Title	Inhibition of class I histone deacetylases by romidepsin potently induces Epstein-Barr virus lytic cycle and mediates enhanced cell death with ganciclovir
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Citation	International Journal of Cancer, 2016, v. 138 n. 1, p. 125–136
Issued Date	2016
URL	http://hdl.handle.net/10722/216730
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Inhibition of class I histone deacetylases by romidepsin potently induces Epstein-Barr virus lytic cycle and mediates enhanced cell death with ganciclovir

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Inhibition of class I histone deacetylases by romidepsin potently induces Epstein-Barr virus lytic cycle and mediates enhanced cell death with ganciclovir

Short title: EBV lytic cycle reactivation by romidepsin

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Abbreviations: EBV - Epstein-Barr virus, GCV - ganciclovir, HDAC - histone deacetylase, SAHA -suberoylanilide hydroxamic acid, NPC - nasopharyngeal carcinoma and GC - gastric carcinoma

Journal Category: Cancer therapy

Novelty and Impact: Pharmacological reactivation of Epstein-Barr virus (EBV) lytic cycle is being explored as a potential therapeutic strategy against EBV-associated diseases. This study demonstrates that a class I histone deacetylase (HDAC) inhibitor, romidepsin, can potently induce EBV lytic cycle through inhibition of HDAC-1/-2/-3 and activation of protein kinase C-δ and confer cytotoxicity of ganciclovir in EBV-positive epithelial malignancies. The data warrant further application of romidepsin for the investigation of EBV life cycle and treatment of EBV-associated cancers.

Abstract

Pan-histone deacetylase (HDAC) inhibitors, which inhibit eleven HDAC isoforms, are widely used to induce EBV lytic cycle in EBV-associated cancers *in vitro* and in clinical trials. Here, we hypothesized that inhibition of one or several specific HDAC isoforms by selective HDAC inhibitors could potently induce EBV lytic cycle in EBV-associated malignancies such as nasopharyngeal carcinoma (NPC) and gastric carcinoma (GC). We found that inhibition of class I HDACs, particularly HDAC-1, -2 and -3, was sufficient to induce EBV lytic cycle in NPC and GC cells *in vitro* and *in vivo*. Among a panel of selective HDAC inhibitors, the FDA-approved HDAC inhibitor romidepsin was found to be the most potent lytic inducer, which could activate EBV lytic cycle at ~0.5 to 5 nM (versus ~800 nM achievable concentration in patients' plasma) in more than 75% of cells. Up-regulation of $p21^{WAF1}$, which is negatively regulated by class I HDACs was observed prior to the induction of EBV lytic cycle. The up-regulation of $p21^{WAF1}$ and induction of lytic cycle were abrogated by a specific inhibitor of PKC-δ but not the inhibitors of PI3K, MEK, p38 MAPK, JNK, or ATM pathways. Interestingly, inhibition of HDAC-1, -2 and -3 by romidepsin or shRNA knockdown could confer susceptibility of EBV-positive epithelial cells to the treatment with ganciclovir. In conclusion, we demonstrated that inhibition of class I HDACs by romidepsin could potently induce EBV lytic cycle and mediate enhanced cell death with ganciclovir, suggesting potential application of romidepsin for the treatment of EBV-associated cancers.

Introduction:

Epstein-Barr virus (EBV) is a gamma herpesvirus, which infects more than 90% of the world population. It is closely associated with several types of malignancies, such as endemic Burkitt's lymphoma, Hodgkins lymphoma, nasal NK/T-cell lymphoma, nasopharyngeal carcinoma (NPC), and gastric carcinoma (GC).[1, 2] In these EBV-associated malignancies, the virus persists in tightly latent forms with restricted expression of viral latent proteins in every tumor cell.[1, 3] Reactivation of the latent virus into lytic cycle can lead to expression of over 70 viral proteins of higher immunogenicity and replication of viral genomes inside host cells. Despite the strong association with various cancers, current chemotherapy regimens ignore the EBV-positive status of the associated tumours due to difficulty in targeting the latent virus in these tumours.

Pharmacological reactivation of EBV lytic cycle to confer susceptibility to antiviral drugs is being explored as a potential therapeutic strategy against the EBV-associated diseases.[4] Studies have shown that a nucleoside-type antiviral drug ganciclovir (GCV) could be converted from an inactive form to cytotoxic form, when they are phosphorylated by the viral lytic protein kinase BGLF4.[5] The concept of this lytic induction therapy began in the treatment of EBV-positive lymphoma with combination of histone deacetylase (HDAC) inhibitors such as arginine butyrate or valproic acid (VPA) with GCV.[6] Feng *et al.* further showed that combination of chemotherapy and VPA could induce EBV lytic cycle and sensitize EBV-positive epithelial tumours to GCV treatment.[7] Recently, a clinical trial of the lytic induction therapy using combination of gemcitabine, VPA, and GCV was carried out in three NPC patients and demonstrated improved clinical outcomes.[8] Since the killing effect of GCV relies on expression of BGLF4, the efficacy of this treatment strategy greatly depends on the efficiency and specificity of lytic induction. The development of more

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specific and potent lytic inducing agents will aid its translation into clinical use.

Histone deacetylases (HDACs) are over-expressed in various types of cancer cells. The HDACs can repress the transcription of different cellular and viral genes. Mammalian HDACs can be categorized into four groups, including the class I (HDAC-1, -2, -3 and -8), class II (HDAC-4, -5, -6, -7, -9 and -10), class III (SIRT-1-7) and class IV HDACs (HDAC-11).[9-12] HDAC inhibitors targeting different combinations of HDAC isoforms have been engaged into different stages of clinical trials for the treatment of cancers.[13] To date, two HDAC inhibitors, including suberoylanilide hydroxamic acid (SAHA) and romidepsin have been approved by the Food and Drug Agency (FDA) for treatment of cutaneous T-cell lymphoma and peripheral T-cell lymphoma, respectively.[14, 15] Pan-HDAC inhibitors, which inhibit class I, II and IV HDAC isoforms, including trichostatin A, sodium butyrate, and VPA, are widely used to induce EBV lytic cycle in EBV-associated cancers *in vitro*.[7, 8, 16, 17] We have reported that pan-HDAC inhibitors such as SAHA can trigger the switch of EBV from latent to lytic cycle and mediate enhanced killing of EBV-positive epithelial cancer cells, including those of NPC and GC.[16, 17] However, the effect of several potentially more potent and selective HDAC inhibitors on EBV lytic cycle reactivation is still unknown.

In this study, we hypothesized that inhibition of one or several specific HDAC isoforms by selective HDAC inhibitors can potently induce EBV lytic cycle in EBV-positive NPC and GC cells. We investigated the effects of different selective HDAC inhibitors, which target different combinations of HDAC isoforms, for their abilities to induce EBV lytic cycle in EBV-positive NPC and GC cells. We also examined the potential mechanisms related to the EBV lytic reactivation and investigated the combinatorial effects of the specific HDAC inhibitors with ganciclovir on the killing of EBV-positive NPC and GC cells.

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Materials and Methods:

Cell cultures

AGS and AGS-BDneo are paired Epstein-Barr virus (EBV)-negative and recombinant EBV infected GC cell lines, respectively.[18] SNU-719 is a GC cell line containing native EBV genomes (purchased from the Korean Cell Line Bank).[19, 20] HONE1 and HA are paired EBV-negative and recombinant EBV infected NPC cell lines, respectively.[21, 22] C666-1 is an EBV-positive NPC cell line that harbors native EBV genomes.[23] AGS-BX1 and HONE1-EBV are generated by infecting AGS and HONE1 cells with recombinant EBV genomes expressing green fluorescent protein.[18, 21, 22] The cell lines were authenticated with an AmpF/STR Identifiler PCR Amplication Kit (Applied Biosystems, Foster City, CA), according to the manufacturer's protocol. The data were analyzed by GeneScan and GeneMapper[TM] ID Software (Applied Biosystems, Foster City, CA). The cells were cultured as previously described.[17, 24] G418 was added to maintain the recombinant EBV in AGS-BDneo, AGS-BX1, HA and HONE1-EBV cells in culture flasks. To avoid any unexpected interaction between G418 and drugs, G418 was not added during drug treatment.

