Medicinal chemistry

Agents and Approaches for Lytic Induction Therapy of Epstein-Barr Virus Associated Malignancies

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Abstract

Epstein-Barr virus (EBV) is causally associated with multiple cancers of epithelial and lymphoid origin. Generally, EBV establishes a lifelong quiescent homeostasis (latency) exerting its immune modulating and oncogenic properties only under certain conditions. Lytic replicative EBV infection is restricted to differentiated oropharyngeal epithelia and plasma B-cells. In EBV-associated tumor cells viral gene expression is limited to defined latency programs, without overt virus "lytic" replication. Artificial triggering of EBV lytic replication with chemical agents (drugs) forms the basis of cytolytic virus activation (CLVA) therapy currently under clinical investigation for treating EBV-positive malignancies. Reactivation from latency requires expression of viral transactivator proteins BZLF1 (Zta) and BRLF1 (Rta). In tumor cells, the BZLF1 promoter (Zp) is highly methylated and generally inactive. However, Zp can be triggered into a self-enhancing activation loop in response to chemical or biological agents, including cytostatic drugs and epigenetic modifiers. This process is orchestrated by Zp-binding cellular transcription factors, such as myocyte enhancer factor 2 (MEF2), specificity protein 1 (SP1), and zinc finger E-box binding homeobox (ZEB) proteins. Understanding the mechanism of lytic induction by chemical inducers is essential for developing effective therapies. This review provide a short overview of EBV biology and will focus on branded and novel agents (drugs) used for EBV lytic induction and discuss molecular mechanisms by which the EBV lytic switch is triggered and controlled.

Introduction

Epstein Barr virus (EBV) is a gamma herpes virus that is ubiquitous in humans, infecting more than 90% of the world population [1]. The most common disease manifestation is infectious mononucleosis (IM), also known as glandular fever or kissing disease, a self-limiting inflammatory illness associating with primary infection. Importantly, besides it's link with several chronic and autoimmune diseases which will not to be discussed here, EBV is associated with a variety of epithelial and lymphoid malignancies, including undifferentiated nasopharyngeal carcinoma (NPC), 10% of all gastric carcinomas (GC) worldwide, endemic Burkitt lymphoma (BL), 50-90% of classic Hodgkin's Disease, various T-/NK-/B-cell non-Hodgkin lymphoma's and B-cell lymphoma's in immune suppressed individuals [1]. The virus exists in the host in either a reproductive lytic or a quiescent latent state. During primary infection, EBV infects and transforms naive B cells into rapidly growing B-cell blasts, which are effectively eliminated by strong anti-EBV T-cell immune responses [2-4]. This strong early-stage T-cell response, comprising both CD4+ and CD8+ T-cells, is directed against viral peptide epitopes particularly of the lytic proteins of EBV [4,5]. The associating cytokine storm causes the symptoms characteristic of IM [2,4,5].

Following primary infection, a lifelong dynamic balance is maintained between sporadic proliferation of virus-immortalized B cells and the controlling cytotoxic T cell response, which is highly effective in eliminating proliferating EBV transformed B-blasts [4,5]. In EBV positive cancers viral gene expression is limited to only a few latency-associated gene products which actively contribute to tumor cell growth, apoptosis resistance and immune escape [1,6].

