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Dehydroxymethylepoxyquinomicin selectively ablates T-CAEBV cells

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1. ABSTRACT

Chronic active Epstein-Barr virus infection (CAEBV) represents a new subtype of lymphoproliferative disorders characterized by high morbidity and mortality rates and often leads to malignant transformation of infected cells. Efficient therapeutic strategies are presently unavailable; therefore, the development of therapies to prevent CAEBV-mediated transformation and disease progression is crucial. Here, we used microarray analysis and luciferase reporter assays to reveal the potential role of activated nuclear factor kappa B (NF-κB) in T cell type of-CAEBV infection. Using a series of cellular and molecular experiments, we demonstrated that dehydroxymethylepoxyquinomicin (DHMEQ), a novel NF-κB inhibitor, can selectively induce apoptosis in SNT-16 cells infected with CAEBV. Mechanistic studies suggested that DHMEQ induces SNT-16 cell apoptosis through NF-κB inhibition coupled with oxidative stress generation. Thus, activated NF-κB could be a new target for CAEBV therapeutics. Owing to its selective targeting ability, DHMEQ may be a candidate for a novel therapeutic regimen to control the progression of CAEBV infections.

2. INTRODUCTION

As a ubiquitous and often sub-clinically infecting human herpesvirus (1), Epstein-Barr virus (EBV) contributes to the global cancer burden (2). EBV is the causal agent of certain malignancies, including nasopharyngeal carcinoma (NPC), Burkitt's lymphoma, and Hodgkin's lymphoma (1,3). EBV is also believed to be responsible for the occurrence of certain lymphoproliferative disorders (LPD), which are a prophase of lymphoid malignancies. Chronic active Epstein-Barr virus (CAEBV) infection is a new subtype of EBV-associated LPD characterized by chronic/recurrent infectious mononucleosis–like symptoms and unusually increased levels of anti-EBV antibodies. CAEBV infection has high mortality and morbidity with life-threatening complications; two-thirds of patients die within 4–5 years and nearly one-third develop virus-associated hemophagocytic syndrome, leukemia, or lymphoma (4,5). No standard effective treatment regimens have yet been defined for CAEBV infection. Many therapeutics, including antiviral agents (acyclovir and gancyclovir) and immunomodulating agents (vidarabine, interferon-α, and interleukin (IL)-12) have been tried unsuccessfully (6,7). Therefore, novel therapies to eliminate cancerous proliferative cells must be developed.

The nuclear factor kappa B (NF-κB) signaling pathway plays important roles in a wide variety of biological processes, including proliferation, apoptosis, and transformation (8,9). NF-κB is activated by several stimuli, including infection by viruses (rhinovirus, influenza virus, EBV, cytomegalovirus, and adenovirus), cytokines (TNFα, IL-1β, and IL-17), and extrinsic factors (lipopolysaccharides and ultraviolet radiation) (10). The pathogenic mechanisms of CAEBV remain to be identified; however, recent reports have provided important insights into its abnormal pathogenesis. For instance, emerging evidence indicates that abnormal cytokine secretion plays a role in the pathogenesis of CAEBV infections and CAEBV-associated transformation. Of note, the transcription and serum levels of TNFα and IL-1β are higher in patients infected with CAEBV than in normal volunteers (5,11-13). Furthermore, CAEBV often exhibits a latency II or III phenotype. Latent membrane protein 1 (LMP1), an EBV-encoded oncogenic protein expressed in CAEBV, has been found to participate in transformation in various tumor types by activating the NF-κB signaling pathway (14-16). EBV also participates in transformation in human cancer through NF-κB activation. For example, NF-κB is activated in the presence of EBV gene expression in primary NPC cells (17) and Burkitt's lymphoma cells (18). Moreover, NF-κB activation is a common feature of LPD (19,20). Together, these findings prompted us to hypothesize that NF-κB activation may participate in the pathogenesis of CAEBV infections. If indeed NF-κB is constitutively activated in CAEBV, it may become a therapeutic target for this disease.

Recently, dehydroxymethylepoxyquinomicin (DHMEQ), which is derived from epoxyquinomicin C (21), has been identified as a novel NF-κB inhibitor with therapeutic potential against hematological malignancies (22,23), breast cancer (24), prostate cancer (25), NPC (26), and hepatocellular carcinoma (27). Mechanistic studies have shown that DHMEQ can selectively inhibit the nuclear translocation of activated NF-κB (28,29), induce G0/G1 cell cycle arrest and then trigger apoptosis (30), and enhance reactive oxygen species (ROS) production (31). Interestingly, no significant hematopoietic toxicity has been observed in DHMEQ treatment (23).

These findings suggest that DHMEQ might be a candidate for CAEBV therapy. However, the efficacy of DHMEQ on CAEBV has not been reported. Hence, in this study, we set out to define the role of NF-κB activation in CAEBV and determine its effects on CAEBV-infected cells by using a series of cellular and molecular assays.

