REVIEW ARTICLE

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# Therapies based on targeting Epstein-Barr virus lytic replication for EBV-associated malignancies

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In recent years, Epstein-Barr virus (EBV) lytic infection has been shown to significantly contribute to carcinogenesis. Thus, therapies aimed at targeting the EBV lytic cycle have been developed as novel strategies for treatment of EBV-associated malignancies. In this review, focusing on the viral lytic proteins, we describe recent advances regarding the involvement of the EBV lytic cycle in carcinogenesis. Moreover, we further discuss 2 distinct EBV lytic cycle-targeted therapeutic strategies against EBV-induced malignancies. One of the strategies involves inhibition of the EBV lytic cycle by natural compounds known to have anti-EBV properties; another is to intentionally induce EBV lytic replication in combination with nucleotide analogues. Recent advances in EBV lytic-based strategies are beginning to show promise in the treatment and/or prevention of EBV-related tumors.

**KEYWORDS**
carcinogenesis, EBV lytic replication, lytic induction, natural compound, vaccine

## 1 | INTRODUCTION

Epstein-Barr virus (EBV) is one of the most common human viruses and infects approximately 95% of the world's population. EBV was the first human tumor virus to be identified, accounting for more than 200 000 cases of cancer each year, and its infection is associated with 1.8% of all cancer deaths.[1] Currently, EBV is known to be strongly associated with the development of several human cancers,

including nasopharyngeal carcinoma (NPC), Burkitt's lymphoma (BL), Hodgkin's lymphoma, and gastric cancer.[2]

Similar to all herpesviruses, EBV establishes 2 alternative modes of infection in its host, latent and lytic. During latency, EBV expresses only a limited number of products. Years of research results have shown that latent infection plays an essential role in carcinogenesis. Several latent proteins, such as LMP1, LMP2A, and EBNA1, are considered to show obvious carcinogenic activities.[3] Upon reactivation induced by various stimuli, EBV undergoes 3 consecutive lytic stages, including immediate early (IE), early (E), and late (L) stages. The IE proteins, Zta and Rta, activate transcription from the promoters of lytic E genes, which trigger EBV genomic DNA replication from the lytic origin of replication, *oriLyt*. Then the lytic L genes that encode structural proteins are expressed, followed by viral genome packaging into infectious virion particles. In recent years, the EBV lytic cycle has been reported to contribute to carcinogenesis of several cancer types. In the present study, we highlight recent findings related to the roles of EBV lytic proteins in carcinogenesis. Furthermore, we discuss therapeutic strategies aimed at targeting the viral lytic cycle for patients with EBV-associated malignancies.

## 2 | EPSTEIN-BARR VIRUS LYTIC PROTEINS IN CARCINOGENESIS

Accumulating data suggest the contribution of the EBV lytic cycle to carcinogenesis through the induction of oncogenic cytokine secretion and genome instability.[4,5] A small subset of lytically infected cells is commonly detected in clinical tissue samples of EBV-associated malignancies,[6,7] suggesting a potential role for viral lytic replication in promoting tumor growth in vivo. Fang et al[8] first reported that compared to latent infection, the recurrent induction of the EBV lytic cycle contributed more profoundly to NPC carcinogenesis. Also, epidemiological studies have shown that individuals with elevated antibody titers against EBV lytic antigens (viral capsid antibody [VCA] and early antigen [EA] antibody) and increased plasma EBV DNA load have a higher risk of developing NPC.[9,10] Recently, detecting the titers of IgA antibodies against VCA and Zta in serum has been widely studied and even used in clinical practice for NPC diagnosis and screening in high-risk populations.[11,12]

During the lytic cycle, EBV expresses a series of intriguing proteins. These proteins show homology to a wide variety of cytokines and anti-apoptotic proteins. They can also enhance genomic instability or target tumor suppressors, thereby making a significant contribution to human pathology (Table 1).[5,13–31]

### 2.1 | Zta

Zta is a transcription factor that binds to and activates the EBV lytic E gene promoters, thereby triggering EBV reactivation from latency. Enhanced Zta expression has been shown to cause lymphomas in vivo.[13] Zta contributes to secretion of immunosuppressive cytokines, including interleukin-10 (IL-10)[14] and IL-13,[15] and also plays an

