 로미뎁신을 단독 처리하였을 때와 비슷한 정도의 효과였다. 게다가 LMP1 과 c-myc 을 이중으로 억제하거나, 로미뎁신을 단독으로 처리하였을 때에 EBV 양성 세포에서 EBV 용해주기의 활성화가 일어났다.

결론: 결론적으로, 로미뎁신은 LMP1 과 c-myc 의 억제를 통해 EBV 양성 DLBCL 에서 강력한 항종양효과를 보여주었다. 우리의 결과는 로미뎁신이 EBV 양성 DLBCL 에서 유망한 치료제로서의 가능성을 보여주었다.

주요어: c-myc, 미만성 B대세포 림프종, 엡스타인-바 바이러스, latent membrane protein-1, 로미뎁신

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