Chemicals

The selective HDAC inhibitors including romidepsin, apicidin, MS-275, droxinostat and PCI34051 were purchased from Selleck (Selleck Chemicals, Houston, TX); CAY10603 and SAHA were purchased from Cayman (Cayman Chemicals, Ann Arbor, MI). The anti-viral drug ganciclovir (GCV) was purchased from Sigma (Sigma-Aldrich, St. Louis, MO). The pathway blockers including LY294002 (PI3K inhibitor), PD98059 (MEK inhibitor), SP600125 (JNK inhibitor), SB202190 (p38 MAPK inhibitor), and rottlerin (PKC-δ inhibitor) were purchased from Calbiochem

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(Calbiochem, San Diego, CA); KU-55933 (ATM inhibitor) was purchased from Selleck (Selleck Chemicals).

Western blot analysis

To analyze the expression of EBV lytic proteins, NPC and GC cells were treated with various concentrations of selective HDAC inhibitors for 48 hr. After treatment, the cells were pelleted and washed once with PBS. Proteins from the cell pellets were extracted and western blot analyses were performed as described previously.[16] EBV latent and lytic proteins were detected with anti-EBNA1, anti-Zta, anti-Rta, anti-BMRF1, anti-VCA p18, and anti-gp350/220 antibodies; cell cycle regulatory proteins, were detected with anti-p21[WAF1], anti-cyclin-B1, anti-p-cdc2, anti-cyclin-D1 and anti-p-Rb antibodies; histone acetylation was detected with an anti-acetyl-H3 and anti-acetyl-H4 antibodies; apoptosis was detected with anti-PARP rabbit polyclonal antibody as described previously.[16, 17, 24, 25] Expression of cellular kinases was detected with anti-p-PKC-δ, anti-p-JNK, anti-p-p38 and anti-p-ATM antibodies (1:1000; Cell Signaling Technology, Beverly, MA). Expression of human β-actin was detected with anti-β-actin antibody (1:10000; Sigma-Aldrich, St. Louis, MO) as a loading control. The proteins were visualized with HRP-conjugated anti-mouse IgG (1:5000; Zymed, San Francisco, CA), anti-rabbit IgG (1:5000; Zymed, San Francisco, CA) or anti-rat IgG (1:5000; Zymed, San Francisco, CA) goat polyclonal antibodies and Pierce ECL Western Blotting Detection Reagent (Thermo Fisher Scientific, Rockford, IL). All proteins were identified according to their corresponding molecular weights.

Immunocytochemistry

EBV-positive HA cells grown on cover slips coated with 0.1% gelatin were treated with various selective HDAC inhibitors for 48 hr. The cells were fixed with acetone

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for 10 minutes and then stained with a combination of anti-Zta mouse monoclonal (1:50) and anti-VCA-p18 rat polyclonal (1:50) antibodies overnight at 4°C. Zta and VCA-p18 proteins were visualized with Alexa Fluor 488 goat anti-mouse IgG antibody (1:500; Invitrogen, Carlsbad, CA) and Alexa Fluor 594 goat anti-rat IgG antibody (1:500; Invitrogen), respectively, under fluorescence microscopy. Nuclei of cells were stained with 4',6-diamidino-2-phenylindole (DAPI) (Roche, Mannheim, Germany).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide(MTT) assay

NPC and GC cells were treated with various concentrations of selective HDAC inhibitors for various time points in 96-well cell culture plates and incubated at 37°C in 5% CO_2. MTT assay was performed as previously described.[16]

Nude mice experiment

HA, SNU-719, HONE1 and C666-1 (1×10^7) cells were resuspended in 200 μl serum-free culture medium. The cells were subcutaneously injected at the right flanks of female BALB/c nude (nu/nu) at 6-7 weeks of age. When the tumours became palpable, 375 or 750 μg/kg romidepsin (days 1 & 4 each week) dissolved in DMSO in 10 μl was administered to the nude mice of the treatment groups (n=5) by intraperitoneal (IP) injection for 4 weeks. Equal volume of DMSO was administered to the nude mice of the control group (n=5). For the combination treatment with GCV (Roche), 50 mg/kg GCV (5 days per week) dissolved in PBS in 50 μl was administered to the mice by IP injection for 4 weeks. The mice were weighed twice weekly to assess the toxicity of the drugs. All the mice were euthanized by IP injection of 200 mg/kg pentobarbital at the end of experiment, when the control group of mice had tumours greater than 1.7 cm in diameter. The size and the weight of the

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tumours were measured as described previously.[17, 24]

Cell cycle analysis

HA and AGS-BDneo cells were treated with romidepsin for 12, 24, 48, and 72 hr. The treated cells were subjected to cellular DNA content analysis by flow cytometry as described previously.[16]

Flow cytometric analysis

EBV-positive HA and AGS-BDneo cells were treated with various concentrations of romidepsin for 5 days. The treated cells were then fixed and stained with combinations of LIVE/DEAD fixable aqua dead cell stain kit (Invitrogen, Carlsbad, CA), cleaved caspase-3 rabbit monoclonal antibody (1:200) and either anti-Zta (1:50), anti-BMRF1 (1:800) or anti-gp350/220 (1:50) mouse monoclonal antibodies overnight at 4°C. Co-expression of the proteins was detected with Alexa Fluor 488 goat anti-rabbit IgG (1:500; Invitrogen, Carlsbad, CA) and Alexa Fluor 647 goat anti-mouse IgG (1:500; Invitrogen) antibodies using flow cytometry (LSRII, BD Biosciences, San Jose, CA) as previously described. Data were analyzed by FlowJo software (Tree Star, San Carlos, CA).

Cell viability studies

EBV-negative and -positive NPC and GC cells were seeded in 24-well cell culture plates. Cells grown to 70% confluence were treated with combinations of various concentrations of romidepsin and GCV for 7 days. Cell viability was determined by trypan blue exclusion assay.

Quantitative PCR assay

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To quantitate viral DNA replication during induction of EBV lytic cycle in NPC and GC cells, HA and AGS-BDneo cells were treated with different concentrations of romidepsin for 48 hr. After treatment, the cells were pelleted and washed once with PBS. DNA from the cell pellets was extracted and quantitative PCR was performed as described previously.[16] EBV viral load was presented as number of viral genomes per cell. Data were determined in triplicate in a 96-well plate format.

EBV infection assay

To examine the production of infectious viral particles, AGS-BX1 and HONE1-EBV cells, which were infected with GFP-expressing recombinant EBV, were treated with romidepsin with or without GCV for 5 days. The culture supernatants were collected and EBV infection assay was performed as previously described.[16]

Knockdown constructs for HDACs

A pLKO TRC cloning vector (Addgene no. 10878) mammalian expression lentiviral plasmid was used to establish the HDAC-1, -2, and -3 knockdown constructs. For each HDAC, two sets of oligonucleotides were utilized to perform knockdown experiments. The knockdown constructs were designed according to the information of the Public TRC portal: HDAC-1 (TRCN0000195672 and TRCN0000004816), HDAC-2 (TRCN0000195547 and TRCN0000196590), and HDAC-3 (TRCN0000004825 and TRCN0000196267). The scramble vector (Addgene 1864) served as a control.

Statistical analysis

In vitro experiments were performed in triplicate and repeated at least 3 times. Data were analyzed for statistical significance using One-way ANOVA Dunnett's Multiple Comparison Test. P value < 0.05 was considered statistically significant. All statistical

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analyses were performed with GraphPad Prism Version 5.0 software.