3. MATERIALS AND METHODS

3.1. Cell lines and separation of primary cells

This study was approved by the institutional ethics committee of Xinhua Hospital, Shanghai Jiao Tong University School of Medicine (SJTU-SM) and was conducted in accordance with the Declaration of Helsinki. Complete written informed consent was obtained from the volunteers or their guardians. Peripheral blood mononuclear cells (PBMCs) were isolated from four healthy peripheral blood samples and mononuclear cells (MNCs) were isolated from three umbilical cord blood (UCB) samples by Ficoll-Paque density gradient separation (Lymphoprep™, Fresenius Kabi Norge AS, Norway). UCB-MNCs were further used to separate CD34+ stem/progenitor cells with a magnetic-activated cell sorter (MACS) according to the manufacturer's manual (CD34 isolation kit; Miltenyi Biotech, Auburn, CA). SNT-15 (γδ-T CAEBV) and SNT-16 (αβ-T CAEBV)

cells were gifts from JH Ohyashiki (32,33). K562 cells and Jurkat cells were obtained from Xinhua Hospital, SJTU-SM. CAEBV cells were maintained in RPMI 1640 with 10% heat-inactivated fetal bovine serum (FBS) and 700 U/ml of IL-2. PBMCs, $CD34^+$, K562, and Jurkat cells were incubated with RPMI 1640 with 10% FBS.

3.2. Reagents

DHMEQ, gifted by Kazuo Umezawa, was dissolved in DMSO (21). Vitamin C was purchased from Sigma Chemical Company (St Louis, MO). RPMI-1640 and FBS were purchased from Gibco (Grand Island, NY). CD3-phycoerythrin (PE), CD19-allophycocyanin (APC), and the Annexin V-fluorescence isothiocyanate (FITC)/7-amino-actinomycin D (7AAD) apoptosis detection kit were purchased from Becton Dickinson (San Jose, CA). Polyclonal anti-p65 (sc-372), anti-p50 (sc-1190), and anti-Nrf-2 (SC-13032X) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-PARP (556494) and CD95-FITC (555673) were purchased from BD Pharmingen (San Diego, CA). The caspase-3 activity assay kit, BAY 11-7082, and pNF-κB-luc plasmid were purchased from Beyotime (China). 2',7'-Dichlorofluorescin diacetate (DCFH-DA) was purchased from Invitrogen (Carlsbad, CA). IL-2 was purchased from PeproTech (Rocky Hill, NJ). The caspase-3 inhibitor Z-Asp-Glu-Val-Aap (DEVD)-FMK was purchased from Calbiochem (San Diego, CA).

3.3. Flow cytometry

For apoptosis detection, cells were harvested after DHMEQ or BAY 11-7082 treatment, washed, and resuspended in 200 µl of Annexin V binding buffer. PBMCs were incubated with CD3 and CD19 antibodies prior to further staining. Annexin V-FITC and 7AAD were added, and the samples were incubated at room temperature in the dark for 20 minutes. As an anti-ROS agent, 100 µM vitamin C was added to the culture media for 3 hours before DHMEQ treatment. SNT-16 cells were pretreated with 20 µM Z-DEVD-FMK for 2 hours before DHMEQ treatment. For intracellular ROS measurement, cells were collected, centrifuged, and labeled with 10 µM DCFH-DA at 37°C for 20 minutes. The cell pellet was washed twice and resuspended in 500 µl of RPMI 1640. For CD95 expression, cells were harvested, washed, and stained with CD95-FITC for 20 minutes in the dark. The cell pellet was washed twice and resuspended in 200 µl of PBS. All flow cytometry experiments were performed using a Cytomics FC 500 system (Beckman-Coulter, Fullerton, CA).

3.4. Caspase-3 activity assay

To evaluate the activity of caspase-3, cells were homogenized in a reaction buffer containing caspase-3 substrate (Ac-DEVD-pNA; 2 mM) after DHMEQ treatment. The lysates were incubated at 37°C for 2 hours and measured with an enzyme-linked immunosorbent assay (ELISA) reader at an absorbance of 405 nm.

3.5. Immunoblot assays

Cytosolic and nuclear fractions were extracted and blots were probed with anti-p65, anti-p50, and anti-PARP antibodies. GAPDH and lamin B were used as internal cytosolic and nuclear fraction controls, respectively. Western blots were visualized with enhanced chemiluminescent (ECL) reagent (GE Healthcare, Piscataway, NJ, USA) and a LAS-4000 imager (FujiFilm, Tokyo, Japan).

3.6. Immunofluorescent assays

Cells were fixed in methanol at -20°C and permeabilized with 0.2% Triton-100. The cells were stained using anti-Nrf-2 in blocking buffer for 2 hours at room temperature and then washed and stained with goat anti-rabbit Alexa 546 (Invitrogen, Carlsbad, CA). Slides were mounted and left to dry overnight. Images were acquired using Leica LAS AF Lite 1.7.0. build 1240 (Leica, Germany).

3.7. Real-time RT-PCR assays

Total RNA was extracted from cells using an RNeasy kit from Qiagen (Chatsworth, CA). Reverse transcription was performed using a Superscript II reagent set (Invitrogen) with random hexamer primers. Quantitative real-time PCR was performed using an ABI Prism 7900HT detection system (Applied Biosystems, Foster City, CA). The relative expression level for each target compared to the internal control GAPDH was calculated according to the following equation: ΔCt = Ct (target) - Ct (GAPDH), where the relative mRNA expression = $2^{-\Delta Ct}$. Each assay was performed in triplicate. The primers used in this study are listed in Table 1.

