Inhibition of the Epstein–Barr virus lytic cycle by protoapigenone

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Epstein–Barr virus (EBV) expresses two transcription factors, Rta and Zta, during the immediate–early stage of the lytic cycle to activate the transcription of early and late genes. This study finds that 0.31 mM protoapigenone from Thelypteris torresiana (Gaud.) inhibits the expression of EBV lytic proteins, including Rta, Zta, EA-D and VCA, in P3HR1 cells after lytic induction with 12-O-tetradecanoylphorbol-13-acetate and sodium butyrate. The lack of expression of EBV lytic proteins after protoapigenone treatment is attributed to the inhibition of the transactivation function of Zta because protoapigenone reduces the transactivation activity of Zta and Gal4–Zta, which contains the transactivation domain of Zta fused with Gal4. In contrast, protoapigenone does not affect the ability of Rta to activate a promoter that contains an Rta-response element, showing that the inhibition is unrelated to Rta. Furthermore, in a lactate dehydrogenase assay, protoapigenone is not toxic to P3HR1 cells at the concentrations that inhibit the function of Zta, showing that protoapigenone is valuable for studying the function of Zta and preventing EBV lytic proliferation.

INTRODUCTION

Epstein–Barr virus (EBV) is a human herpesvirus that causes infectious mononucleosis (Henle & Henle, 1970) and is closely associated with many malignant diseases, including nasopharyngeal carcinoma, B-cell and T-cell lymphomas, and Hodgkin’s disease (Jones et al., 1988; Weiss et al., 1989; Wolf et al., 1973). After infecting B-lymphocyte cells, EBV is typically maintained under latent conditions. However, the virus must enter a lytic cycle to reproduce to establish new infections. Although the mechanism by which the virus activates its lytic cycle in humans is unclear, treatment of cells that are latently infected by EBV with sodium butyrate (SB) and 12-O-tetradecanoylphorbol-13-acetate (TPA) activates the lytic cycle (Datta et al., 1980; Luka et al., 1979). The activation of the lytic cycle by TPA is attributed to activation of the ERK signal transduction pathway (Fenton & Sinclair, 1999; Gao et al., 2001), which ultimately causes the phosphorylation of AP-1 and ATF1/2 (Adamson et al., 2000; Wang & Prywes, 2000; Wang et al., 1997). The activated AP-1 and ATF1/2 then promotes the transcription of the Zta gene, BZLF1, by binding to the TPA-response elements in the promoter (Adamson et al., 2000; Wang & Prywes, 2000; Wang et al., 1997). The expression of Zta is critical to EBV lytic development because the transcription of many EBV lytic genes depends on Zta (Chiu et al., 2007; Miller et al., 2007). Meanwhile, initiation of EBV lytic DNA replication requires the binding of Zta to the seven Zta-response elements (ZRE) in the lytic origin of replication (Schepers et al., 1996), showing the importance of Zta in EBV lytic development. During the immediate–early stage of the lytic cycle, EBV also expresses another transcription factor, Rta, which is encoded by BRLF1 (Chevallier-Greco et al., 1986). Rta and Zta not only influence each other’s transcription but also cooperate to activate the transcription of EBV lytic genes synergistically (Chang et al., 2010b; Miller et al., 2007). Rta and Zta are known to bind to their respective response elements, Rta-response element (RRE) and ZRE, to activate transcription (Chen et al., 2005; Cox et al., 1990; Feederle et al., 2000). Rta also interacts with MCAF1 and RanBPM to activate transcription that is mediated by Sp1 and Zta (Chang et al., 2005, 2008b, 2010b). Without Rta or
Zta, EBV cannot complete its lytic cycle (Chiu et al., 2007; Feederle et al., 2000).

A number of compounds that are purified from plants are known to inhibit EBV. For instance, glycyrrhizic acid from liquorice root (Glycyrrhiza radix) inhibits EBV infection by interfering with viral attachment and penetration (Lin, 2003). (−)-Epigallocatechin gallate (EGCG) from green tea and andrographolide from Andrographis paniculata inhibit the EBV lytic cycle (Chang et al., 2003; Lin et al., 2008). Moronic acid, a triterpenoid keto acid from propolis, and resveratrol, a non-flavonoid polyphenol present in Polygonum cuspidatum, inhibit the functions of Rta and Zta as well as the EBV lytic cycle (Chang et al., 2010a; Yiu et al., 2010). This study finds that protoapigenone, which is present in Thelypteris torresiana, inhibits the transactivation function of Zta to prevent the virus entering the lytic cycle.