EBV Life Cycles

EBV persists for life in a low number metabolically quiescent memory-B-cells as a methylated and transcriptionally silenced nuclear dsDNA episome of 184 kilobases, expressing only non-coding small and microRNAs and being invisible to immune responses (Latency-0, also named true latency; Figure 1, left side) [1,6]. Dividing memory-B-cells may express a single EBV encoded protein, EBNA1, essential for maintenance of the latent viral genome in dividing cells (Latency-I). Some EBV+ B-cells may proliferate upon passage through lymphoid tissues, expressing two additional latent membrane proteins LMP1 and LMP2, which are barely immunogenic (Latency-II or Default Program). The full expression of EBV latent genes (Latency-III) is only seen during primary infection of B-cells, and sporadically in immunosuppressed individuals. Occasionally, antigenic triggering of an EBV carrying memory B-cell into antibody-producing plasma cell will reactivate the virus from latency into lytic reproduction (Figure 1, right side), a process that mainly occurs at oro-nasopharyngeal lymphoid tissues such as the tonsils, which are considered the homing site of most EBV carrying B-cells [6]. Virus from B-cells may transfer into submucosal epithelial cells [7-9], leading to abundant virus production and shedding [10]. Although EBV epithelial latency has been demonstrated in primary tonsil explant cultures [11], direct evidence of EBV latency in normal epithelia is rare [12]. In immunocompromised individuals, a lytic infection can persist manifesting as oral hairy leukoplakia (OHL) and uncontrolled proliferation of latent EBV-infected B cells can lead to the development of malignant lymphoma, illustrating the importance of immune control over both arms of the viral life cycle [13].
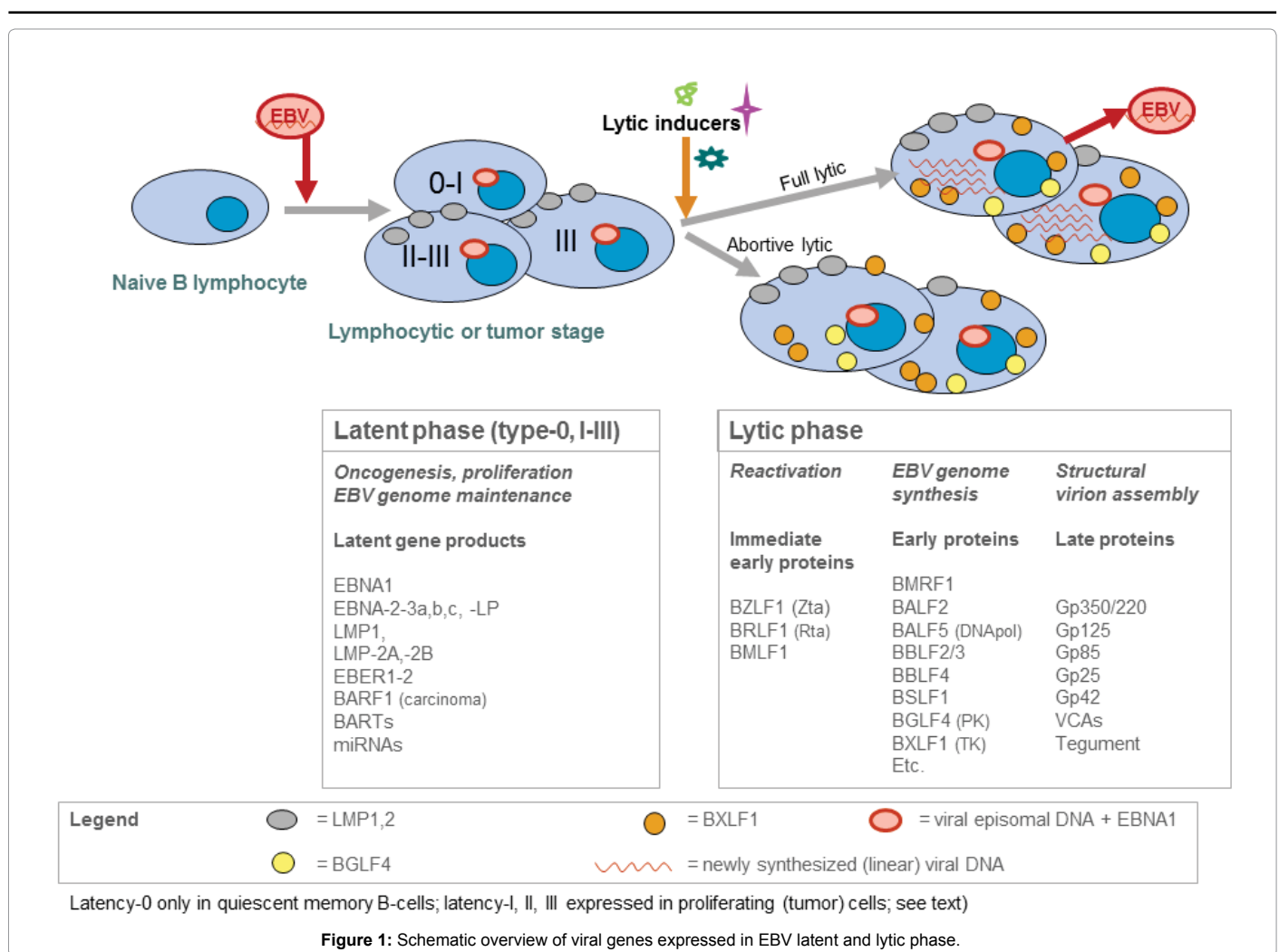
Besides infecting B cells and causing lymphoproliferative disorders,

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Figure 1: Schematic overview of viral genes expressed in EBV latent and lytic phase.