3.8. Gene expression analysis

Gene expression data (GSE 13906) collected by Ohyashiki K *et al*. (32) were retrieved from NCBI Gene Expression Omnibus (GEO). Expression data were normalized using robust multiarray averaging (RMA) with quantile normalization. We selected genes with a fold change ≥2 compared to normal γσT cells for further analysis. These genes were uploaded into DAVID v6.7. (Database for Annotation, Visualization and Integrated Discovery)

Table 1. Primers for real-time RT-PCR used in this study

Gene name	Direction	Sequence (5' - 3')
BCL-2	Forward	TTCGGAAGAACAGAATGGCTC
	Reverse	GACCTGAAACATCTTCTGTTGG
XIAP	Forward	CGTGCGGTGCTTTAGTTGTCA
	Reverse	GGTTCCTCGGGTATATGGTGTCT
c-IAP1	Forward	GATTGTGGCCTTCTTTGAGT
	Reverse	CAAACTGAGCAGAGTCTTCA
c-IAP2	Forward	TGCCAAGTGGTTTCCAAGGT
	Reverse	CTAAAGCCCATTTCCACGGC
BAK	Forward	TGCAACCTAGCAGCACCATG
	Reverse	GCCATGCTGGTAGACGTGT
Survivin	Forward	GACCACCGCATCTCTACATTCAA
	Reverse	TCTCCGCAGTTTCCTCAAATTCTT

to identify enriched gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Benjamini and FDR < 0.05) (34).

3.9. Gene set enrichment analysis

GSEA was performed using GSEA v2.0.7 software with 1,000 phenotype permutations (35). The gene sets used in this study are listed in Table S2. Gene sets were considered to be significantly enriched at a false discovery rate (FDR) <25% (q-value < 0.25).

3.10. Reporter gene assays

A κB site-dependent luciferase Germany). Luciferase activities were analyzed using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI).

3.11. Statistical analysis

Data are presented as mean ± SEM. Statistical analyses were performed using GraphPad Prism software (GraphPad, San Diego, CA). For two-group comparisons, significance was determined by two-tailed *t* tests.

4. RESULTS

4.1. NF-κB is constitutively activated in CAEBV-infected cells

Constitutive NF-κB activation is a characteristic feature of LPD, such as acute T cell leukemia (ATL), lymphoma, and chronic lymphocytic leukemia (CLL) (22,23,36). To assess the NF-κB profile in T-CAEBV, we compared data sets for T-CAEBV (GSE13906) (32) and T cell lymphoma (SNT-8 cells). Relative to normal γδ-T cells, 3533 and 4094 genes with >2-fold changes were selected for SNT15 and SNT-8 cells, respectively (Figure 1A). Among these, 2052 genes (59% of SNT-15 regulated genes) were co-regulated in SNT-8 and SNT-15 cells, indicating that SNT-15 cells had a similar gene expression profile to SNT-8 cells (T/NK lymphoma). To reveal the underlying pathogenesis, we performed GO/KEGG analysis using DAVID. Of note, genes involved in the negative regulation of apoptosis/programmed cell death and transcription factor binding were significantly enriched in the upregulated genes ($p < 0.05$, FDR < 0.1), while those for immune response, natural killer cell-mediated cytotoxicity, apoptosis, lymphocyte activation, and cell defense response were enriched in the downregulated genes (Figure 1B). We further probed the *NF-κB* profile, and found that its functional units (*RELA, RELB*, and *NFKB2*) and target genes (*BAX*, *MYB*, *PCNA*, *CCND2*, *BIRC2*, and *BIRC3*) were upregulated compared to normal γδ -T cells (Figure 1C). To further evaluate the role of NF-κB in T-CAEBV, we transfected K562, Jurkat, SNT-15, and SNT-16 cells with 100 ng of pNF-κB-luc plasmid and studied the transcriptional activity of *NF-κB*. Compared with the K562 control cell line without NF-κB activity, T-CAEBV cells (SNT-15 and SNT-16) showed an activated NF-κB pattern, similar to that observed in Jurkat cells treated with TNFα (10 ng/ml) (Figure 1D). These data suggest that NF-κB might be activated in T-CAEBV.

4.2. DHMEQ selectively induces death in CAEBV-infected cells

DHMEQ, a novel NF-κB inhibitor, has been shown to effectively target several LPD (ATL, CLL), multiple myeloma, and certain solid tumors, while its efficacy in T-CAEBV cells remains undetermined. Thus, we performed a CCK-8 assay to observe the cytotoxic effect of DHMEQ on T-CAEBV cells. The IC50 value of DHMEQ was approximately 9 μg/ml for SNT-16 cells and 15 μg/ml for SNT-15 cells (Figure 2A). DHMEQ had an inhibitory effect on the proliferation of SNT-15 and SNT-16 cells (Figure 2B). A 24-hour treatment with 10 μg/ml DHMEQ had a greater cytotoxic effect on SNT-16 cells than on SNT-15 cells (73.1% ± 1.7% and 57.4% ± 2.7% viable cells for SNT-15 and SNT-16, respectively), suggesting that SNT-16 cells were more sensitive to DHMEQ than