**RESULTS**

**Inhibition of expression of EBV lytic proteins by protoapigenone**

Six anti-cancer and anti-microbial compounds commonly found in plants, e.g. gelomulide K, ovatodiolide, oxopurpureine, protoapigenone, ursolic acid and zederone (Supplementary Fig. S1, available in JGV Online), were studied to determine their capacity to inhibit the EBV lytic cycle. These compounds were added to P3HR1 cultures during lytic induction of EBV. The effect of these compounds on the expression of an EBV early protein, EA-D, which is expressed during the lytic cycle and is involved in EBV lytic DNA replication, was determined by immunoblotting. As expected, expression of EA-D by EBV in P3HR1 cells was undetected if the cells were not treated with TPA and SB (Fig. 1, lane 1). However, the expression of EA-D was detected at 24 h following lytic induction (Fig. 1, lane 2). Immunoblotting also established that 10 μM gelomulide K, ovatodiolide and oxopurpureine inhibited the expression of EA-D (Fig. 1a, lane 9). However, the expression of EA-D was unaffected by treatment with 10 μM ursolic acid or zederone (Fig. 1a, lane 9). This study also found that protoapigenone inhibited the expression of EA-D at a concentration that was considerably less than that of gelomulide K, ovatodiolide and oxopurpureine. At 0.16 μM, protoapigenone substantially reduced the expression of EA-D and two EBV immediate–early proteins, Rta and Zta (Fig. 1b, lane 6). At a concentration of 0.31 μM protoapigenone, the expression of these proteins was further reduced to background levels (Fig. 1b, lane 7), indicating that protoapigenone is more effective than the other five compounds in inhibiting the EBV lytic cycle. Additionally, treatment of P3HR1 cells with these six compounds did not result in the expression of EA-D (Fig. 1, lane 3), indicating that these compounds did not activate the EBV lytic cycle.

**Flow cytometric analysis of expression of Zta, Rta, EA-D, VCA and EBNA-1**

The expression of EBV lytic proteins, including Rta, Zta and EA-D, in P3HR1 cells at 24 h after lytic induction was analysed by flow cytometry. The expression of VCA was studied at 48 h after lytic induction. When P3HR1 cells were treated with neither TPA nor SB, the percentages of the population that expressed Rta, Zta, EA-D and VCA were 0.06, 0.14, 0.02 and 0.02%, respectively (Fig. 2), showing that EBV does not express these proteins during latency. On the other hand, 99.98% of the cell population expressed EBNA-1, a protein that is required for EBV DNA replication at the latent stage. As expected, lytic induction of EBV using TPA and SB greatly increased the proportions of the populations that expressed these four proteins to 38.17, 37.8, 31.49 and 30.72%, respectively (Fig. 2). Meanwhile, lytic induction did not appear to influence the expression of EBNA-1 (Fig. 2). The study also showed that treating the cells with protoapigenone at the time of lytic induction inhibited the population that expressed these four proteins in a dose-dependent manner (Fig. 2). For example, the population that expressed Zta dropped from 37.8% to 21.91, 11.89 and 6.01% after the concentration of protoapigenone increased from none to 0.08, 0.16, and 0.31 μM, respectively (Fig. 2). When the cells were treated with 0.31 μM protoapigenone, the population that expressed Zta, Rta, EA-D and VCA...
decreased to 6.01, 7.27, 3.93 and 6.23 %, respectively (Fig. 2). A similar reduction in the expression of VCA was also observed (Fig. 2). Meanwhile, protoapigenone treatment did not affect the expression of EBNA-1 (Fig. 2).