EBV has the ability to infect epithelial cells by cell-to-cell contact which can lead to epithelial malignancies such as NPC and GC, where all cells harbour the virus, making it a valuable target for therapy [14]. The majority of tumour cells contain the virus in its latent form, producing only a few proteins necessary for EBV genome maintenance and tumour growth [15]. These latent proteins are low in immunogenicity and therefore difficult to be recognized by the immune system. This allows EBV-carrying tumour cells to successfully evade the host immune system.

EBV Latency Gene Products and Functions

The roles of EBV latent genes are summarized in recent reviews [15-17]. Via EBNA-1, the circular viral DNA episome is directly bound to host chromosomes ensuring reliable duplication by host DNA polymerases and enabling maintenance of viral genome in dividing cells. EBNA-1, EBNA-LPs, EBNA-2, EBNA-3A, EBNA-3C and LMP1 are individually essential for *in vitro* transformation of primary B cells to lymphoblastoid cell lines [1,6,16,17]. The "higher EBNAs" (i.e., all EBNAs besides EBNA1) are involved in initial B-cell immortalization, but otherwise not expressed in human malignancies, with exception of lymphoproliferative diseases in severely immunosuppressed patients.

The key EBV oncogene, LMP1, is interacting with several host adapter proteins triggering multiple cellular signaling pathways such as NF-kB, JNK, p38 and JAK/STAT, P53/ATM. This is mediated by at least three distinct signal activating domains in C-terminal sequence

of LMP1 (CTAR1-3) [18]. For example, in B-cells LMP1 is mimicking T-cell stimulatory signaling by CD40 mediated NF-kB activation and signal transduction pathway through interaction with TNFR-associated factors (TRAF's), especially TRAF1 and TRAF3 and TNFR-associated death domain protein (TRADD), preventing apoptosis and enhancing cell growth and oncogenic transformation [18-20]. The activated NF-κB translocates from cytoplasm to the nucleus and regulates target genes essential for cell proliferation and apoptosis resistance. When Janus kinase 3 (JAK3) binds to C-terminal domain of LMP1, it activates signal transducer activator of transcription (STAT) to upregulate transcription [21]. Furthermore, LMP1 activates the PI3-K /Akt pathway resulting enhanced cell survival and remodeling actin filament [22]. Importantly, LMP1 induces selective host DNA methylation, thereby limiting the expression of tumor suppressor genes and pro-apoptotic signaling and modulating "danger signaling" in general [23]. In order to limit oncogenic signaling, LMP1 rapidly associates with CD63 to be incorporated into intraluminal vesicles, where LMP1 loses its signaling capacity and is prepared for secretion in exosomes [24]. LMP2A mimics antigen-independent B-cell receptor (BCR) signaling by constitutive binding of Syk/Lyn and activation of the ERK/MAPK pathway thus contributing to cell survival [25-27].

EBV Lytic Reactivation

Under certain circumstances the latent virus can be reactivated, changing from its dormant non-replicating stage into its lytic phase

which ultimately results in production of new infectious virions. The 3-step lytic cascade starts with activating the expression of immediate early (IE) transactivator proteins encoded in BZLF1 (ZEBRA or Zta) and BRLF1 (Rta) (Figure 1, middle) [28]. Many cellular and viral proteins modulate the induction and function of ZEBRA and Rta in disruption of latency and this is cell type dependent, as will be discussed later in more detail. The BZLF1 gene encodes a sequence specific DNA-binding protein (ZEBRA), which is a member of the bZIP family of leucine-zipper transactivators. The BZLF1 promoter (Zp) is a 233 bp long sequence on the EBV genome which generally is heavily methylated and transcriptionally silenced in the viral latent state. Its activity is controlled by the balance between (host) (de-) methylating and (de-)acetylating enzyme activities regulating access and activities of cellular transcription factors on Zp. Murata et al. summarized cis- and *trans*-elements playing a role in Zp regulation and the balanced interaction between positive and negative regulators that governs the switch from latent to lytic phase (Figure 2) [28]. For Zta expression and activity the methylation of EBV DNA is required [29-32]. ZEBRA can bind to at least 32 variants of ZEBRA-responsive elements (ZRE's) present in the Zp and in promoters of EBV early genes [33,34]. Thus, ZEBRA together with endogenous host cell specific cellular transcription factors activate Zp in an auto-stimulatory loop [35]. The BZLF1 gene can also be activated by the BRLF1 encoded transactivator Rta. Reversely, ZEBRA drives expression of Rta (and Na - another viral protein encoded by BRRF1), which results in Zta-Rta-BRRF1 synergy forming a positive feedback loop to activate Zp [36-38]. The BMLF1 gene product (EB2 or I'ta) is another IE protein, which is involved in nuclear export and cytoplasmic stabilization of non-spliced RNA transcripts. It increases the level of viral and cellular protein(s) required for stable Zta expression [39].