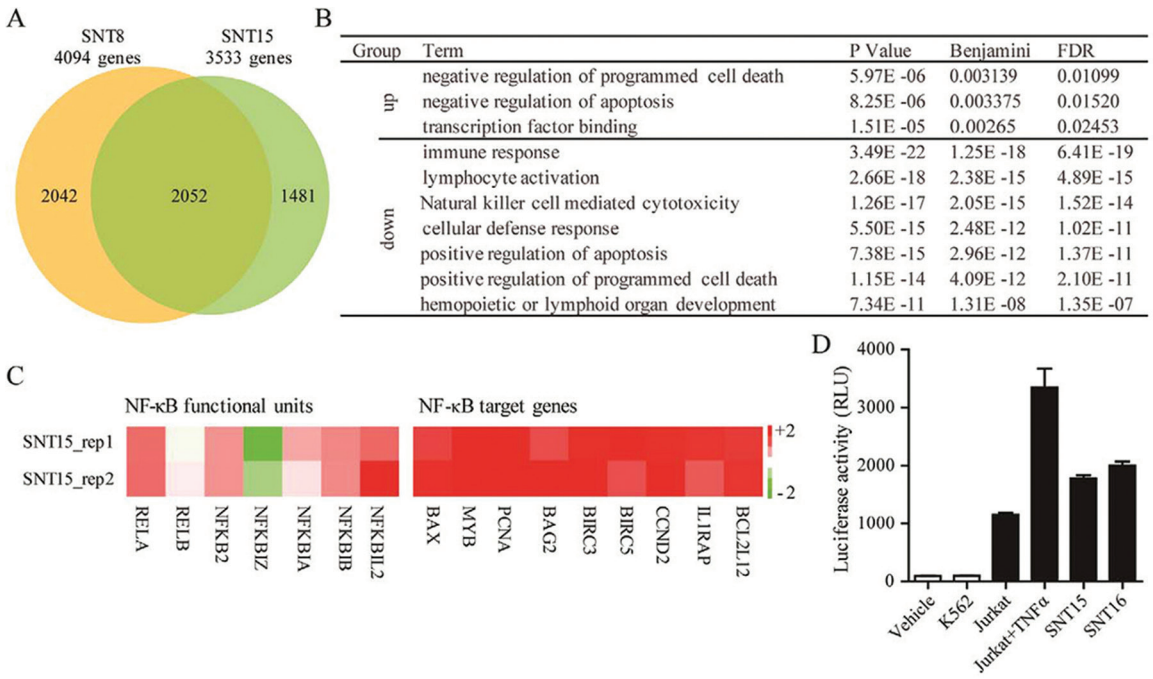

Figure 1. NF-κB might be activated in T-CAEBV cells. (A) Results on transcriptome changes of γσ-T-CAEBV representative cell line (SNT-15, represented in green) and NK/T lymphoma cell line (SNT-8, represented in orange). (B) Table of functional GO/KEGG analysis of >2-fold changed genes in SNT-15 cells. (C) Major functional genes associated with NF-κB signaling pathway in SNT-15 cells. The color bar stands for expression values (log ratio with base 2). (D) Reporter gene assays for detecting NF-κB transcriptional activity in K562, Jurkat, SNT-15 and SNT-16 cells.

SNT-15 cells (Figure 2C). Therefore, we selected SNT-16 cells for further analysis. No toxic effect of DHMEQ was detected in normal hematopoietic cells, normal PBMCs, T or B lymphocytes, or $CD34^+$ hematopoietic stem/progenitor cells (Figure 2D). These initial studies indicated that DHMEQ induces cell death in T-CAEBV cell lines but not in normal hematopoietic cells.

4.3. DHMEQ targets CAEBV-infected cells through induction of apoptosis

To investigate the mechanism of the observed selective killing of T-CAEBV cells, we tested apoptosis induction via flow cytometry analysis after 12, 24, 36, and 48 hours of 10 μg/ml DHMEQ treatment. As shown in Figure 3A, DHMEQ exerted a time-dependent apoptotic effect on SNT-16 cells (33.2% ± 0.5%, 37.2% ± 3.4%, 47.2% ± 1.3%, and 56.1% ± 2.6% apoptotic cells after 12, 24, 36, and 48 hours, respectively). To further identify the apoptosis-inducing effect of DHMEQ, we evaluated caspase-3 activity by determining p-nitroaniline (pNA) content. Caspase-3 activity was enhanced approximately 1.48-fold after DHMEQ treatment (Figure 3B). Moreover, PARP was upregulated after DHMEQ treatment (Figure 3C). Together, these data suggest that DHMEQ induced SNT-16 cell apoptosis in a caspase-dependent manner. To verify the role of caspase-3, we pretreated SNT-16 cells with 20 μM caspase-3 inhibitor z-DEVD-FMK for 2 hours and then treated them with 10 μg/ml DHMEQ for 24 hours. As shown in Figure 3D, z-DEVD-FMK pretreatment prevented DHMEQ-induced apoptosis in SNT-16 cells. Therefore, we conclude that DHMEQ selectively kills CAEBV-infected cells via apoptosis induction.

4.4. The role of NF-κB inhibition in DHMEQ-induced apoptosis

We next performed cellular and molecular experiments to identify the mechanisms of DHMEQ-induced apoptosis. First, we performed GSEA analysis to characterize the relationship between NF-κB and apoptosis in SNT-15 cells. This analysis revealed that the downregulated genes in SNT-15 cells were highly correlated with the cluster of apoptosis via NF-κB (Figure 4A). This prompted