**TABLE 1** Overview of EBV lytic proteins and oncogenic functions

| EBV lytic proteins | Lytic phase | Oncogenic functions | References |
|---|---|---|---|
| Zta | IE | Induces production of oncogenic cytokinesand VEGF | 13–17 |
| BHRF1 | E | Anti-apoptotic function | 18, 19 |
| BALF1 | E | Anti-apoptotic function | 20–22 |
| BARF1 | E | Malignant transformation and anti-apoptotic activity | 23–25 |
| BGLF4 and BGLF5 | E | Leads to genomic instability | 26, 27 |
| BALF3 | E | Induces genomic instability | 5, 28 |
| BCRF1 | E | Homologue of cellular IL-10 | 29 |
| BILF1 | E | Transforms cells and induces VEGF secretion | 30, 31 |

E, early; EBV, Epstein-Barr virus; IE, immediate early; IL-10, interleukin-10; VEGF, vascular endothelial growth factor.

important role in tumor growth through release of vascular endothelial growth factor (VEGF).[16] Beyond that, Zta reportedly inhibits tumor necrosis factor alpha (TNFα)-induced apoptosis by downregulating TNF receptor 1 (TNF-R1).[17]

### 2.2 | BHRF1

BHRF1 is an early lytic protein that shows both sequence and functional homology to the human anti-apoptotic protein Bcl-2 and inhibits apoptosis of EBV-positive cancer cells. Recent studies suggest that BHRF1 exerts its anti-apoptotic activity by interacting with the pro-apoptotic BH3-only protein, Bcl-2 interacting mediator (Bim), and by upregulating Bcl-2 to amplify the anti-apoptotic effect.[18] Inducible BHRF1 expression protects BL cells from apoptosis, which contributes to EBV-associated lymphomagenesis in vivo.[19]

### 2.3 | BALF1

In addition to BHRF1, the EBV genome encodes a second Bcl-2 homolog, BALF1. In an earlier study, Marshall et al[20] showed that BALF1 suppresses apoptosis by associating with 2 cellular pro-apoptotic proteins, Bax and Bak. Recently, BALF1 was shown to increase cell survival by suppressing serum starvation-induced apoptosis,[21] thereby playing an important role in oncogenesis of EBV-associated tumors. BALF1 also enhances cellular metastasis and invasion in vitro and in vivo, which suggests a key role for BALF1 in facilitating the potential of increased tumor metastasis.[22]

### 2.4 | BARF1

BARF1, reported as an early lytic gene,[23] is a homologue to the colony-stimulating factor-1 (CSF-1) receptor. Sakka et al[24] showed that BARF1 is capable of inducing malignant transformation in human B cell lines and rodent fibroblasts. They then found that BARF1 leads to activation of the cell cycle from passage of G1 to S phase in nude

mice, suggesting that it could act as a growth factor to induce tumor formation in vivo.[24] Moreover, BARF1 promotes cancer cell survival by activating the expression of the anti-apoptotic Bcl-2 protein.[25]

## 2.5 | BGLF4 and BGLF5

The lytic E genes, *BGLF4* and *BGLF5*, encode a Ser/Thr kinase and an alkaline DNase, respectively. Recent studies indicate that both BGLF4 and BGLF5 induce the DNA damage signal that eventually leads to genomic instability, which consequently contributes to the carcinogenesis of human epithelial cells.[26,27]

## 2.6 | BALF3

BALF3 is a homologue of the enzyme, terminase, which is involved in viral DNA synthesis and packaging.[28] Chiu et al[5] provided evidence showing that BALF3 contributes effectively to the induction of genomic instability and mediates NPC relapse by enhancing the expression of the oncogenes, *EVI1*, *FIGF*, *SOX2* and *TP63*.

## 2.7 | BCRF1

BCRF1 is a homologue of cellular IL-10. Recently, Han et al[29] provided evidence showing that the distribution of the vIL-10 variant types is tumor-specific and is found in lymphoma, NPC, and EBV-associated gastric carcinoma (EBVaGC), implying a pathogenic role in these diseases.