Results:

The class I HDAC inhibitor romidepsin significantly induced EBV lytic cycle in EBV- positive NPC cells

Pan-HDAC inhibitors, which inhibit eleven HDAC isoforms, were widely used to induce EBV lytic cycle *in vitro* and in clinical study. We have reported that SAHA, an FDA approved HDAC inhibitor, can significantly induce EBV lytic cycle in EBV-positive epithelial malignancies such as NPC cells.[16, 17] Interestingly, SAHA, which was previously recognized as a pan-HDAC inhibitor, is selective against several HDAC isoforms, including HDAC-1, -2, -3, -6 and -8.[26] We, therefore, set out to test several selective HDAC inhibitors for their abilities to induce EBV lytic cycle in EBV-positive NPC cells. Expression of an EBV immediate-early lytic protein, Zta, was detected as a marker to indicate the induction of EBV lytic cycle in an NPC cell line, HA. We found that class I HDAC inhibitors targeting HDAC-1, -2 and -3 isoforms (romidepsin, MS-275 or apicidin) could significantly induce NPC cells into EBV lytic cycle, whereas those targeting HDAC-3, -6 and -8 (droxinostat), HDAC-6 (Cay10603), or HDAC-8 (PCI34051) could not (Fig 1a). Among the selective HDAC inhibitors, romidepsin was the most potent HDAC inhibitor, which can induce EBV lytic cycle in more than 70% of cells at nanomolar concentrations (Fig. 1b). Romidepsin also induced a comparable level of EBV lytic protein expression and histone acetylation at a 2000-fold lower concentration, when compared with SAHA (2.5 nM vs 5 μM), whilst it did not cause acetylation of α-tubulin, a substrate of HDAC6 (Fig. 1c). These data demonstrated the potency of romidepsin on the induction of EBV lytic cycle and specificity of romidepsin against HDAC-1, -2 and -3 in EBV-positive epithelial cells.

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Romidepsin inhibited proliferation and induced EBV lytic cycle in multiple EBV-positive NPC and GC cell lines

We further tested the effects of romidepsin on cell proliferation and EBV lytic cycle induction in different NPC and GC cell lines. EBV-positive NPC (HA and C666-1) and GC (AGS-BDneo and SNU-719) cells were either untreated (U) or treated with romidepsin at different concentrations. After 2 days, the percentage of proliferating cells was determined by MTT assay (Supplementary Fig. 1a). The proliferations of all these cancer cell lines were inhibited in a dose dependent manner. We also analyzed the expression of Zta in these cell lines upon treatment with romidepsin by western blotting. Interestingly, we found that romidepsin can potently induce EBV lytic cycle in all these cell lines from 600 picomolar to 5 nM concentrations (Supplementary Fig. 1b). Among the four EBV-positive epithelial cell lines, HA and AGS-BDneo cells are relatively more sensitive to the lytic cycle reactivation by romidepsin. In these two cell lines, the expression of Zta could be detected upon treatment with romidepsin at non-cytotoxic concentrations. These two sensitive cell lines were chosen for subsequent analyses of mechanisms of EBV lytic reactivation.

Romidepsin induced EBV lytic cycle and suppressed growth of EBV-positive NPC and GC xenografts in nude mice

We examined the *in vivo* effects of romidepsin on NPC and GC cells. HA and SNU-719 cells (AGS-BDneo failed to grow in nude mice) were inoculated subcutaneously at the right flanks of nude mice. The mice (n=5) were either treated with DMSO (vehicle control), 0.375 mg/kg or 0.75 mg/kg romidepsin for days1 & 4 each week over 4 weeks by intraperitoneal injection (Fig. 2a). No significant weight

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loss or other apparent toxicity was observed in the nude mice following the drug treatment (Fig. 2b). Growth of tumors was measured twice weekly by using a caliper. Mean increase in tumor volume of NPC and GC in DMSO vehicle-treated and romidepsin-treated mice during the experimental period was shown in Fig. 2c and 2d, respectively. Suppression of the NPC and GC tumor growth was first discerned on Day 8 and continued through to Day 22 following treatment with romidepsin. On Day 22, we observed a dose-dependent killing of NPC and GC cells by romidepsin (Fig. 2e and 2f). Western blot analysis showed that romidepsin could induce the cleavage of PARP and expression of Zta in both NPC and GC tumors in the nude mice (Fig. 2g). However, the level of Zta expression seemed to be weaker when compared with that observed *in vitro*. It might be due to the reason that most of the tumor cells which expressed EBV lytic proteins had already been killed before the detection of Zta at the end of the *in vivo* experiments.

Romidepsin induced acetylation of histone H3 and up-regulation of p21 prior to the reactivation of EBV lytic cycle

Romidepsin is a class I HDAC inhibitor, which can selectively inhibit HDAC-1, -2 and -3 isoforms.[27, 28] Acetylation of histone H3 and up-regulation of p21^{WAF1} are two well-known markers following inhibition of HDAC-1, -2 or -3.[29-31] We compared the kinetics of expression of these two markers with those of EBV lytic proteins upon treatment with romidepsin by western blotting (Fig. 3a). Expression of EBV immediate-early lytic protein (Zta) and early lytic protein (BMRF1) was first detected 12 hr post-treatment in AGS-BDneo cells and at 24 hr in HA cells. Expression of EBV late lytic protein, gp350/220, was clearly detected in HA cells at 48 hr post-treatment, whilst that in AGS-BDneo was only barely detected. Interestingly, both acetylation of histone H3 and up-regulation of p21^{WAF1} were observed prior to the expression of Zta.

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The up-regulation of p21^{WAF1}, which is a cyclin-dependent kinase inhibitor, led to down-regulation of several cell cycle regulatory proteins (cyclin B1, p-cdc2, cyclin D1 and p-Rb) and subsequent cell cycle arrest at G2/M phase in both HA and AGS-BDneo cells (Fig. 3b). Collectively, our data suggest that the effects of HDAC inhibition, including histone acetylation and p21^{WAF1} up-regulation, could be observed prior to the reactivation of EBV lytic cycle.

Induction of EBV lytic cycle was abrogated by a specific inhibitor of PKC-δ but not the inhibitors of PI3K, MEK, p38 MAPK, JNK or ATM pathways

We attempted to further characterize the mechanisms of EBV lytic cycle reactivation by romidepsin. Various reports showed that the phosphatidylinositol 3'-kinase (PI3K), MAPK/ERK kinase (MEK), c-Jun amino terminal kinase (JNK), p38 stress mitogen-activated protein kinase (MAPK), ataxia telangiectasia-mutated (ATM) kinase, and protein kinase C-delta (PKC-δ) pathways were involved in EBV lytic cycle induction.[4, 5, 32-37] We, therefore, examined the roles of these pathways in the romidepsin's induction of EBV lytic cycle by using specific pathway blockers (Fig. 4a and 4b). HA and AGS-BDneo cells were pre-treated with either 15 μM LY294002 (PI3K inhibitor), 50 μM PD98059 (MEK inhibitor), 50 μM SP600125 (JNK inhibitor), 20 μM SB202190 (p38 MAPK inhibitor) or 10 μM rottlerin (PKC-δ inhibitor) for 1 hr, followed by incubation with romidepsin for 24 hr. Expression of EBV lytic proteins, including Zta and Rta, in the HA and AGS-BDneo cells were examined by western blot analysis. Only rottlerin, but not other pathway blockers, was able to inhibit the expression of EBV lytic proteins, indicating that PKC-δ, rather than the PI3K, MEK, JNK, and p38 MAPK pathways, was required for EBV lytic cycle reactivation by romidepsin. We further found that rottlerin could suppress the expression of early lytic protein BMRF1 and up-regulation of p21^{WAF1} (Fig. 4c). In the same experiment,

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rottlerin could specifically reduce the phosphorylation of PKC-δ but not other kinases such as JNK and p38 MAPK. We also analyzed the effect of pharmacological inhibition of ATM pathway by KU-55933 (Fig. 4d). Our data showed that KU-55933 could effectively reduce the phosphorylation of ATM expression in both HA and AGS-BDneo cells. However, inhibition of the ATM pathway could only weakly affect the expression of Zta. Taken together, our results suggest that only PKC-δ, but not other reported pathways, was involved in the reactivation of EBV lytic cycle by romidepsin.