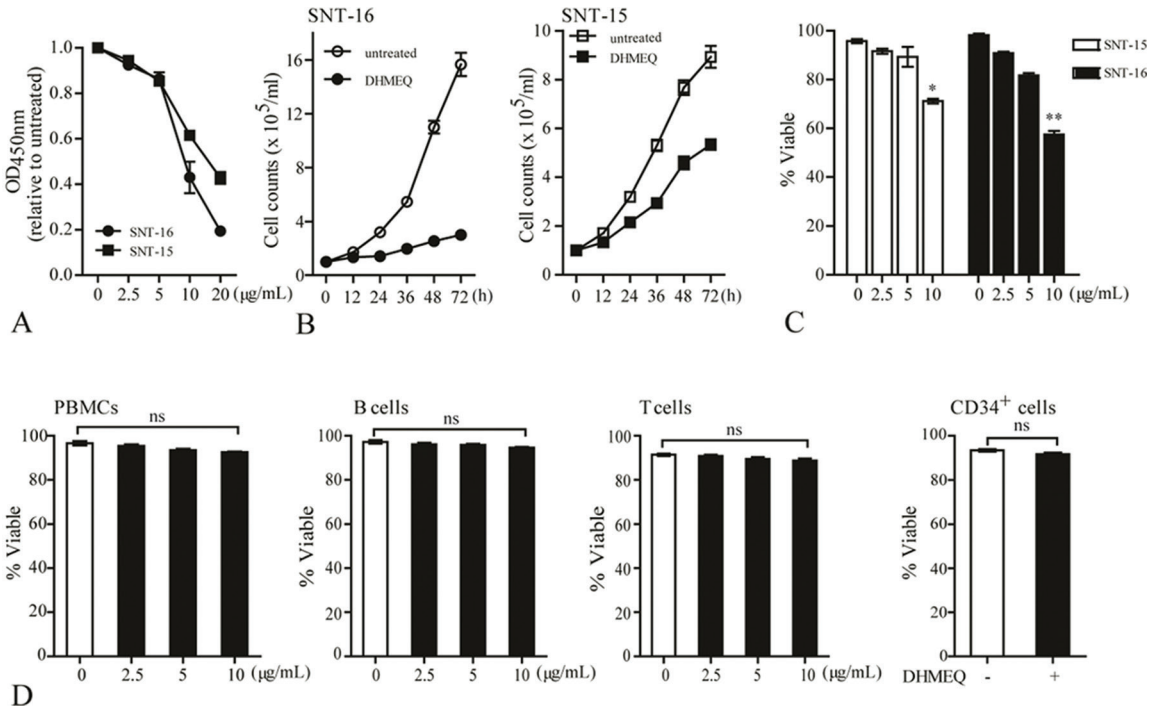

Figure 2. DHMEQ selectively affects T-type CAEBV cells but not normal hematopoietic cells. (A) Relative OD_{450nm} value of SNT-15 and SNT-16 cells exposed to 2.5., 5, 10 and 20 μg/ml DHMEQ for 24 hours. (B) Cell density of SNT-15 and SNT-16 cells exposed to 10 μg/ml DHMEQ for 0, 12, 24, 36, 48, 72 hours. (C) Percentage of viable SNT-15 and SNT-16 cells after 2.5., 5 and 10 μg/ml DHMEQ treatment for 24 hours. (D) Percentage of viable PBMCs, T, B cells (n = 4) and $CD34^+$ cells (n = 3) after treatment with 2.5, 5 and 10 μg/ml DHMEQ for 24 hours. The horizontal bars represent the mean and each error bar represents the SEM. **, P < 0.0.1; ns, no significance by the two-tailed t test.

us to test whether NF-κB inhibition participated in DHMEQ-induced apoptosis. By extracting total protein and nuclear protein, we determined that DHMEQ inhibited p65 and p50 nuclear translocation (Figure 4B). Next, we transfected SNT-16 cells with a pNF-κB-luc reporter plasmid (100 ng) and found that DHMEQ inhibited NF-κB transcriptional activity in a dose-dependent manner (Figure 4C). To validate this result, we treated SNT-16 cells with DHMEQ and performed real-time RT-PCR assays on NF-κB target genes. As shown in Figure 4D, the mRNA levels of NF-κB target genes (*BAK, BCL2*, *XIAP*, *c-IAP1*, *c-IAP2*, and *SURVIVIN*) were reduced, further supporting the role of NF-κB inhibition in DHMEQ-induced apoptosis. To determine the specificity of NF-κB inhibition, we used BAY 11-7082 (a known NF-κB inhibitor) as a positive control to perform parallel cytotoxic experiments. Intriguingly, 1–4 μM BAY 11-7082 had a lesser effect than DHMEQ on cell viability (Figure 4E). Together, these findings suggest that DHMEQ-induced apoptosis is partially mediated by NF-κB inhibition.

4.5. Oxidative stress plays an important role in DHMEQ-induced apoptosis

Since NF-κB inhibition could not fully explain DHMEQ-induced apoptosis, we believed that other underlying mechanisms might be involved. As reported by Lampiasi *et al*., ROS-mediated apoptosis is another feature of DHMEQ anti-tumor activity (31); thus, we tested whether ROS exerted a role in DHMEQ-induced apoptosis. GSEA analysis showed a global downregulation of stress in SNT-15 cells (Figure 5A), while DCFH-DA staining to measure intracellular ROS production showed a ~2.1-fold increase in intensity after 30 minutes of exposure to DHMEQ (Figure 5B). These data confirmed the previous finding that DHMEQ was a ROS inducer. To further determine the role of ROS, we pretreated SNT-16 cells with 100 μM vitamin C for 3 hours prior to DHMEQ treatment and observed a near-complete rescue of DHMEQ-induced apoptosis (Figure 5C). Moreover, we found that NRF2, an important oxidative stress transcription factor, was upregulated during the DHMEQ treatment (Figure 5D). The FAS signaling pathway was globally downregulated in