## 2.8 | BILF1

BILF1 is an EBV-encoded constitutively active G protein-coupled receptor (GPCR) that modulates various intracellular signaling pathways, including CRE (cAMP response element) and nuclear factor kappa B (NF-κB), through $G\alpha_i$.[30] Lyngaa et al[31] suggested that BILF1 can transform cells and induce VEGF secretion in a constitutively active way. Moreover, BILF1 promotes tumor formation in 90% of cases in nude mice, suggesting that BILF1 could be involved in the pathogenesis of EBV-associated malignancies.[31]

# 3 | INHIBITORY EFFECTS OF SMALL MOLECULES ON EBV LYTIC REPLICATION

By inhibiting the EBV lytic cycle, anti-EBV agents may block the transmission of the virus from cell to cell and are therefore valuable in chemoprevention or clinical treatment of EBV-associated malignancies. This leads to the search for new compounds that may prevent EBV lytic replication. Until now, many small molecules have been identified to effectively suppress EBV lytic replication. Earlier studies demonstrated that nucleoside analogs can inhibit EBV lytic replication.[32] Daigle et al recently reported that EBV reactivation can be blocked by valproic acid (VPA)[33] and its amide derivative valpromide (VPM)[34] in B lymphoma cells. The inhibitory effect of the multifunction drug maribavir (MBV) on EBV lytic replication is produced mainly by attenuating viral DNA replication as well as by suppressing EBV lytic gene expression.[35] Rapamycin, a specific inhibitor of mTOR activity, was reported to decrease EBV lytic gene expression in B cell lines, but not in epithelial cell lines.[36]

In contrast to chemicals, compounds from natural sources are more attractive for inhibiting the EBV lytic cycle. Before 2000, only genistein, found in soy, was reported to downregulate Zta expression in Akata cells.[37] Currently, a wide variety of active phytochemicals or dietary ingredients have been identified as inhibitors of the EBV lytic cycle (Table 2).[7,37-50]

**TABLE 2** Anti-EBV reagents based on natural products

| Natural compounds family | | Mechanisms of anti-EBV action | References |
| --- | --- | --- | --- |
| Polyphenolics | EGCG | Inhibits the MEK/ERK1/2 and PI3-K/Akt pathways | 7, 38 |
| | Curcumin | Inhibits *BZLF1* gene transcription | 39 |
| | Resveratrol | Suppresses the activation of the redox-sensitive transcription factors NF-κB and AP-1 | 40 |
| | Sulforaphane | Reduces the transactivation activity of the *BRLF1* gene | 41 |
| Flavonoids | Genistein | Blocks the activation of IgG-mediated tyrosine kinase | 37 |
| | Protoapigenone | Reduces the transcriptional function of Zta | 43 |
| | Luteolin | Represses the promoter activities of the *BZLF1* and *BRLF1* genes | 44 |
| Terpenoids | Andrographolide | Inhibits Zta and Rta expression | 45 |
| | Moronic acid | Suppresses the transactivation function of Rta | 46 |
| Lignans | Emodin | Inhibits EBV IE protein expression and DNA replication | 47 |
| Saponins | Glycyrrhizic acid | Inhibits EBV IE protein expression and DNA replication | 48 |
| Adenosine analogues | Cordycepin | Suppresses EBV lytic replication | 49 |
| Antioxidants | Vitamin C | Reduces EBV EA IgG and VCA IgM antibody levels | 50 |

AP-1, activator protein 1; EA, early antigen; EBV, Epstein-Barr virus; EGCG, epigallocatechin gallate; IE, immediate early; NF-κB, nuclear factor kappa B; VCA, viral capsid antigen.