Romidepsin dose-dependently induced co-expression of EBV lytic protein and cell death marker in both NPC and GC cells

We have previously reported that induction of EBV lytic cycle by HDAC inhibitors could mediate enhanced killing of EBV-positive NPC and GC cells.[16, 17] Here, we investigated the co-expression of cleaved caspase-3 and EBV lytic protein in the HA and GC cells upon treatment with increasing concentrations of romidepsin for 5 days by flow cytometry. We found that romidepsin could dose-dependently induce the co-expression of EBV lytic proteins (Zta, BMRF1, and gp350/220) in both HA and AGS-BDneo cells. At 2.5 and 5 nM romidepsin, the percentages of AGS-BDneo and HA cells co-expressing cleaved caspase-3 and Zta were 50.9% and 24.4%, respectively (Fig. 5a and 5b). The data indicated that the majority of cells expressing EBV lytic proteins were undergoing apoptosis. Upon the same treatment, there were 27.1% of AGS-BDneo and 26.2% of HA cells expressed cleaved caspase-3 but not Zta. These apoptotic populations probably represented the cells killed directly by the general toxicity of romidepsin to cancer cells. Interestingly, upon treatment with relatively non-toxic concentrations of romidepsin in the GC and NPC cells (0.625 nM in AGS-BDneo and 1.25 nM in HA), there were high percentages of cells expressing

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Zta without cleaved caspase-3 (34.7% in AGS-BDneo and 10.6% in HA). These concentrations would be interesting for the investigation of combinatorial effect with anti-viral agents such as ganciclovir (GCV). We also stained dying AGS-BDneo and HA cells with aqua blue dye prior to staining for Zta, BMRF1 or gp350/220, which showed similar results to those of caspase-3 and lytic protein's co-staining (Fig. 5c and 5d). When we compared the effect of romidepsin with another FDA-approved HDAC inhibitor SAHA, we found that romidepsin can induce similar percentages of cells to co-express EBV lytic protein and cleaved caspase-3 at a 2000-fold lower concentration (Fig. 5e). These data suggest the potential application of romidepsin for the treatment of EBV-associated cancers.

Inhibition of HDAC-1, -2, or -3 by romidepsin conferred susceptibility of EBV-positive epithelial malignancies to ganciclovir treatment

Studies have shown that induction of EBV lytic cycle could confer susceptibility of EBV-associated cancer to treatment with ganciclovir.[5, 6, 8, 38] In this study, we also investigated the effect of combination of romidepsin and ganciclovir on the killing of EBV-negative and EBV-positive NPC and GC cells (Fig. 6a and 6b). According to our data, treatment with romidepsin alone was sufficient to induce enhanced killing of EBV-positive HA and AGS-BDneo cells, when compared with their EBV-negative counterparts. Interestingly, romidepsin could also confer susceptibility of the EBV-positive epithelial cells to the treatment with ganciclovir. Of note, the concentrations of romidepsin, which induced the expression of EBV lytic proteins without causing substantial cell death (refer to Fig. 5a and 5b), could mediate the most significant combinatorial killing effects with ganciclovir. We also analyzed the viral DNA replication and infectious virus production by quantitative PCR and EBV infection assays, respectively. Our data showed that treatment with ganciclovir could

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effectively suppress the viral DNA replication and infectious virus production induced by romidepsin (Fig. 6c and 6d). The weak virion production in AGS-BX1 cells corresponds to the relatively weak expression level of EBV late lytic protein upon treatment with 2.5 nM romidepsin (refer to Fig. 5a). We also tested the anti-tumor effects of combination of romidepsin and GCV on different EBV-negative and -positive xenografts in nude mice (Fig. 6e). Our data showed that combination of romidepsin and GCV, when compared with either drug alone, mediated enhanced growth suppression of EBV-positive xenografts (HA, C666-1 and SNU-719). However, such enhanced growth suppression by combination of romidepsin and GCV could not be observed in EBV-negative xenografts (HONE1). To assess the toxicity of the drug combination, we measured the weight of mice throughout the period of drug treatment and observed no significant weight loss in the mice treated with combination of romidepsin and GCV or either drug alone (Fig. 6f). To investigate whether the enhanced killing by combination of romidepsin and GCV is related to inhibition of HDAC-1, -2 or -3, we performed short hairpin RNA (shRNA) knockdown experiments to individually knockdown either HDAC-1, -2 or -3 and analyzed the reactivation of EBV lytic cycle and susceptibility of EBV-positive cells to GCV (Fig. 6g and 6h). We found that knockdown of either HDAC-1, -2 or -3 could be sufficient to induce the expression of Zta in HA cells. Our data also showed that GCV could induce significant percentages of cell death in cells transfected with shRNA targeting either HDAC-1, -2, or -3. Collectively, our data demonstrated that inhibition of HDAC-1, -2 and -3 by romidepsin was sufficient to induce EBV lytic cycle and confer susceptibility of EBV-positive epithelial cells to the treatment with ganciclovir.

Discussion:

Pharmacological reactivation of EBV into lytic cycle from its latency is being explored as a potential therapeutic strategy against the EBV-associated diseases.[4-8] Pan-HDAC inhibitors, which inhibit eleven HDAC isoforms, are widely used to induce EBV lytic cycle in EBV-associated cancer cells *in vitro* and in clinical trials.[4-8] We have also reported that pan-HDAC inhibitors including trichostatin A, VPA, sodium butyrate, and SAHA, could induce EBV lytic cycle in EBV-positive epithelial malignancies, including NPC and GC, and mediate enhanced cell death.[16, 17] Newly synthesized selective HDAC inhibitors, which target different combinations of specific HDAC isoforms, were reported to have more potent effects than pan-HDAC inhibitors against different types of cancers.[27, 28] In this study, we hypothesized that inhibition of one or several specific HDAC isoforms by selective HDAC inhibitors could more potently induce EBV lytic cycle in EBV-positive NPC and GC cells. Among the selective HDAC inhibitors we have tested, romidepsin, which is FDA-approved for the treatment of cutaneous T-cell lymphoma and peripheral T-cell lymphoma,[15] was found to be the most potent lytic cycle inducer. It could activate early phase of EBV lytic cycle at ~0.5 to 5 nM. These concentrations are much lower than the clinically achievable concentration in patients' plasma, which is around 800 nM.[28] We further tested the effect of romidepsin *in vivo* and found that romidepsin could also significantly induce EBV lytic cycle and suppress the growth of NPC and GC xenografts in nude mice. All these preclinical data suggest the potential application of romidepsin for the treatment of EBV-associated cancers such as NPC and GC.

Although HDAC inhibitors have been widely used to induce EBV lytic cycle in various types of cancer cells, the essential role of HDAC inhibition in the regulation of EBV lytic cycle reactivation is still unknown. According to our data, only the HDAC inhibitors targeting HDAC-1, -2 and -3 (romidepsin, MS-275 or apicidin)

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could significantly induce EBV lytic cycle in NPC cells. To further evaluate the involvement of HDAC inhibition on EBV lytic reactivation, we examined the expression kinetics of two typical markers of inhibition of HDAC-1, -2 and -3, including the acetylation of histone H3 and up-regulation of p21^{WAF1}, upon treatment with romidepsin. Both acetylation of histone H3 and up-regulation of p21^{WAF1} were observed prior to the reactivation of EBV lytic cycle. The up-regulation of p21^{WAF1} also led to the down-regulation of several cell cycle regulatory proteins and subsequent cell cycle arrest at G2/M phase, suggesting a potential link between the G2/M arrest and the reactivation of EBV lytic cycle. Liu *et al.* also recently reported that nocodazole, which could induce mitotic arrest in various types of cell lines, could reactivate EBV lytic cycle in NPC cells.[39] The exact relationship of cell cycle arrest and lytic cycle reactivation in EBV-positive cancer cells deserves further investigation. To further evaluate the role of HDAC inhibition in the lytic reactivation by HDAC inhibitors, we performed shRNA knockdown experiments to specifically inhibit HDAC-1, -2 and -3. Interestingly, we found that inhibition of either HDAC-1, -2 or -3 was sufficient to induce EBV lytic cycle in the EBV-positive epithelial malignancies. The data is consistent with the reported finding that overexpression of HDAC-2 could suppress the lytic cycle reactivation in NPC cells by HDAC inhibitors.[35] We also observed that class I HDACs such as HDAC-2 and -3 were overexpressed in various EBV-positive epithelial cells, when compared with lymphoma cells (unpublished data). The data might explain the previously reported differential responses of epithelial cells and lymphoma cells towards lytic cycle reactivation by HDAC inhibitors.[16, 40]

To further investigate the mechanisms of EBV lytic cycle reactivation by romidepsin, we examined several kinase pathways, including the PI3K/Akt, ERK, JNK, p38 MAPK, ATM and PKC-δ pathways[4, 5, 32-37] which were demonstrated to

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regulate EBV lytic cycle in various cancer cell types. We found that only pharmacological inhibition of PKC-δ, rather than the PI3K, MEK (activator of ERK), JNK, p38 MAPK, and ATM pathways, could significantly inhibit the EBV lytic cycle reactivation by romidepsin. The PKC-δ pathway has an established role in induction of lytic cycle upon treatment by HDAC inhibitors in EBV-positive epithelial cancer cells. For instance, Tsai *et al.* has shown that the activation of PKC-δ and Sp1 was required for the lytic cycle reactivation in NPC cells by HDAC inhibitors.[35] Feng *et al.* also showed that PI3K, p38 MAPK, and PKC-δ signaling were involved in EBV lytic cycle induction in GC cells following treatment with 5-FU and cisplatin.[5] However, the involvement of cellular pathways in mediating lytic induction seems to be varying in different cell types and upon treatment with different stimuli. For instance, although the ATM pathway was shown to be crucial for the regulation of EBV lytic cycle reactivation,[36] other findings have also suggested that ATM was only involved in the stage of viral DNA replication rather than the initial step of lytic reactivation.[41, 42] Our data seem to support the latter argument since ATM inhibitor did not abrogate the lytic cycle reactivation by romidepsin in both NPC and GC cell lines in the present study (refer to Fig. 5d). More investigations are still required to reconcile such contradictory data and further elucidate the mechanisms of EBV lytic cycle induction in EBV-positive epithelial cells.