Once induced, these IE products cause cell-cycle arrest in G1 phase and cooperate to induce the second step in the reactivation cascade, i.e., the early (E) phase [35,40,41], ultimately inducing the entire lytic cascade activation and virus replication (Figure 1, right side) [42]. It has been demonstrated that in some EBV-associated malignancies, sporadic tumor cells show detectable ZEBRA expression. Since true late lytic gene transcription in EBV associated tumor cells is mostly absent, these low levels of ZEBRA expression probably reflect partial (abortive) activation of the lytic phase.

During reactivation a plethora of viral gene products is expressed, starting with early gene products, such as EBV-DNA binding proteins, helicase (together forming the complex with early antigen diffuse protein EA-D), thymidine kinase (TK), protein kinase (PK) and other enzymes involved in nucleotide metabolism, all preparing the host cell for viral DNA replication by the viral DNA-polymerase (DNApol; BALF5) [28,41]. In the third phase of viral reactivation,

and closely related to synthesis of new non-methylated viral DNA templates, the late structural proteins are expressed that travel into the nucleus to incorporate newly synthesized linear viral DNA into stable capsid structures. These are subsequently released into the cytoplasm to acquire an envelope by budding into Golgi membranes and forming new mature infectious EBV virions that are secreted into the extracellular space. The newly synthesized viral genome is linear and epigenetically unmodified. The lytic phase is essential part of EBV life cycle, since the production of infectious viral particles that allows the virus to be transmitted from cell to cell and host to host.

Molecular Triggers for EBV Reactivation

Although in EBV-associated malignancies as well as in infected memory B cells of healthy carriers latency is the mainstream of EBV infection, there are indications that bursts of spontaneous (recurrent) reactivation in fact may play a role in EBV persistence and pathogenesis [41]. Distinct cellular differentiation factors regulate spontaneous EBV reactivation and this is highly cell-type dependent [43]. Activation pathways will be detailed later in this review. BLIMP1 is one of the key players in both epithelial and B-cell differentiation and was recently shown to facilitate EBV reactivation from latency in both cell backgrounds [44]. Together with BLIMP1, another differentiation-dependent cellular transcription factor, Kruppel-like factor4 (KLF4) was recently reported to play a critical role in epithelial cells [45]. Modifications of EBV genome, such as 5-hydroxymethylation of cytosine (important in epithelial-cell differentiation) may also affect EBV lytic reactivation and is regulated by cellular TET enzymes [46]. Expression of virus maintenance factor EBNA1 inhibits spontaneous reactivation, but facilitates lytic cycle after initial reactivation in gastric carcinoma cells AGS-BX1 [47]. Serological studies in NPC-risk populations suggest that EBV reactivation occurs a long time before clinical diagnosis of NPC with increasing EBV-specific IgA antibody responses paralleling NPC tumor development [48-50]. It has been reported that certain nutritional habits and/or environmental risk factors may have substantial impact on NPC development [51-54]. A recent study demonstrated increased tumorogenicity after N-nitroso compounds (commonly found in food in high-risk NPC regions) induced recurrent EBV reactivation in NPC cells and mouse model [55]. In addition, regular consumption of rancid fat and certain spicy food components have been linked to NPC risk [56].

Throughout the years, multiple agents have been described to trigger EBV lytic induction, such as radiation and different chemotherapeutical agents - alone or in combination with histone deacetylase inhibitors HDACi-s [57,58]. Proteins produced in this phase (viral neo-antigens) are highly immunogenic and can be recognised and targeted by the immune system [4,5]. Furthermore, the reactivation of the EBV lytic

The horizontal orientated rectangles indicate the *cis*-acting regulatory elements. The vertical orientated ovals indicate their *trans*-acting factors. ZEB1 binds via its two zinc-finger domains to ZV and ZV'. Image based on the figure of Yu et al, PLOS Path 2007.
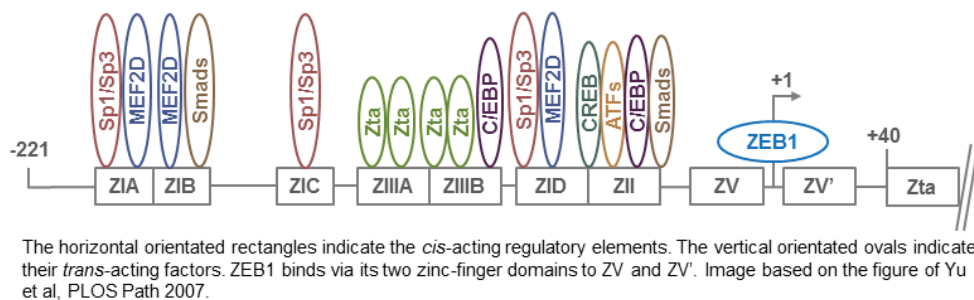
Figure 2: Schematic close-up of the regulatory elements within the Zebra promoter (Zp).