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## 3.1 | Polyphenolics

Chang et al[38] first reported the inhibitory effect of epigallocatechin gallate (EGCG) on the EBV lytic cycle. Using NPC and lymphoma as models, our group showed that the inhibition of EBV spontaneous lytic replication by EGCG involves suppression of the MEK/ERK1/2 and PI3-K/Akt pathways.[7] Curcumin, the main yellow bioactive component of turmeric, suppresses *BZLF1* gene transcription.[39] Resveratrol (RV) was shown to inhibit EBV lytic gene expression and production of viral particles by suppressing the activation of redox-sensitive transcription factors NF-κB and activator protein 1(AP-1).[40] At the concentrations used for inhibition of EBV reactivation, RV inhibits the proliferation of BL cells without increasing cell death.[40]

The histone deacetylase (HDAC) inhibitor sulforaphane (SFN) has the potential to be consumed as a dietary compound for prevention of EBV reactivation by reducing the transcription of the *BRLF1* gene.[41]

## 3.2 | Flavonoids

Koyama et al[42] first found a correlation between the antioxidant potential of flavonoids and inhibition of EBV lytic replication. Protoapigenone reduces the ability of Zta but not Rta to activate transcription from lytic promoters in B lymphoma cells.[43] A recent study indicated that luteolin could inhibit EBV reactivation by decreasing the promoter activities of both *BZLF1* and *BRLF1* genes, and thus repress NPC tumorigenesis in a mouse model.[44]
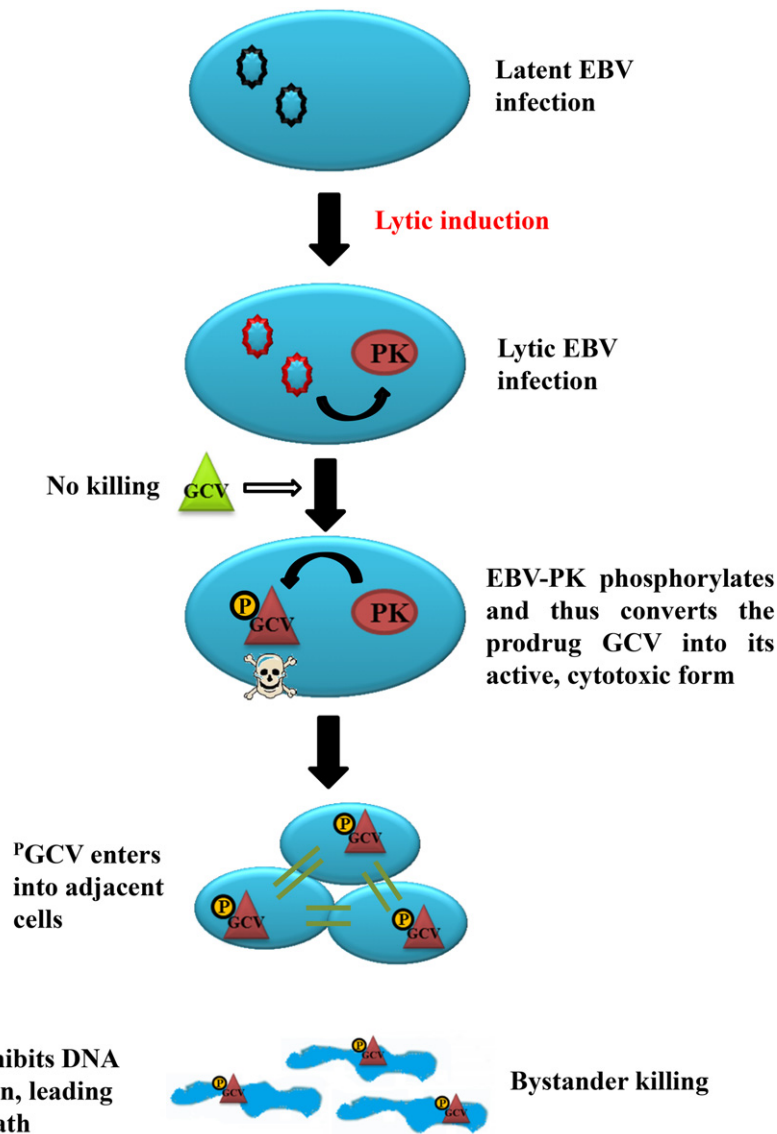