**Inhibition of production of EBV viral particles by protoapigenone**

EBV particles that were generated by 5 ml of P3HR1 cells (3 × 10^6 cells) after 5 days of lytic induction were harvested from the culture medium by ultracentrifugation. After purifying EBV DNA from the pellet fraction, the copy number of EBV DNA was determined by quantitative PCR (qPCR). The results revealed that the background level of EBV genome detected in 1 ml of uninduced cells was 1.02 × 10^5 copies, which was set at 0 % (data not shown). Meanwhile, cells treated with TPA and SB yielded approximately 1.9 × 10^7 copies of EBV DNA, which was set at 100 % (Fig. 3). Although 0.04 μM protoapigenone did not reduce the copy number of EBV DNA (Fig. 3), treating cells with 0.08, 0.16, 0.31 and 0.63 μM protoapigenone reduced the genome percentages to 90.1, 88.1, 64.0 and 26.6 %, respectively (Fig. 3). These results showed that protoapigenone reduces the production of EBV particles.

**Inhibition of BZLF1, BRLF1 and BMRF1 transcription by protoapigenone**

Since protoapigenone inhibited the expression of EBV lytic proteins, this study further examined whether this inhibition manifests at the transcriptional level. Reverse transcription qPCR (RT-qPCR) analysis indicated that EBV synthesized little BRLF1, BZLF1 and BMRF1 mRNA under latent conditions (Fig. 4). Treating the cells with DMSO or protoapigenone at 0.31 or 0.63 μM did not influence the mRNA levels of these genes (Fig. 4).
However, lytic induction with TPA and SB markedly increased the mRNA levels of these genes (Fig. 4). After the cells were treated with 0.31 or 0.63 μM protoapigenone upon lytic induction, the amount of \( BZLF1 \) mRNA was 81.3 or 95.6 % lower than that of the cells untreated with protoapigenone, respectively (Fig. 4a). Protoapigenone at 0.31 or 0.63 μM also reduced the amount of \( BRLF1 \) mRNA by 78 and 92 %, respectively (Fig. 4b), and that of \( BMRF1 \) by 87.5 and 98 % (Fig. 4c), respectively.

**Protoapigenone and ERK signalling**

An earlier study showed that protoapigenone activates ERK signalling in MDA-MB-231 cells, a human breast cancer line (Chen et al., 2010). If this is also the case for P3HR1 cells, protoapigenone should trigger the EBV lytic cycle because the activation of the pathway activates the transcription of \( BZLF1 \) (Fenton & Sinclair, 1999; Gao et al., 2001; Lee et al., 2008b). However, the inability of protoapigenone to activate the EBV lytic cycle (Fig. 1) strongly suggests that the compound may not influence ERK signalling in P3HR1 cells. Accordingly, this study conducted an immunoblot study to examine how protoapigenone influences ERK signalling in P3HR1 cells. As the studies on ERK signalling are commonly conducted using cells that are under starvation conditions, in this study, we cultured P3HR1 cells in RPMI 1640 medium containing 0.1 % FBS for 24 h before treating them with TPA and protoapigenone. Meanwhile, the phosphorylation of MEK and ERK were examined at 15 and 60 min after the treatment, respectively, according to the method of Naranatt et al. (2003). Immunoblotting revealed that phosphorylated MEK and ERK were not detected if the cells were not treated with TPA (Fig. 5, lanes 1–4). Treating the cells with TPA, as expected, caused the phosphorylation of MEK and ERK (Fig. 5, lane 5). On the other hand, treating the cells with TPA and 0.31–1.25 μM protoapigenone did not affect the phosphorylation of MEK and ERK (Fig. 5, lanes 6–8), indicating that protoapigenone...
Accordingly, BJAB cells were co-transfected with pCMV-R in order to study the inhibition of the transactivation functions of Rta and Zta. This study investigated whether the prevention of activation of the transcription of the mRNA levels of the Rta, Zta and EA-D genes (Fig. 4). The inhibition is verifiable by flow cytometric analysis (Fig. 2) and RT-qPCR analysis of the mRNA levels of the Rta, Zta and EA-D genes (Fig. 4).

To investigate whether protoapigenone inhibits the transactivation of Zta, we co-transfected BJAB cells with pM-Zta, which expresses a protein that contains the transactivation domain of Zta fused with the DNA-binding domain of Gal4, and a reporter plasmid, pLG5, which contains a promoter with five copies of the Gal4-binding site. Results showed that Gal4–Zta activated the GAL4 promoter 472-fold. The activity of the promoter decreased to 342- and 73-fold after the cells were incubated with 0.08 and 0.63 μM protoapigenone, respectively (Fig. 7), showing that protoapigenone inhibits the transactivation activity of Zta.