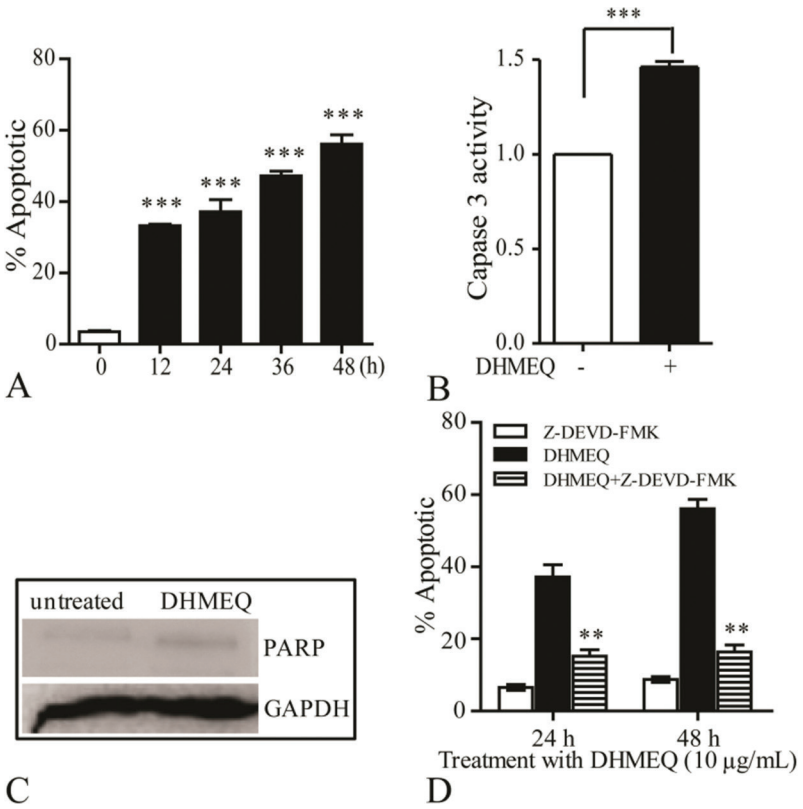

Figure 3. DHMEQ targets SNT-16 by apoptosis induction manner.(A) Percentage of apoptotic cells for SNT-16 cells exposed to 10 μg/ml DHMEQ for 12, 24, 36 and 48 hours. (B) Caspase 3 activity of the DHMEQ effects on SNT-16 cells, as measured by the content of pNA. (C) The expression of PARP after DHMEQ treatment. (D) The protective effect of z-DEVD-FMK on DHMEQ-induced apoptosis. The horizontal bars represent the mean and each error bar represents the SEM. ***, $P < 0.0.01$; **, $P < 0.0.1$ by the two-tailed t test.

SNT-15 cells; however, DHMEQ did not induce CD95 expression, indicating that the FAS signaling pathway did not participate in DHMEQ-induced apoptosis (Figure 5E-F). These data suggest that DHMEQ overcomes the weak response to oxidative stress and induces SNT-16 cell apoptosis through elevation of ROS levels. Collectively, the above mechanistic studies demonstrate that DHMEQ functions via a combination of NF-κB signaling inhibition and oxidative stress generation to induce SNT-16 cell apoptosis.

5. DISCUSSION

Utilizing currently available agents to selectively target EBV-infected cells, especially CAEBV-infected cells, is of great significance for several reasons. First, as a carcinogenic virus, EBV contributes to the global burden of infection-related cancers and closely relates to malignant diseases (NPC, Burkitt's lymphoma, HPS, etc.) (1,37). Accordingly, successful translational medicine in this area will improve the clinical prognosis of herpesvirus-associated tumors. Second, as a rare subtype of EBV-associated diseases characterized by ultimate progression to lymphoma or leukemia, CAEBV-associated malignancies are classified into mature T- and NK-cell neoplasms (38). Therefore, preventing CAEBV-associated malignant transformation can decrease tumor incidence. Third, other than hematopoietic stem cell transplantation, conventional chemotherapeutic regimens cannot efficiently cure CAEBV infection, and they produce several side effects and recurrence (7). In the present study, we report the anti-SNT-16 cell capability of DHMEQ.

To reveal the role of NF-κB signaling in the pathogenesis of T-CAEBV infection, we performed transcriptome analysis and found that SNT-15 cells had a similar gene expression profile to T/NK lymphoma (SNT-8) cells. Consistent