cascade leads to the expression of viral enzymes (thymidine and protein kinases, TK and PK respectively) that may sensitize tumour cells to antiviral treatment [35]. These findings have led to the development of novel therapeutic approaches aiming at EBV reactivation.

Therapeutic Options Exploiting the EBV Lytic Cycle

New strategies are emerging for virus-targeted therapy to treat EBV-associated malignancies, generally consisting of two components. The first step is aimed at initiating virus reactivation and triggering recognition of the tumour cells by the immune system [originally described by Moolten in 1986] [59]. The second step is the administration of an antiviral drug which can be metabolised exclusively in cells expressing viral lytic cycle proteins [60].

In previous studies, variable combinations of drugs/reagents were tested on different EBV-driven tumour cell lines *in vitro* and *in vivo* to investigate the most potent effect on reactivating the latent viral genome into lytic gene expression. For example, plasmids encoding immediate early lytic genes were transfected in a truly latent EBV-infected Burkitt Lymphoma (BL) cell line. After expression of these lytic proteins an antiviral compound was administered, which resulted in a decreased growth of cells [60]. Activation of Ganciclovir (GCV) requires conversion to monophosphate by EBV-encoded kinases, mainly BXLF1 encoded TK and BGLF4 encoded PK [61], followed by two additional phosphorylations by host cell enzymes and formation of cytotoxic triphosphate form which is incorporated in the growing DNA strand causing a "DNA chain termination". Therefore, only lytically infected but not latently infected EBV-positive tumor cells are sensitive to the cytotoxic effects of GCV [62]. Chemotherapy combined with HDACi-s reduced the growth of EBV-positive tumours in a mouse lymphoma model [63]. This effect is amplified by the addition of an antiviral drug [64]. We and others have studied EBV reactivation in the naturally infected NPC epithelial cell line C666.1 and observed synergistic cytotoxic effects of the combination of gemcitabine (GCb), valproic acid (VPA) for inducing viral reactivation and (val)Ganciclovir as antiviral compound [65-67]. This Cytolytic Virus Activation Therapy (CLVA) for EBV-associated malignancies was subsequently used with promising results in three Dutch NPC patients [65]. A recent continuation of this Phase-I/-II study on eight end-stage NPC patients showed that drug combination was well tolerated and induced clinical responses in patients naïve to GCb [68].

In this review effective (combinations of) EBV lytic inducers are presented. The chemical structure of compounds used as virus lytic inducers are shown in (Supplementary) Figure 3a-c. Effectiveness was demonstrated by various techniques (presented in Tables 1-4) including detection of EBV lytic protein and mRNA expression, as well as functional characteristics such as apoptosis induction and sensitivity to antiviral treatment. The mechanism of action of the various components is indicated and the consequence of adding an antiviral drug is also described. This systematic overview focuses on efficacy of compounds for viral reactivation in various *in vitro* models. Highlighted are DNA damage responses and host-susceptibility factors. Recently more clinically oriented overviews on EBV targeted therapeutic strategies were published [69,70].

Pharmacological Inducers of EBV Lytic Phase

Histone deacetylase inhibitors

Histone deacetylase inhibitors (HDACi-s) represent a well-known group of drugs that modify the acetylation status of several cellular (and viral) proteins. According to their chemical structure they can be categorized into different classes, including short chain fatty acids,