Because romidepsin could potently induce EBV lytic cycle *in vitro* and *in vivo*, we further evaluated the potential clinical value of using romidepsin for lytic induction therapy in EBV-positive epithelial malignancies. We found that romidepsin could induce co-expression of Zta and cleaved caspase-3 in a significant proportion of cells, suggesting the majority of cells induced into early phase of EBV lytic cycle would eventually proceed to cell death. We also found that induction of EBV lytic cycle by romidepsin could mediate enhanced killing of EBV-positive cells versus their

EBV-negative counterparts. These findings are similar to those observed in NPC cells upon treatment with SAHA.[16, 17] However, romidepsin could induce EBV lytic cycle in a high percentage of cells at 2000-fold lower concentration when compared with SAHA. Moreover, in contrast to the effect of SAHA, romidepsin was able to induce EBV lytic cycle in EBV-positive epithelial cells at concentrations that were not cytotoxic to the cells. These two properties would favor the application of romidepsin for the scientific investigation of EBV life cycle and clinical treatment of EBV-associated cancers. Since induction of EBV lytic cycle was shown to confer susceptibility of EBV-associated cancer to treatment with GCV,[5, 6, 8, 38] we also investigated the combinatorial effect of romidepsin and GCV *in vitro* and *in vivo*. Our *in vitro* data showed that romidepsin, especially when administered at non-cytotoxic concentrations, could induce much enhanced killing of EBV-positive NPC and GC cells when combined with ganciclovir. The drug combination, when compared with either drug alone, could also mediate stronger growth suppression of EBV-positive NPC and GC xenografts in nude mice. While exerting potent cytotoxic effects with romidepsin in the EBV-positive cancer cells, GCV significantly suppressed the viral replication in the cancer cells, thus could serve to minimize the production of the potentially harmful oncogenic virus in the patients.

In conclusion, this is the first study to demonstrate that romidepsin could potently induce EBV lytic cycle through inhibition of HDAC-1, -2 or -3 and confer cytotoxicity of GCV in EBV-positive epithelial malignancies. The data suggest further application of romidepsin for the investigation of EBV lytic cycle reactivation and clinical treatment of EBV-associated cancers.

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Figure legends:

Figure 1. Inhibition of HDAC-1/-2/-3, but not HDAC-6/-8, by selective class I HDAC inhibitors (e.g. romidepsin) was sufficient to induce EBV lytic cycle in NPC cells. *(a)* HA cells were treated with increasing concentrations of either apicidin (HDAC1-3), MS-275 (HDAC1-3), romidepsin (HDAC1-3), droxinostat (HDAC-3,-6 and -8), cay10603 (HDAC-6), PCI34051 (HDAC-8) or 5 μM SAHA (HDAC1-3,-6 and -8) for 48 hr. Expression of an immediate-early lytic protein of EBV, Zta, was analyzed by western blotting. *(b)* HA cells were treated with either 1.25 μM Apicidin, 2.5 μM MS-275, 2.5 nM romidepsin, 25 μM droxinostat, 2 nM Cay10603 or 20 μM PCI34051 for 48 hr. Expression of Zta (green signals) and a late lytic protein of EBV, VAC-p18, (red signals) was analyzed by immunofluorescence staining. DAPI (blue signals) stained cell nuclei. *(c)* HA and BD-neo cells were treated with either 5 μM SAHA or 2.5 nM romidepsin for 48 hr. Expression of EBV lytic protein, Zta, and cellular proteins, acetyl-histone H4 and acetyl-α-tubulin (substrate inhibited by HDAC 6), was analyzed by western blotting. Cellular α-tubulin served as loading control.

Figure 2. Romidepsin induced EBV lytic cycle and suppressed growth of NPC xenografts in nude mice. Five million HA NPC or SNU-719 GC cells resuspended in 100 μl RPMI were subcutaneously injected into the right flanks of nude mice. One week later (when the tumors were palpable), the mice were either treated with DMSO, 0.375 or 0.75 mg/kg romidepsin for 2 days/week over 4 weeks by intraperitoneal injection. (*a*) Resected tumours of mice of control and romidepsin-treated groups were illustrated. (*b*) The weights of mice of control and romidepsin-treated groups were shown. (*c & d*) The size of tumors (HA and SNU-719) during the period of experiment was measured twice weekly using a caliper. Data are presented as the

mean tumor volumes of mice in both treatment and control groups on the days

post-treatment. (*e & f*) Average tumor masses (HA and SNU-719) of mice of control

and romidepsin-treated groups were shown. Error bars represent the standard error of

mean (SEM) of tumor masses. (*g*) Protein samples extracted from the tumors were

tested for expression of PARP and Zta proteins by western blot analysis. Cellular

α-tubulin served as loading control.

**Figure 3. Romidepsin induced the expressison of p21^{WAF1} and G2/M arrest in

NPC and GC cells prior to EBV lytic cycle reactivation.** HA and AGS-BDneo cells

were treated with 5 or 2.5 nM romidepsin, respectively, for 0, 1, 2, 4, 8, 12, 24, 48 and

72 hr. (*a*) The kinetics of expression of the EBV lytic proteins including EBV

immediate-early (Zta), early (BMRF1) and late (VCA p-18 and gp350/220); EBV

latent protein EBNA1; cellular acetyl-H3, p21^{WAF1}, cyclin-B1, p-cdc2, cyclin-D1 and

p-Rb was analyzed by western blotting. α-tubulin was detected as a loading control.

(*b*) Cell cycle status was analyzed by flow cytometry.

**Figure 4. The up-regulation of p21^{WAF1} and induction of EBV lytic cycle were

abrogated by a specific inhibitor of PKC-δ but not the inhibitors of PI3K, MEK,

JNK, p38 MAPK or ATM pathways.** (*a*) HA and (*b*) AGS-BDneo cells were

pre-treated with either 15 μM LY294002 (PI3K inhibitor), 50 μM PD98059 (MEK

inhibitor), 50 μM SP600125 (JNK inhibitor), 20 μM SB202190 (p38 MAPK inhibitor)

or 10 μM rottlerin (PKC-δ inhibitor) for 1 hr, and then incubated with 2.5 (for

AGS-BDneo) or 5 nM (for HA) romidepsin for 48 hr. Expression of EBV lytic

proteins, including Zta and Rta, and acetyl-histone H3 proteins, were analyzed by

western blot analysis. (*c*) HA and AGS-BDneo cells were pre-treated with 10 μM

rottlerin or (*d*) 10 μM KU-55933 (ATM inhibitor) for 1 hr before treatment with 5 nM

romidepsin for 48 hr. Expression of EBV proteins, including Zta, Rta and BMRF1, and cellular proteins including p21^{WAF1}, p-PKC-δ, p-JNK and p-p38 were analyzed by western blotting. α-tubulin was detected as a loading control.