Toxicity of protoapigenone to P3HR1 cells

We conducted LDH assays to evaluate the toxicity of protoapigenone to P3HR1 cells and found that protoapigenone at concentrations <0.63 μM had little toxicity towards P3HR1 cells after 24 h of incubation (Fig. 8a). When the concentration was increased to 5 μM, the viability decreased to 54.8%; at 10 μM the viability decreased to 24.5% (Fig. 8a). After treating the cells with protoapigenone at concentration <0.16 μM for 48 h, >99% of the cells were still viable. When the concentration of protoapigenone was increased to 0.63 and 1.25 μM, the viability decreased to 74.5 and 48.1%, respectively (Fig. 8b). If the cells were treated with 5 or 10 μM protoapigenone for 48 h, <15.7 or <12.6%, respectively, of the cells were viable (Fig. 8b). The CC_{50} values, which are defined as the concentration of protoapigenone that kills 50% of the cell population, against P3HR1 cells at 24 and 48 h were 5.74 and 1.31 μM, respectively (Fig. 8).

**DISCUSSION**

This study screens anti-cancer and anti-microbial compounds from plants for their ability to inhibit the EBV lytic cycle. According to our immunoblot study, protoapigenone effectively inhibits the expression of Rta, Zta and EA-D at the level of 0.31 μM (Fig. 1). The inhibition is verifiable by flow cytometric analysis (Fig. 2) and RT-qPCR analysis of the mRNA levels of the Rta, Zta and EA-D genes (Fig. 4). This study also shows that the abilities of Zta and Gal4–Zta to activate a ZRE and a GAL4 promoter, respectively, are reduced by protoapigenone (Figs 6b and 7). As is generally known, the transcription of many EBV lytic genes is dependent on Zta (Chiu et al., 2007; Giot et al., 1991; Miller et al., 2007). Zta also autoregulates its own transcription by directly binding to the BZLF1 promoter, which is critical for the expression of Zta at a sufficient level to activate the lytic cycle (Flemington & Speck, 1990), explaining why inhibition of the transactivation function of Zta by protoapigenone decreases the transcription of BZLF1, BRLF1 and BMRF1 (Fig. 4), and inhibits the EBV lytic cycle. Rta is another transcription factor that strongly influences the transcription of lytic genes (Chiu et al., 2007; Giot et al., 1991; Miller et al., 2007). However, protoapigenone does not seem to interfere with the functioning of this transcription factor (Fig. 6a).

TPA is a potent compound that activates the EBV lytic cycle (Gao et al., 2001). This activation is attributed to the activation of the ERK signalling pathway (Fenton & Sinclair, 1999; Gao et al., 2001; Lee et al., 2008b), which ultimately phosphorylates AP-1 and ATF1/2 (Adamson et al., 2001; Giot et al., 1991; Lee et al., 2008b). The result showed that adding protoapigenone at concentrations between 0.08 and 0.63 μM to the culture did not appear to influence the ability of Rta to activate the promoter (Fig. 6a). A similar study was performed using pCMV-Z and a reporter plasmid, pZRE, which contains a ZRE from the BRLF1 promoter (Chang et al., 2010b). Adding 0.08, 0.16, 0.31, and 0.63 μM protoapigenone reduced the transactivation activity of Zta by 15.4, 36, 52.1, and 87.9% (Fig. 6b), respectively, indicating that protoapigenone interferes with the function of Zta. Furthermore, immunoblot analysis revealed that protoapigenone did not destabilize Rta or Zta (Fig. 6), showing that the reduction of the activity of Zta is not attributed to the stability of Zta.

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**Fig. 5.** Effect of protoapigenone on ERK signalling. Lysates were prepared from P3HR1 cells that had been cultured in RPMI 1640 medium containing 0.1 % FBS and were subsequently treated with protoapigenone and TPA. Phosphorylated MEK and ERK were observed in the cells that had been treated with TPA and protoapigenone for 15 and 60 min, respectively. Proteins in the lysates were immunoblotted using anti-MEK, anti-phospho-MEK (pMEK), anti-ERK, anti-phospho-ERK (pERK) and anti-β-actin antibodies.