hydroxamates, benzamides, cyclic tetrapeptides and epoxides [71]. These compounds modulate chromatin structure via inhibition of histone acetylation, but also target several non-histone substrates. HDACi-s are structurally diverse compounds with biological effects ranging from cell death induction, differentiation, angiogenesis inhibition to modulation of immune response. In general, HDACi-s have been shown to have potent anticancer activities including reversing aberrant epigenetic changes associated with cancer [72,73]. Several combination strategies vis-à-vis with HDACi-s have been tested and validated in preclinical studies, either as chemo sensitizing agents in standard chemotherapy regime or in combination with DNA methyltransferase inhibitors (DNMTi-s) in the context of "epigenetic therapies" aiming to revert epigenetic alterations found in cancer cells. Pan-HDACi-s, which inhibit 11 HDAC isoforms, are widely used to induce EBV lytic cycle. Here we discuss EBV-specific lytic induction capacities of commonly used HDACi-s (VPA, NaB, TSA and SAHA) and results are summarized in Table 1. HDAC-inhibition does not necessarily lead to virus reactivation in all EBV-positive cell lines, as the EBV reactivation process by chromatin modulation is cell type dependent. Chemically-triggered expression of viral transactivator (Zta/Rta) induces chromatin remodelling at target promoters, leading to restructuring of nucleosomes and loss of polycomb-repression which initiates the EBV lytic cascade [74]. Thus, HDACi-s can selectively modulate viral gene expression by influencing the accessibility of enhancer elements in viral promotors for host transcription factors, see Figure 2. The level and presence of these regulatory elements and host HDACi and DNMT activities all influence lytic induction response and are largely cell type specific.

Valproic acid

VPA, 2-propylpetanoic acid, sodium valproate is a short-branched-chain fatty acid that has been widely used in the clinic as anticonvulsant and mood stabilizer for decades. Currently, there are numerous VPA-based clinical trials for treatment of a wide range of malignancies. Although other mechanisms may also contribute to VPA-induced anti-cancer effects, the inhibition of histone deacetylases (HDACs) appears to play a central role. VPA is a class I and II HDACi with preference for class I inhibition, but it has also been reported as inducer of DNA demethylation in non-dividing cells [75-77]. Due to its HDACi-property, VPA treatment can induce lytic viral gene expression in some EBV positive tumor models and this effect appears to be dose and time-dependent. VPA itself only weakly activates lytic EBV gene expression, but it strongly enhances the ability of chemotherapeutic agents (such as 5-FU and gemcitabine) to reactivate EBV in both epithelial and lymphoid tumour cells [63,65]. VPA may promote lytic induction by increasing the acetylation state of the chromatin surrounding the episomal viral genome, thus providing opening of the genome for access by cellular transcription factors and by epigenetic reprogramming via demethylation of host regulatory gene promoters [63,76,78,79]. However opening of chromatin structure is not equally effective for EBV lytic reactivation in different cell backgrounds. *In vitro* studies showed the ability of VPA to regulate epigenetic modifications of the EBV genome and chromatin modulating proteins in breast cancer cells [80]. VPA alone is not sufficient to induce lytic phase in truly latent HH514 BL cells, despite inducing hyperacetylation of H3 in contrast to TSA and AZC and we found the same for C666.1 NPC cells [65,79].

Iwata et al. analyzed EBV-positive T and NK cell lines for expression of lytic genes encoding Zta and gp350/220, after exposure to VPA for 24 and 48 hours [78]. RT-PCR showed no significant difference between VPA-treated cells and controls. Although VPA induced apoptosis and cell cycle arrest, it did not trigger a lytic switch. Since the killing effect of