Figure 5. Romidepsin induced co-expression of EBV lytic proteins and cleaved caspase-3 in EBV-positive epithelial cells. AGS-BDneo and HA cells were treated with 0.625, 1.25, 2.5 and 5 nM romidepsin for 5 days. Flow cytometric analysis was performed to quantify the percentages of *(a)* AGS-BDneo and *(b)* HA cells co-expressed either Zta, BMRF1 or gp350/220 and cleaved caspase-3. Following the same treatment, flow cytometric analysis was performed to quantify the percentages of live (aqua blue -) or dead (aqua blue +) *(c)* AGS-BDneo and *(d)* HA cells cells which expressed Zta, BMRF1 or gp350/220. *(e)* AGS-BDneo cells were treated with either 2.5/5 nM romidepsin or 2.5/5 μM SAHA for 5 days. Flow cytometric analysis was performed to quantify the percentages of cells expressed Zta and cleaved caspase-3 or incorporated aqua blue dye.

Figure 6. Romidepsin conferred susceptibility of EBV-positive epithelial cancer cells to the treatment with ganciclovir. EBV-negative and -positive NPC (HONE1 and HA) and GC (AGS and AGS-BDneo) cells were treated with increasing concentrations of romidepsin, 10 μg/ml GCV or their combinations for 7 days or untreated. Number of viable *(a)* NPC and *(b)* GC cells was determined by trypan blue exclusion assay. Results are presented as percentages of viable cell populations among treated cells compared with those of untreated control. Error bars represent the standard error of mean (SEM) of data obtained in at least three independent experiments. *(c)* HA and AGS-BDneo cells were treated with either romidepsin (2.5 and 5 nM for AGS-BDneo and HA, respectively), 10 μg/ml GCV or their combination

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for 3 days. Quantitative PCR was performed to analyze the induction of EBV DNA replication in both HA and AGS-BDneo cells. Data are presented as number of viral genomes per cell. *(d)* HONE1-EBV and AGS-BX1 cells were treated with either romidepsin (2.5 and 5 nM for AGS-BX1 and HONE1-EBV, respectively), 10 μg/ml GCV or their combination for 5 days. EBV infection assay was performed to analyze the production of infectious viral particle. Culture supernatants, which would contain GFP-expressing recombinant EBV particles released by HONE1-EBV and AGS-BX1 cells, were used to infect Daudi cells for 48 hr. The amount of infectious EBV particles was estimated by the level of GFP expression detected by flow cytometry. The shaded areas represent the percentage of Daudi cells infected with fresh medium. The open areas represent the percentage of Daudi cells infected with medium cultured with HONE1-EBV and AGS-BX1 cells upon treatment with either romidepsin, GCV or their combination. *(e)* Five million HONE1, HA, C666-1 or SNU-719 GC cells resuspended in 100 μl RPMI were subcutaneously injected into the right flanks of nude mice. When the tumours were palpable, the mice were either treated with 0.375 mg/kg romidepsin for 2 days/week, 50 mg/kg GCV for 5 days/week, or their combination over 4 weeks by intraperitoneal injection. Percentages of tumor masses of mice of the treatment groups when compared with the control group were shown. Error bars represent the standard error of mean (SEM) of tumor masses. (*f*) The weights of mice of control and treatment groups were shown. *(g)* HA cells were stably infected with 2 sets of shRNA oligonucleotides targeting HDAC-1, -2 or -3 or scramble shRNA control vector. Western blotting was performed to analyze the expression of HDAC-1, -2, -3 and Zta in the stable knockdown cell lines. *(h)* The stable knockdown cell lines were treated with 10 μg/ml GCV for 7 days or untreated. Number of viable cells was determined by trypan blue exclusion assay.
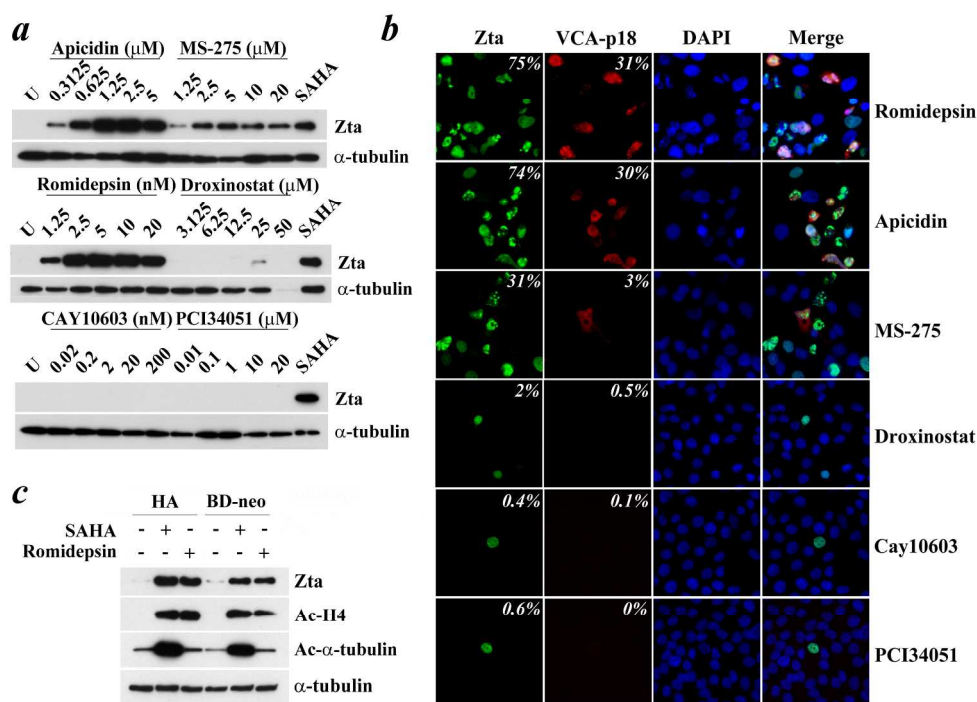
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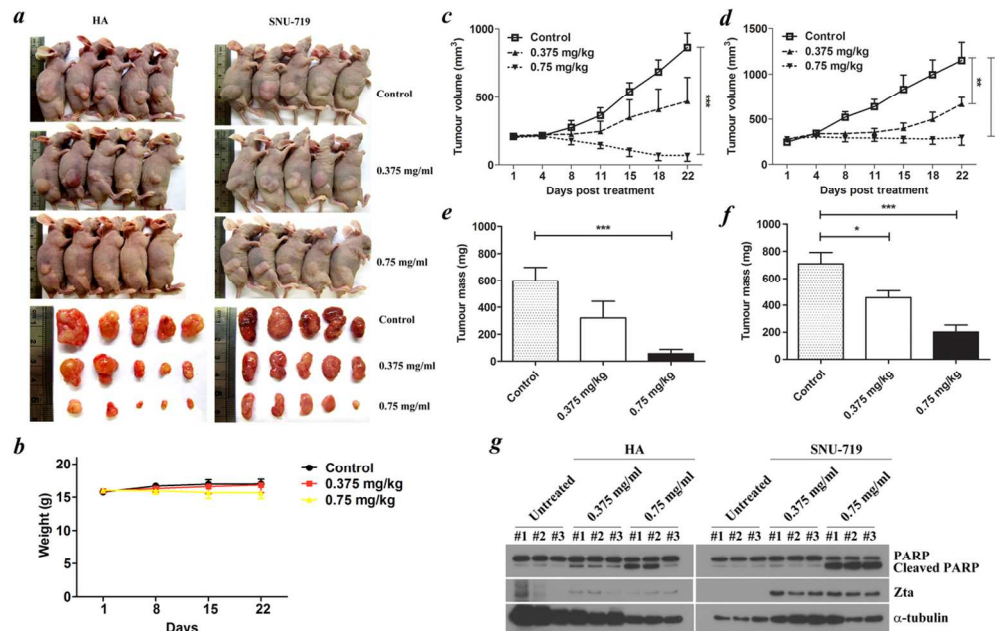
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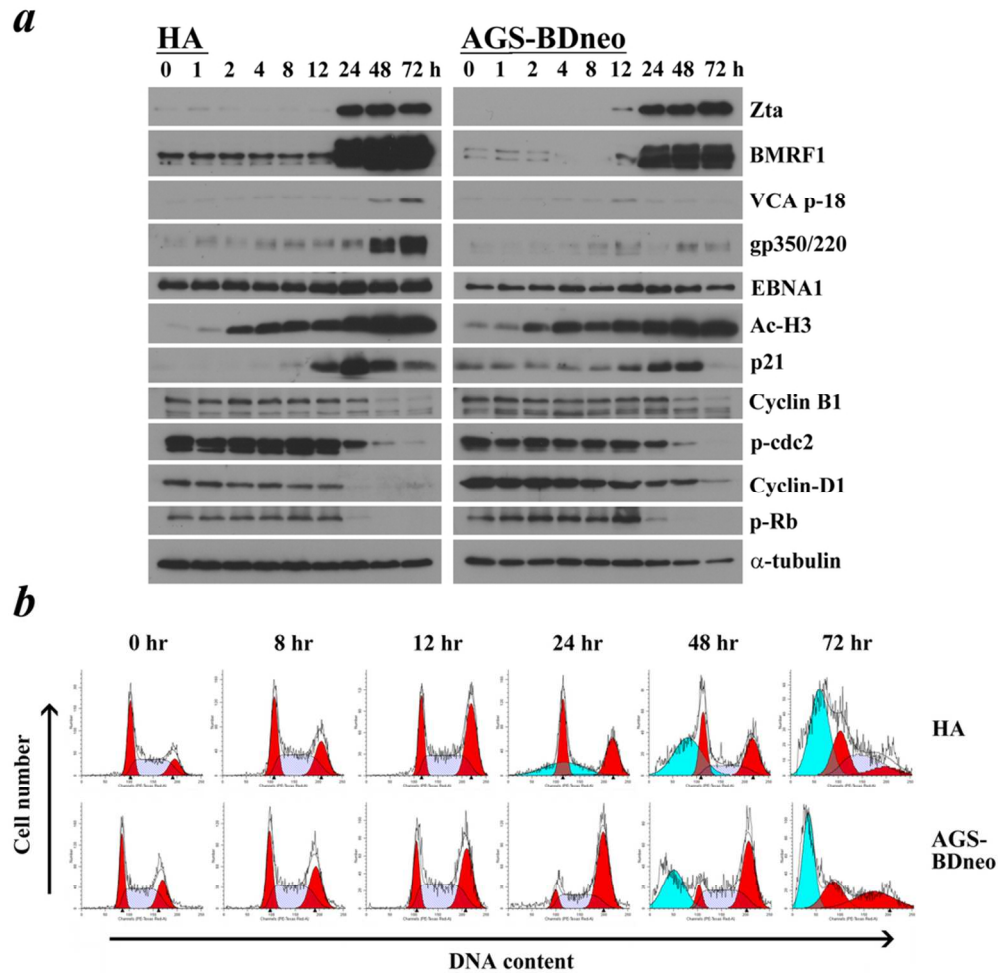116x75mm (300 x 300 DPI)