**FIGURE 1** Lytic-inducing therapy for Epstein-Barr virus (EBV)-positive tumors. Upon reactivation by lytic-inducing agents, EBV enters into the lytic form of infection. During the lytic cycle, EBV expresses EBV-PK, a Ser/Thr protein kinase, which phosphorylates and thus converts the prodrug, ganciclovir (GCV), into its active, cytotoxic form in EBV-infected cells. Phosphorylated GCV (^PGCV) interferes with host DNA polymerase, leading to early termination of DNA replication and thus cell death. Phosphorylated GCV can be transferred to adjacent cells, thus inducing "bystander" killing. The intentional induction of the EBV lytic cycle can enhance GCV-induced cytotoxicity, which has been developed as a novel therapy against EBV-positive tumors

## 3.3 | Terpenoids

Andrographolide is extracted from *Andrographis paniculata*. Lin and colleagues reported that andrographolide effectively inhibited EBV lytic replication by reducing the expression of Zta and Rta in P3HR1 cells.[45] Moronic acid, found in the galls of *Rhus chinensis* and *Brazilian propolis*, suppresses the ability of Rta but not Zta to transactivate lytic early promoters.[46]

## 3.4 | Lignans and saponins

Lignans such as emodin[47] and saponins such as glycyrrhizic acid[48] were also reported to inhibit the expression of EBV IE proteins and EBV DNA replication.

Additionally, cordyceps has been shown to be a novel chemical suppressor of EBV lytic reactivation.[49] A recent clinical study suggests that high-dose vitamin C results in the reduction of EBV EA IgG and VCA IgM antibody levels in patients with EBV infection.[50]

## 4 | LYTIC-INDUCTION THERAPY FOR EBV-POSITIVE TUMORS

It is already well established that expression of the herpes simplex virus thymidine kinase gene allows tumor cells to be killed by ganciclovir (GCV).[51] During the lytic phase of infection, EBV expresses 2 specific kinases, thymidine kinase (TK) and protein kinase (PK). EBV-PK rather than EBV-TK phosphorylates and converts the prodrug GCV into its active, cytotoxic form in EBV-infected cells.[52] Active GCV interferes with the viral and host DNA polymerase to lead to early termination of DNA replication and cell death of tumor cells, which enables exposure of EBV to host immune surveillance. Also, phosphorylated GCV can be transferred to adjacent cells, thus inducing "bystander" killing.[53] Thus, the intentional induction of the EBV lytic cycle in EBV-positive tumor cells may be developed as a novel therapy for EBV-related tumors (Figure 1).

For this purpose, searching for agents that induce EBV reactivation seems imperative. Diverse small molecules have been identified as stimulators of EBV lytic replication (Table 3). For example, TPA, sodium butyrate (SB),[54] trichostatin A (TSA),[55] and 5-aza-2′-deoxycytidine (5-AZA)[56] serve as common agents to induce EBV lytic gene expression. TPA, an agonist of protein kinase C (PKC), ultimately promotes activation of transcription factors of *BZLF1* and *BRLF1* genes.[57] Epigenetic inhibitors such as SB, TSA, and 5-AZA can open chromatin at promoters of both genes, accounting for the effective and widely used strategy to induce the EBV lytic cycle by TPA in combination with the epigenetic inhibitors. Recent studies have shown that various specific anti-tumor agents induce EBV lytic replication and, by doing so, sensitize cancer cells to nucleoside antiviral agents. Suberoylanilide hydroxamic acid (SAHA), a HDAC inhibitor, promotes EBV reactivation by disrupting *BZLF1* and *BRLF1* gene silencing to facilitate the access of transcription factors, and the addition of GCV enhances the ability to kill EBV-positive lymphoma cells in vitro and in lymphoma-bearing nude mice.[58,59] A series of apoptotic inducers, including gemcitabine, doxorubicin, etoposide, and icaritin, have been reported to induce EBV reactivation. Both gemcitabine and doxorubicin activate transcription from promoters of the *BZLF1* and *BRLF1* genes, and the effects require the early growth response protein 1 (EGR-1) motif in the *BRLF1* promoter and the CRE (ZII) and myocyte enhancer factor 2D (MEF-2D) (ZI) binding sites in the *BZLF1* promoter.[60,61] The p38 MAPK, PI3K, and MAPK/ERK kinase (MEK) signaling pathways are also involved in the