Drug	Cell line (tumor origin)	Assays	Detected proteins	+GCV?	Result on lytic induction	(Optimal) drug concentration	Exposure time	Ref.
VPA	AGS-BX1 (GC)	WB	BZLF1, BRLF1, BMRF1	No	+	1-10 mmol/L	48 h	[84]
		IF	BZLF1	No	+	1-10 mmol/L	48 h	[84]
VPA+5-FU	C666.1 (NPC); AGS-EBV (GC)	WB	BZLF1, BRLF1, BMRF1	No	+	0.3 mmol/L VPA, 1 µg/mL 5-FU	48 h	[63]
VPA+CDDP	AGS-EBV (GC)	IF	BMFR1	No	+	0.3 mmol/L VPA, 0.25 µg/mL CDDP	48 h	[63]
NaB	AGS-BX1(GC)	WB	BZLF1, BRLF1, BMRF1	No	+	0.5-3 mmol/L	48 h	[84]
		IF	BZLF1	No	+	1.5 mmol/L	48 h	[84]
	AGS-Akata (GC)	WB	BZLF1, BRLF1, BMRF1	No	+	3 mmol/L	48 h	[123]
NaB/TSA/TPA	HH514-16 (BL)	NB	BZLF1, BRLF1	No	+ +	3 mmol/L NaB, 5 µmol/L TSA	8 h	[85]
	B95-8 (LCL)	NB	BZLF1, BRLF1	No	+ TPA, - NaB	3 mmol/L NaB, 20 ng/mL TPA	8h	[85]
	BC-1 (EBV/KSHV LCL)	NB	BZLF1, BRLF1	No	+ TPA, - NaB	3 mmol/L NaB, 20 ng/mL TPA	8 h	[85]
	BC-1 (EBV/KSHV LCL); HH514-16 (BL)	qRT-PCR	BRLF1	No	+	5 µmol/L TSA	8 h;9 h	[85]
	BC-1 (EBV/KSHV LCL)	WB	BRLF1	No	+ TPA, +/- NaB	3 mmol/L NaB, 20 ng/mL TPA	24 h	[85]
NaB/TPA	AGS-BX1 (GC)	WB	BZLF1, BRFL1, BMRF1	No	+	2.5 mmol/L NaB, 20 ng/mL TPA	24 h	[182]
NaB + γ-irradiation	LCLs; EBV + BL (Jijoye, Akata); AGS-EBV (GC)	WB	BZRF1, BMLF1	No	+	2.5 mmol/L	48 h	[57]
TSA	SNU-1103 (LCL)	WB	BZLF1, BRLF1, BMRF1, BALF5, BBLF2/3	No	+	100-300 nmol/L	48 h	[83]
		IF	BZLF1, BMRF1, gp110, gp350	No	+	100-300 nmol/L	48 h	[83]
	AGS-BX1 (GC)	WB	BZLF1,BRLF1, BMRF1 BZLF1	No	+	100 nmol/L	48 h	[84]
		IF		No	+	100 nmol/L	48 h	[84]
	SNU-719 (GC)	WB	BZLF1, BRLF1, BMRF1	Yes (+)	+	0.5 or 5 nmol/L, 10 µg/mL GCV	6 d	[86]
SAHA	AGS-Akata (GC)	WB	BZLF1, BMRF1	No	+	5 µmol/L	48 h	[123]
	HK1-EBV (NPC); HA (NPC); HONE-EBV (NPC)	WB	BZLF1, BRLF1, BMRF1,gp350/220	No	+	2.5-20 µmol/L	48 h	[92]
	C666.1 (NPC)	WB	BZLF1, BRLF1, BMRF1	No	+	20-40 µmol/L	96 h	[92]
	HA (NPC)	IF	BZLF1, BRLF1, BMRF1,gp350/220	No	+	5 µmol/L	48 h	[92]
	HA (NPC)	FACS	BZLF1,BMRF1,gp350/220	No	+	5 µmol/L	2-3d	[92]
	AGS-BX1 (GC)	WB	BZLF1,BRLF1,BMRF1, VCA-p40,gp350/220	No +	+	0.5-10 µmol/L	48 h	[84]
	HONE-EBV (NPC)	IF	BZLF1,BMRF1,gp350/220	No	+	5 µmol/L	48 h	[84]
	AGS-BX1 (GC)	IF	BZLF1,BMRF1,gp350/220	No	+	10 µmol/L	48 h	[84]
	HA (NPC)	WB	BZLF1, BRLF1, BMRF1, gp350/220	No	+	5 µmol/L	24-48 h	[93]
	C666.1 (NPC)	WB	BZLF1, BRLF1, BMRF1, gp350/220	No	-	5 µmol/L	24- 48 h	[93]
SAHA+BTz	HA (NPC)	WB	BZLF1, BRLF1, BMRF1, gp350/220	No	+	5 µmol/L SAHA, 30 nmol/L BTz	24-48 h	[93]

The column "Result" displays the effect of the drug on lytic induction: an increased (+), inhibited (-) or no significant (0) effect. The column "+GCV" indicates whether the drug described is combined with GCV (Ganciclovir) or not. If a "Yes" is noted in this column; the subsequent symbol shows if the addition of GCV has a positive (+), negative (-) or no effect (0) on the amount of cell killing. WB=Western blot; NB=Northern blot; IF=Immunofluorescence.

Table 1: Summary of HDACi-s (alone or in combination with TPA/chemo or other stimuli) effects on EBV lytic induction in cancer cell lines of epithelial and lymphoid origin.

VPA was limited, the combination of VPA and BTz was tested, and an additive killing effect demonstrated. Subsequently, exposure of PBMCs from three patients with EBV-associated T or NK lymphoproliferative diseases to VPA was tested. As expected, VPA resulted in a higher killing of EBV-infected cells compared to uninfected cells, and the effect was increased when VPA was combined with BTz. Xu et al. and Nebbioso et al. summarized HDACi's used in clinical trials focused on mechanisms of HDACi-induced cell death [81,82].