Protoapigenone treatment and the transactivation activity of Rta and Zta

Rta and Zta activate a number of EBV lytic genes to promote the EBV lytic cycle. Therefore, this study investigated whether the prevention of activation of the EBV lytic cycle by protoapigenone can be attributed to the inhibition of the transactivation functions of Rta and Zta. Accordingly, BJAB cells were co-transfected with pCMV-R and pRRE, a reporter plasmid that contains an RRE from the BMLF1 promoter (Chang et al., 2010b). The result showed that adding protoapigenone at concentrations between 0.08 and 0.63 μM to the culture did not appear to influence the ability of Rta to activate the promoter (Fig. 6a). A similar study was performed using pCMV-Z and a reporter plasmid, pZRE, which contains a ZRE from the BRLF1 promoter (Chang et al., 2010b). Adding 0.08, 0.16, 0.31, and 0.63 μM protoapigenone reduced the transactivation activity of Zta by 15.4, 36, 52.1, and 87.9% (Fig. 6b), respectively, indicating that protoapigenone interferes with the function of Zta. Furthermore, immunoblot analysis revealed that protoapigenone did not destabilize Rta or Zta (Fig. 6), showing that the reduction of the activity of Zta is not attributed to the stability of Zta.

Inhibition of transactivation activity of Gal4–Zta by protoapigenone

To investigate whether protoapigenone inhibits the transactivation activity of Zta, we co-transfected BJAB cells with pM-Zta, which expresses a protein that contains the transactivation domain of Zta fused with the DNA-binding domain of Gal4, and a reporter plasmid, pLG5, which contains a promoter with five copies of the Gal4-binding site. Results showed that Gal4–Zta activated the GAL4 promoter 472-fold. The activity of the promoter decreased to 342- and 73-fold after the cells were incubated with 0.08 and 0.63 μM protoapigenone, respectively (Fig. 7), showing that protoapigenone inhibits the transactivation activity of Zta.

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**Fig. 6.** Effect of protoapigenone on the transactivation activities of Rta and Zta. BJAB cells were co-transfected with pRRE and pCMV-R (a) or pZRE and pCMV-Z (b), and then cultured in medium that contained protoapigenone. The activity was then normalized to the results obtained from the assay involving the co-transfection with reporter plasmid and pCMV-3. Luciferase activity was determined at 24 h after transfection. Each transfection experiment was performed three times, and each sample in the experiment was prepared in triplicate. Rta, Zta and -actin in the lysates were also analysed by immunoblotting using anti-Zta, anti-Rta and anti-β-actin antibodies to show the stability of Rta and Zta. The activity of the DMSO-treated cells was set at 100 %. Error bars represent sd. Statistical differences were found by making comparisons with the DMSO-treated group; ***, P<0.001.

**Fig. 7.** Inhibition of the transactivation function of Gal4–Zta by protoapigenone. BJAB cells were co-transfected with pM-Zta, which expresses Gal4–Zta, and a reporter plasmid, pLG5. Cells were then cultured in medium that contained protoapigenone. Activation of the GAL4 promoter in pLG5 by Gal4–Zta was determined at 24 h after lytic induction. Gal4–Zta in the lysates was also analysed by immunoblotting using anti-Zta and anti-β-actin antibodies to show the stability of Gal4–Zta. Each transfection experiment was performed three times, and each sample in the experiment was prepared in triplicate. Statistical differences were found by making comparisons with the DMSO-treated group; **, P<0.01; ***, P<0.001.