**TABLE 3** Agonists of the EBV lytic cycle

| Agonists category | | Mechanisms of lytic-inducing action | References |
|---|---|---|---|
| Epigenetic inhibitors | SB, TSA, and 5-AZA | Opening chromatin at promoters of the BZLF1 and BRLF1 genes | 54–56 |
| | SAHA | Promoting EBV reactivation by disrupting *BZLF1* and *BRLF1* gene silencing | 58, 59 |
| Phorbol esters | TPA | An agonist of PKC, ultimately promoting activation of transcription factors of *BZLF1* and *BRLF1* genes | 57 |
| Apoptotic inducers | Gemcitabine and doxorubicin | Activating transcription of the *BZLF1* and *BRLF1* genes by upregulating expression of EGR-1, CRE, and MEF-2D | 60, 61 |
| | Etoposide | Inducing EBV reactivation | 61 |
| | Icaritin | Inducing the expression of EBV lytic genes by inhibiting the LMP1-mediated STAT3 and Akt pathways | 62 |
| | Rituximab and dexamethasone | Synergistic induction of Zta and EA-D expressions | 63 |
| Anti-inflammatory agent | Aspirin | Upregulating lytic gene expression by suppressing the activity of NF-κB, a corepressor of Zta | 64 |
| Tetrahydrocarboline derivatives | C09, C50, C53, C60, and C67 | Reactivating EBV lytic markers Zta and EA-D in all EBV-positive cell lines | 65 |
| Small organic compounds | E7, E11, C7, C8, and A10 | Induction of lytic proteins involves activation of PKCδ or/and JNK MAPK | 66 |

5-AZA, 5-aza-2′-deoxycytidine; CRE, cAMP response element; EBV, Epstein-Barr virus; EGR-1, early growth response protein 1; MEF-2D, myocyte enhancer factor 2D; NF-κB, nuclear factor kappa B; PKC, protein kinase C; SAHA, suberoylanilide hydroxamic acid; SB, sodium butyrate; TSA, trichostatin A.

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induction of EBV lytic replication. It has been demonstrated that eto-poside causes EBV reactivation in Akata cells.[61] Icaritin induces the expression of EBV lytic genes by inhibiting the LMP1-mediated STAT3 and Akt pathways.[62] Rituximab and dexamethasone synergistically induce lytic EBV infection, rendering EBV-positive lymphoma cells more susceptible to GCV cytotoxicity in vitro and in vivo.[63] Aspirin also plays an important role in inducing high levels of EBV lytic replication. Results from Liu et al[64] showed that aspirin upregulates lytic gene expression by suppressing the activity of NF-κB, a corepressor of Zta. They also showed that treatment with aspirin in combination with GCV amplifies the cytotoxic effect in B95.8 and Raji cells. From 66 840 small molecule compounds, Tikhmyanova and colleagues recently identified 5 tetrahydrocarboline derivatives, referred to as C09, C50, C53, C60, and C67, that efficiently reactivate latent EBV in a variety of cell types. Importantly, these 5 compounds show promise for lytic therapy in combination with GCV.[65] Choi et al[66] carried out high-throughput screening of a chemical library of 50 240 small organic compounds to identify the strongest inducers of the EBV lytic cycle in EBV-positive epithelial malignancies.

## 5 | CONCLUSIONS

Because EBV is a common human tumor virus, understanding the function of EBV-encoded products such as lytic proteins in EBV-associated malignancies is clearly essential for determining the role of viral lytic infection in carcinogenesis. Hence, the presence of EBV infection in malignancies allows the development of lytic cycle-targeted therapeutic strategies. Anti-EBV compounds, especially natural compounds, have been developed to be useful for the prevention of EBV-induced carcinogenesis. Moreover, intentional induction of lytic replication combined with prodrug treatment, such as GCV, has been regarded as a novel strategy for the treatment of EBV-positive tumors. Notably, recurrent EBV reactivation through deregulated expression of Zta or Rta might also be a potential therapy against EBV-related tumors. These strategies show great potential in the prevention and/or treatment of EBV-associated malignancies in the future.

### CONFLICT OF INTEREST

Authors declare no conflicts of interest for this article.

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