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Figure 3. Romidepsin induced the expresison of p21WAF1 and G2/M arrest in NPC and GC cells prior to EBV lytic cycle reactivation. HA and AGS-BDneo cells were treated with 5 or 2.5 nM romidepsin, respectively, for 0, 1, 2, 4, 8, 12, 24, 48 and 72 hr. (a) The kinetics of expression of the EBV lytic proteins including EBV immediate-early (Zta), early (BMRF1) and late (VCA p-18 and gp350/220); EBV latent protein EBNA1; cellular acetyl-H3, p21WAF1, cyclin-B1, p-cdc2, cyclin-D1 and p-Rb was analyzed by western blotting. α-tubulin was detected as a loading control. (b) Cell cycle status was analyzed by flow cytometry.
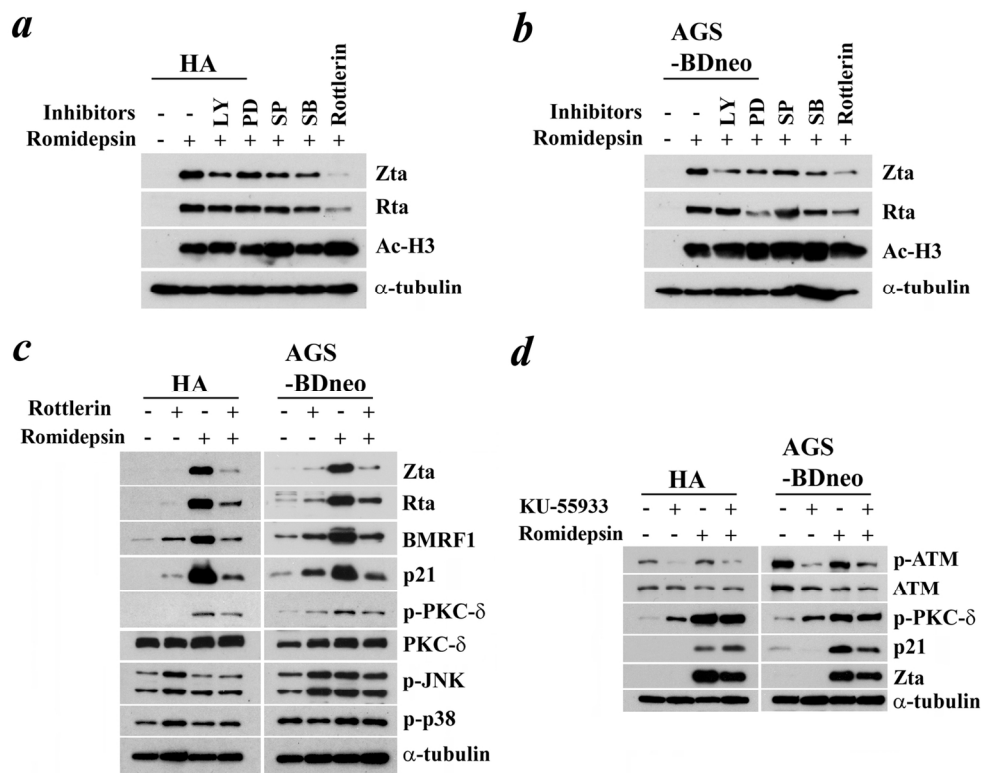88x86mm (300 x 300 DPI)

Figure 4. The up-regulation of p21WAF1 and induction of EBV lytic cycle were abrogated by a specific inhibitor of PKC-δ but not the inhibitors of PI3K, MEK, JNK, p38 MAPK or ATM pathways. (a) HA and (b) AGS-BDneo cells were pre-treated with either 15 μM LY294002 (PI3K inhibitor), 50 μM PD98059 (MEK inhibitor), 50 μM SP600125 (JNK inhibitor), 20 μM SB202190 (p38 MAPK inhibitor) or 10 μM rottlerin (PKC-δ inhibitor) for 1 hr, and then incubated with 2.5 (for AGS-BDneo) or 5 nM (for HA) romidepsin for 48 hr. Expression of EBV lytic proteins, including Zta and Rta, and acetyl-histone H3 proteins, were analyzed by western blot analysis. (c) HA and AGS-BDneo cells were pre-treated with 10 μM rottlerin or (d) 10 μM KU-55933 (ATM inhibitor) for 1 hr before treatment with 5 nM romidepsin for 48 hr. Expression of EBV proteins, including Zta, Rta and BMRF1, and cellular proteins including p21WAF1, p-PKC-δ, p-JNK and p-p38 were analyzed by western blotting. α-tubulin was detected as a loading control.
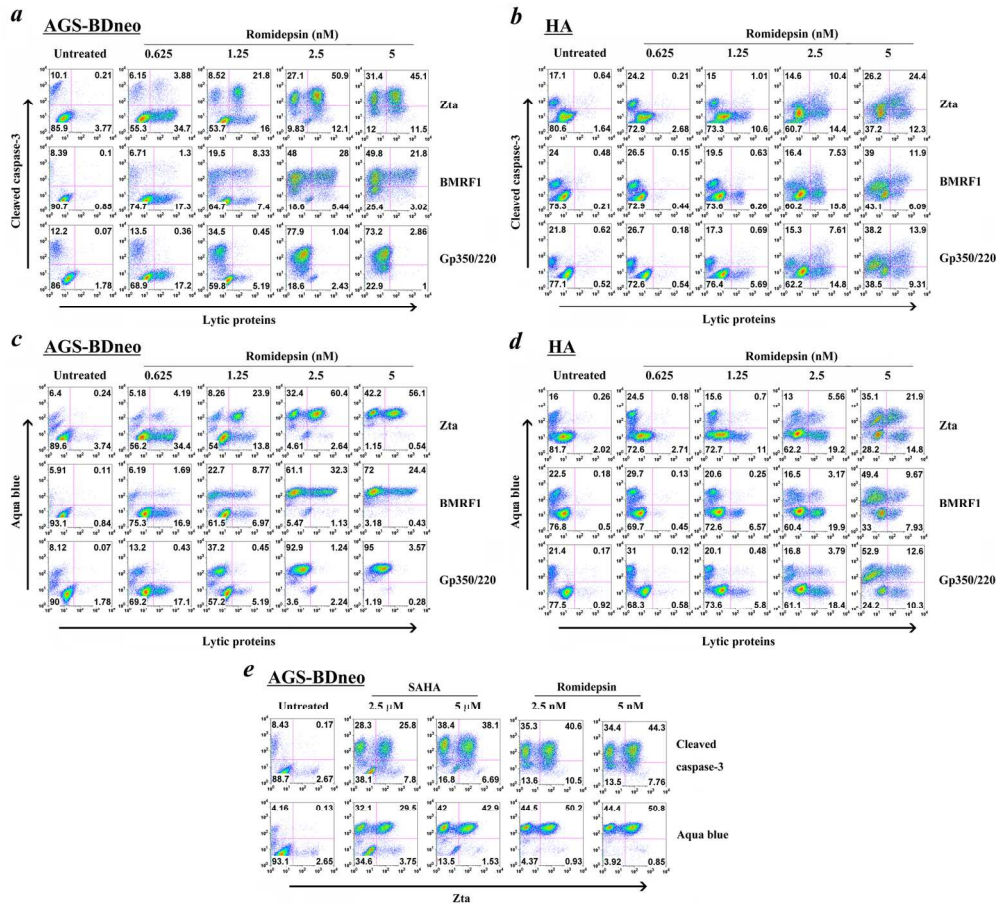142x113mm (300 x 300 DPI)