et al., 2000; Wang & Prywes, 2000; Wang et al., 1997). After phosphorylation, these proteins bind to the BZLF1 promoter at TPA response elements to initiate transcription (Adamson et al., 2000; Wang & Prywes, 2000; Wang et al., 1997). However, protoapigenone, although it is known to activate the ERK pathway in MDA-MB-231 cells (Chen et al., 2010), does not seem to influence the pathway in P3HR1 cells (Fig. 5) in order to inhibit the lytic cycle. An earlier study also established that Rta activates p38 signalling to activate BZLF1 transcription through the ZII region of the promoter (Adamson et al., 2000). However, the fact that protoapigenone activates p38 signalling (Chang et al., 2008a) would seem to be evidence against the hypothesis that the inhibition of the EBV lytic cycle by protoapigenone is related to p38 signalling. Zta is a transcription factor of the bZip family, which is indispensable for the transcription of EBV lytic genes and EBV lytic DNA replication (Chiu et al., 2007; Giot et al., 1991; Schepers et al., 1996). Owing to the importance of Zta, it is often necessary to inhibit its function to elucidate how it influences the EBV lytic cycle. At least two methods have been employed in the past to inhibit the function of Zta in experiments – either by generating mutations in the Zta gene of the EBV genome (Chiu et al., 2007) or by expressing siRNA to inhibit the expression of Zta (Chang et al., 2004). Our current study shows that protoapigenone can also be used for the same purpose. Because protoapigenone is not toxic to cells at the concentration that efficiently inhibits the EBV lytic cycle (Figs 3 and 8), the
compound is valuable for the study of the EBV lytic cycle and preventing lytic proliferation.

**METHODS**

**Compounds, cell lines and lytic induction.** Protoapigenone was synthesized chemically (Lin et al., 2007). Gelomulide K (Lee et al., 2008a), ovatodiolide (Chen et al., 2008), oxopurpureine (Chang et al., 1998a), ursoic acid (Chen et al., 2006) and zederone (Y.-C. Wu, L.-S. Kan and F.-R. Chang, unpublished result) were purified from plant materials: *Gelonium aequorium*, *Antisomeles indica* (L.) Kuntze, *Annona purpurea*, *Hedyotis biflora* and *Curcuma zedoaria*, respectively. These compounds were dissolved in DMSO. *BJAB* is a Burkitt’s lymphoma cell line. P3HR1 is a Burkitt’s lymphoma cell line that is latently infected by EBV. These cells were cultured in RPMI 1640 medium (Biological Industries). The EBV lytic cycle was activated by treating P3HR1 cells with 30 nM TPA (Sigma) and 3 mM SB (Sigma) (Chiu et al., 2007).

**Immunoblot analysis of EBV proteins.** A lysate from P3HR1 cells (3 x 10⁶ cells) that had been treated with TPA and SB for 24 h was prepared by using 100 μl of RIPA buffer [50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1 % NP-40, 0.1 % SDS, 1 % sodium deoxycholate and a complete protease inhibitor cocktail tablet (Roche)] (Chang et al., 2003) and separated by using SDS-PAGE. Immunoblot analysis was performed using a method that has been described previously to detect EBV lytic proteins (Chiu et al., 2007). Anti-Rta and anti-Zta mAbs were purchased from Argene. Anti-EA-D mAb was purchased from Chemicon. Anti-β-actin mAb was purchased from Novus Biologicals.

**Flow cytometric analysis.** P3HR1 cells (5 x 10⁶ cells) were treated with protoapigenone during lytic induction. After incubating for 24 h, they were washed with PBS and fixed with 4 % paraformaldehyde in PBS for 30 min. The cells were then treated with 0.1 % (v/v) Triton X-100 in PBS for 5 min, washed with PBS (pH 7.4), incubated with 1 % (w/v) BSA in PBS for 1 h, and finally incubated for 1 h at room temperature with anti-Rta, anti-Zta, anti-EA-D or anti-VCA (Argene) mAbs, or polyclonal rabbit anti-EBNA-1 antibody, which was provided by Mei Chao (Chang-Gung University). The cells were then washed with 0.5 % (v/v) Tween-20 in PBS, and incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen) or Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen). Finally, they were suspended in 1 % paraformaldehyde in PBS and analysed using a FACSscan cytofluorometer (FACSCalibur E6147; BD Biosciences). Antibodies used in this study were diluted 200-fold with 1 % (w/v) BSA in PBS.