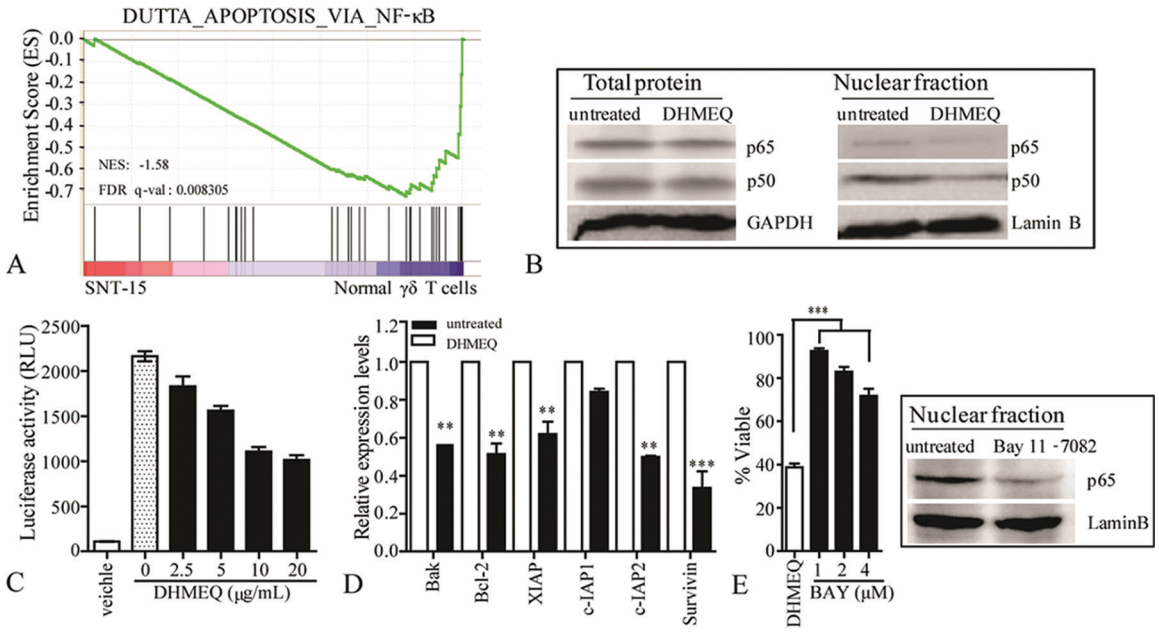

Figure 4. NF-κB inhibition plays certain role in DHMEQ targeting capabilities.(A) GSEA plots revealing correlation between SNT-15 specific genes and apoptosis via NF-κB related-gene set. (B) Immunoblots for untreated and DHMEQ-treated SNT-16 cells. Total proteins and nuclear fractions were extracted for blotting. (C) Reporter gene assays for detecting NF-κB transcriptional activity in untreated and 0 to 20 μg/ml DHMEQ-treated SNT-16 cells. (D) The expression changes of NF-κB target genes before and after DHMEQ treatment was determined using real-time RT-PCR. The relative expression levels were normalized to GAPDH expression. (E) Percentage of viable SNT-16 cells after treatment with 10 μg/ml DHMEQ and 1, 2, 4 μM BAY 11-7082 for 24 hours. Immunoblots for untreated and 4 μM BAY 11-7082-treated SNT-16 cells. The horizontal bars represent the mean and each error bar represents the SEM. ***, P < 0.0.01; **, P < 0.0.1; *, P < 0.0.5 by the two-tailed t test.

with the previous finding reported by Wakiguch, natural killer cell-mediated cytotoxicity was found to be downregulated (39). Besides, apoptosis was downregulated and the functional units and target genes of NF-κB were upregulated (Figure 1). Further, reporter gene assays revealed that the transcription activity of NF-κB was increased in the T-CAEBV cell lines SNT-15 and SNT-16. These findings indicate that activated NF-κB signaling might be an underlying pathogenesis in T-CAEBV cells.

The capacity of DHMEQ for eradicating herpesvirus-related malignancies, such as EBV-positive Burkitt's lymphoma, HTLV-1-induced ATL, EBV-positive NPC, and B cell lymphoma, has been reported in recent years (22-26,30,36,40). By using a series of cellular experiments, we found that DHMEQ induced apoptosis in SNT-16 cells, but not in normal hematopoietic cells including normal mononuclear, mature T/B, and $CD34^+$ progenitor cells (Figure 2 and 3). Together, these findings indicate that DHMEQ is a safe therapeutic agent capable of inhibiting T-CAEBV cells.

Our in-depth mechanistic investigation provides insights into the targeting of CAEBV infection and these data could be used for more efficient exploration of novel potent agents for T-CAEBV infection. Cellular and molecular experiments demonstrated that DHMEQ caused a decrease in nuclear translocation of NF-κB, reduced NF-κB transcriptional activity, and decreased the expression of NF-κB target genes, supporting the notion that NF-κB inhibition played a role in SNT-16 cell killing. BAY 11-7082 exhibited only half the cell-killing ability of DHMEQ (Figure 4), implying that NF-κB inhibition was not the only mechanism involved. Aside from being a novel NF-κB inhibitor, DHMEQ is also a potential ROS inducer dependent on cell type (31). In our study, we found that DHMEQ rapidly increased intracellular ROS production and upregulated NRF2 expression. Moreover, the antioxidant agent vitamin C protected most SNT-16 cells from apoptosis, indicating an ROS role in DHMEQ-induced cell death (Figure 5). NF-κB is a redox-sensitive transcription factor (41) and inhibition of NF-κB can further amplify intracellular ROS