Figure 5. Romidepsin induced co-expression of EBV lytic proteins and cleaved caspase-3 in EBV-positive epithelial cells. AGS-BDneo and HA cells were treated with 0.625, 1.25, 2.5 and 5 nM romidepsin for 5 days. Flow cytometric analysis was performed to quantify the percentages of (a) AGS-BDneo and (b) HA cells co-expressed either Zta, BMRF1 or gp350/220 and cleaved caspase-3. Following the same treatment, flow cytometric analysis was performed to quantify the percentages of live (aqua blue -) or dead (aqua blue +) (c) AGS-BDneo and (d) HA cells cells which expressed Zta, BMRF1 or gp350/220. (e) AGS-BDneo cells were treated with either 2.5/5 nM romidepsin or 2.5/5 μM SAHA for 5 days. Flow cytometric analysis was performed to quantify the percentages of cells expressed Zta and cleaved caspase-3 or incorporated aqua blue dye.
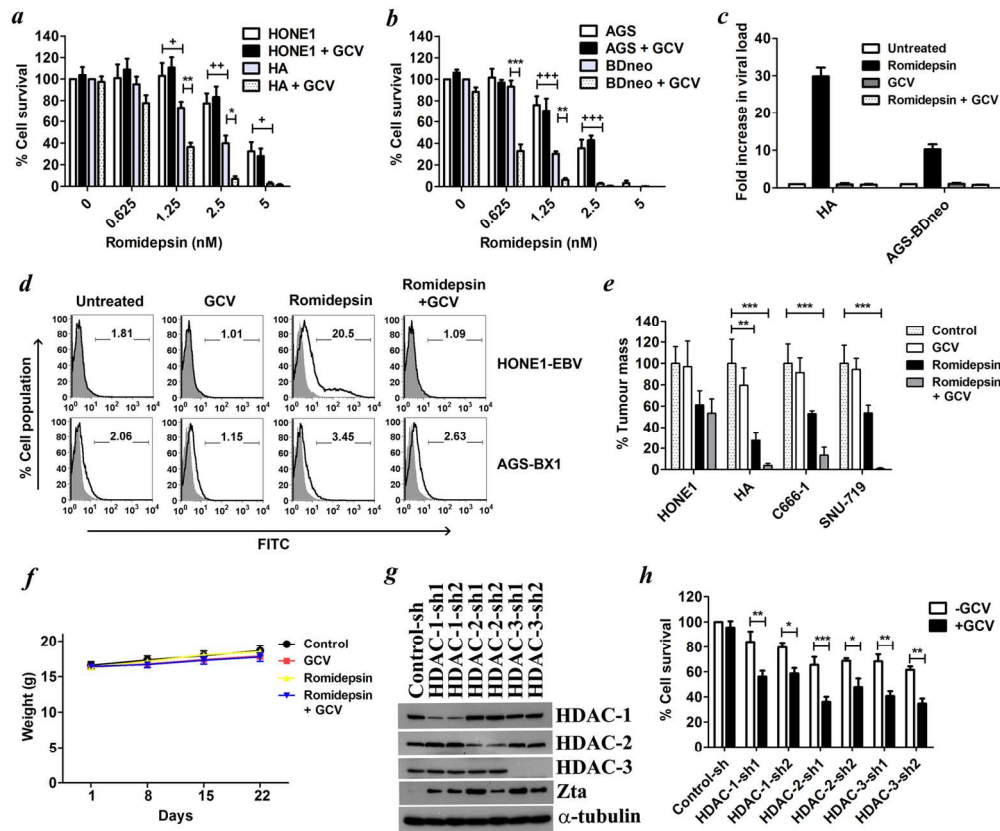156x141mm (300 x 300 DPI)

Figure 6. Romidepsin conferred susceptibility of EBV-positive epithelial cancer cells to the treatment with ganciclovir. EBV-negative and -positive NPC (HONE1 and HA) and GC (AGS and AGS-BDneo) cells were treated with increasing concentrations of romidepsin, 10 μg/ml GCV or their combinations for 7 days or untreated. Number of viable (a) NPC and (b) GC cells was determined by trypan blue exclusion assay. Results are presented as percentages of viable cell populations among treated cells compared with those of untreated control. Error bars represent the standard error of mean (SEM) of data obtained in at least three independent experiments. (c) HA and AGS-BDneo cells were treated with either romidepsin (2.5 and 5 nM for AGS-BDneo and HA, respectively), 10 μg/ml GCV or their combination for 3 days. Quantitative PCR was performed to analyze the induction of EBV DNA replication in both HA and AGS-BDneo cells. Data are presented as number of viral genomes per cell. (d) HONE1-EBV and AGS-BX1 cells were treated with either romidepsin (2.5 and 5 nM for AGS-BX1 and HONE1-EBV, respectively), 10 μg/ml GCV or their combination for 5 days. EBV infection assay was performed to analyze the production of infectious viral particle. Culture supernatants, which would contain GFP-expressing recombinant EBV particles released by HONE1-EBV and AGS-BX1 cells, were used to infect Daudi cells for 48 hr. The amount of infectious EBV particles was estimated by the level of GFP expression detected by flow cytometry. The shaded areas represent the percentage of Daudi cells infected with fresh medium. The open areas represent the percentage of Daudi cells infected with medium cultured with HONE1-EBV and AGS-BX1 cells upon treatment with either romidepsin, GCV or their combination. (e) Five million HONE1, HA, C666-1 or SNU-719 GC cells resuspended in 100 μl RPMI were subcutaneously injected into the right flanks of nude mice. When the tumours were palpable, the mice were either treated with 0.375 mg/kg romidepsin for 2 days/week, 50 mg/kg GCV for 5 days/week, or their combination over 4 weeks by intraperitoneal injection. Percentages of tumor masses of mice of the treatment groups when compared with the control group were shown. Error bars represent the standard error of mean (SEM) of tumor masses. (f) The weights of mice of control and treatment groups were shown. (g) HA cells were stably infected with 2 sets of shRNA oligonucleotides targeting HDAC-1, -2 or -3 or scramble shRNA control vector. Western blotting was performed to analyze the expression of HDAC-1, -2, -3 and Zta in the stable knockdown cell lines. (h) The stable knockdown cell

lines were treated with 10 μg/ml GCV for 7 days or untreated. Number of viable cells was determined by trypan blue exclusion assay.
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