**Determining the number of EBV particles.** P3HR1 cells (3 x 10⁶ cells) were cultured in 5 ml RPMI 1640 medium for 5 days following lytic induction. EBV particles were harvested by ultracentrifugation at 25,000 g for 2 h at 4 °C. The pellet was suspended in 200 μl TNE buffer (10 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, pH 8.0), treated with 2 U of DNase I (Promega), incubated for 30 min at room temperature and then treated with 60 μg Proteinase K (Macherey-Nagel) for 16 h at 55 °C. DNA was extracted using phenol/chloroform, chloroform alone and precipitation with 2-propanol. After washing with 70 % ethanol, the DNA was dissolved in 200 μl of TE buffer. The amount of EBV DNA was determined by a qPCR that amplified *BKRF1* and then used to establish a standard curve for qPCR analysis. The percentage of the EBV genome was calculated according to the equation, [(mean Cn – mean
C_{ab}/(\text{mean } C_{a} - \text{mean } C_{b}) \times 100. The C_{a} value was obtained from cells treated with protoapigenone after lytic induction; C_{b}, from cells treated with protoapigenone alone; C_{a}, from cells treated with TPA and SB; and C_{ab}, cells treated with DMSO.

Quantitative analysis of mRNA by RT-qPCR. mRNA was purified from P3HR1 cells (5 \times 10^6 cells) that had been treated with protoapigenone, TPA and SB for 24 h by using TRIzol reagent and a Dynabeads mRNA Direct kit (Invitrogen). To determine the amount of BRLF1, BZLF1 and ZTA mRNA using RT-qPCR, 1 µg mRNA was reverse-transcribed with random-hexamer primers and 400 U of SuperScript III Reverse Transcriptase (Invitrogen). One-tenth of the cDNA was then amplified by qPCR. The primers that were used to amplify BRLF1 and BZLF1 cDNA are described elsewhere (Chiu et al., 2007; Ryan et al., 2004). BRLF1 cDNA was amplified using primers 5'-ACCTCGTGTTGCTGCTAGCTAGTG-3' and 5'-GGCGGTGTGTTGAGTCCTGTG-3'. A probe, 5'-HEX-TTATTT-AACCAAGGCTCAGCAAGG-3' was used to detect amplified DNA (HEX, hexachlorofluorescein). The amount of GAPDH cDNA was used as an internal standard (Yajima et al., 2000).

ERK signalling. P3HR1 cells (3 \times 10^6 cells) were cultured in RPMI 1640 medium containing 0.1 % FBS (Biological Industries) overnight prior to treating them with protoapigenone and TPA. Lysates were prepared from the cells that were treated with protoapigenone and TPA for 15 min and 60 min, respectively, to analyse MEK and ERK signalling according to the method of Naranatt et al. (2003). Proteins in lysates were immunoblotted following SDS-PAGE by using anti-MEK, anti-phospho-MEK (pMEK), anti-ERK, anti-phospho-ERK (pERK) (Cell Signaling Technology) and anti-β-actin antibodies.

Transient transfection and luciferase assay. Plasmids pRE, pZRE, pCMV-3, pCMV-R, pCMV-Z and pLG5 have been described previously (Chang et al., 1998b, 2005, 2008b, 2010b). A DNA fragment that encodes the region of Zta from aa 1–167 was amplified by PCR and inserted into the EcoRI and SalI sites of plasmid pM, which is a vector that contains the Gal4 DNA-binding domain (Clontech), to generate plasmid pM-Zta. BJAB cells (7.5 \times 10^5 cells) were suspended in 0.4 ml RPMI 1640 medium and mixed with 8 µg each of pRE and pCMV-R, pZRE and pCMV-Z, or pLG5 and pM-Zta in a gene pulser cuvette with a 0.4 cm electrode gap. Electroporation was performed at 975 µF and 0.25 kV using an electroporator (Gene Pulser II; Bio-Rad). Cells were then aliquoted into medium that contained protoapigenone. Luciferase activity was measured at 24 h after transfection using a luminometer (Berthold) (Chang et al., 1998b). Proteins in lysates were also separated by using SDS-PAGE and immunoblotted with anti-Zta, anti-Rta and anti-β-actin antibodies to evaluate the transfection efficiency. Each transfection experiment was performed three times, and each sample in the experiment was performed three times, and each sample in the experiment was prepared in quadruplicate. Error bars represent SD.

Statistics. Data were analysed statistically by one-way ANOVA using the JMP 6.0 (SAS) software package. Data are presented as mean ± SD and a P value of <0.05 was taken as being significant.

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domains of EB1 are required. Transcription factors EB1 and R: both DNA-binding and activation factors, EB1 and EB2, are required to activate transcription from an EBV early promoter.

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