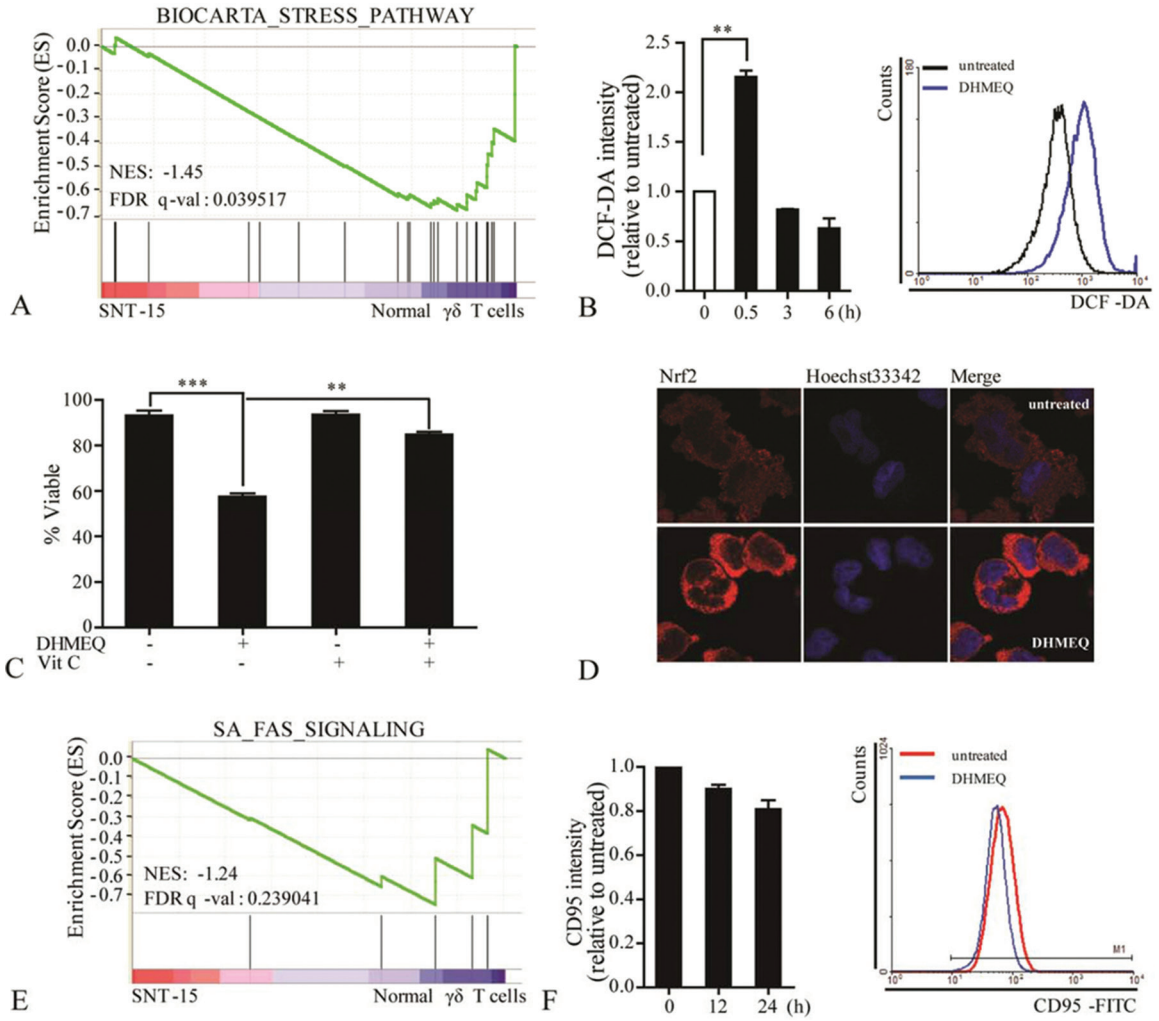

Figure 5. DHMEQ produces ROS-stress in SNT-16 cells. (A) GSEA plots revealing correlation between SNT-15 specific genes and stress related-gene set. (B) ROS production of SNT-16 cells exposed to 10 μg/ml DHMEQ for 0.5., 3 and 6 hours. The intracellular ROS level was determined using DCFH-DA probes, followed by the flow cytometry analysis. The representative histogram was shown in the right panel. (C) Percentage of viable cells after the pretreatment with vitamin C for 3 hours and the following treatment with 10 μg/ml DHMEQ for 24 hours. (D) Immunofluorescent assays for detecting NRF-2 in untreated and DHMEQ-treated SNT-16 cells. (E) GSEA plots revealing correlation between SNT-15 specific genes and Fas signaling related-gene set. (F) CD95 expression of SNT-16 cells exposed to 10 μg/ml DHMEQ for 24 hours. The representative histogram was shown in the right panel. The horizontal bars represent the mean and each error bar represents the SEM. ***, P < 0.0.01; **, P < 0.0.1 by the two-tailed t test.

production, thus synergizing the ROS-inducing effect of DHMEQ.

Besides its anti-tumor activity, recent studies indicate that DHMEQ can sensitize resistant tumor cells to traditional chemotherapy and inhibit metastasis (26,42,43). To date, very few adverse effects of DHMEQ on normal cells have been reported. Though the *in vivo* anti-tumor activity and combinative potential of DHMEQ remain unclear owing to a lack of clinical trials, its diverse effects on tumors and herpesvirus-transformed cells warrants further study of its potential benefits as an anti-cancer agent.

6. ACKNOWLEDGMENTS

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Abbreviation: CAEBV, chronic active Epstein-Barr virus infection; NF-κB, nuclear factor kappa B; ROS, reactive oxidative stress; DHMEQ, dehydroxymethylepoxyquinomicin; LPD, lymphoproliferative diseases; NPC, nasopharyngeal carcinoma; LMP1, latent membrane protein 1; PBMCs, peripheral blood mononuclear cells; MNCs, mononuclear cells; UCB umbilical cord blood; Gene set enrichment analysis, GSEA

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