Trichostatin A

TSA is a"classic" epigenetic modifier and antifungal antibiotic with cytostatic and differentiating properties. TSA treatment is causing (a dose-dependent) cell cycle arrest and apoptosis, and effectively inducing lytic replication (in EBV-transformed B cells) and epithelial cells [83-85]. Enhanced EBV lytic induction by TSA when combined with 5'-azacytidine and GCV in GC cell lines and in tumors originating from epithelial, NK and B cells, was confirmed by expression of BZLF1 and BRLF1 and accompanied by promoter demethylation [86,87]. Dose-dependent TSA induction of EBV lytic phase as well as cell cycle arrest and apoptosis was reported by Seo et al. in a latency type III EBV-transformed LCL cell line [83]. Nishikawa et al. reported increased synthesis of Zta and up-regulation of LMP1 protein expression by HDACi-s (NaB and TSA) in in vitro infected NPC cell line TWO3-EBV [88].

Suberoylanilide hydroxamic acid

(SAHA, Vorinostat, Zolinza) is a potent pan-HDACi, altering gene transcription and inducing cell cycle arrest, differentiation and apoptosis. It binds to the active site of HDAC-s and serves as a chelator for zinc ions, blocking the enzymatic activity. SAHA is very strong inducer of the EBV lytic phase and treatment does not require combination with chemotherapeutics. It is the first HDACi to be approved for clinical use in treating patients with malignancies (e.g., cutaneous T cell lymphoma) [89].

Hui et al. reported significant lytic induction in AGS-BX1 cells (GC, latency II) mediated by SAHA (μM), TSA (μM), NaB (mM) or VPA (mM), with SAHA being the most potent inducer, but found only weak effect at similar drug levels in AK2003 cells (BL, latency I) while no lytic induction was seen in LCLs (B-blast latency III) [84]. In these experiments, SAHA was also found to up-regulate expression of EBV-encoded LMP1. HDACi-s are known to induce LMP1 in lymphoma cells which is mediated via NF-κB activation as suggested by Park and Faller [90]. Treatment of EBV-positive BL cells with NaB-induced apoptosis via induction of the viral lytic program and resulted in an increase in NF-κB DNA binding [91]. Hui et al. further reported that SAHA treatment of various NPC cell lines (HK1-EBV, HONE1-EBV, HA and C666.1) induced Zta/Rta expression and demonstrated its strong pro-apoptotic effects, following early activation of the EBV lytic phase [92]. SAHA treatment was also effective in suppressing C666.1-derived tumor growth in vivo in nude mice. The same group further studied effects of SAHA and/or bortezomib (BTz; inhibitor of 26S-proteasome, pro-apoptotic agent) in NPC cell lines (HA, C666.1) [93]. In these experiments SAHA significantly induced Zta expression and the addition of BTz reduced expression of SAHA-induced Zta (in HA cells). BTz also disrupted SAHA-induced EBV replication, while potentiating SAHA's induction of apoptosis. The authors suggested that use of SAHA/BTz combination could be suitable for treatment of BL and PTLDs (when EBV shows a Cp/Wp-restricted latency) [94]. This combination synergistically induced the apoptosis and killing of Wp-restricted BL and LCLs (latency III) and suppressed the growth of Wp-restricted BL xenografts in nude mice, but did not affect Qp-driven

latency I BL cells.

Romidepsin

(Istodax®, NSC 630176, FR901228, FK228, Depsipeptide) is a bicyclic pentapeptide isolated from the soil bacterium *Chromobacterium violaceum*. This class I specific HDACi has been FDA approved for the treatment of cutaneous T cell lymphoma (CTCL) [95,96]. It is very efficient in inducing EBV lytic induction in vitro and in vivo in both NPC and GC epithelial tumor cells (nM concentrations), and in GCV mediated enhanced cell death [97]. However, in recent pilot study in patients with extranodal natural killer (NK)/T-cell lymphoma (ENKTL), serious adverse events were reported and suggested that romidepsin may cause too rapid EBV reactivation [98]. Further trials should include simultaneous administration of GCV with romidepsin.

Sodium butyrate

(NaB/SB) is a short-chain fatty acid which is altering gene expression and arresting cell proliferation by inhibition of the chromatin-remodeling activity of HDAC-s and histone hyperacetylation. It is commonly used as EBV lytic inducer often in combination with other agents, such as TPA or TSA. The early experiments showed that a low-level of lytic induction (EA) was obtained after the addition of NaB (or TPA alone) in Raji and NC37 cell lines, while the simultaneous presence of both compounds resulted in a significantly higher percentage of EBV reactivating cells [99]. In combination with GCV, butyrate is shown to induce killing of EBV-positive tumor cells in vitro and beneficial effects were obtained in lymphoma treatment [58,100].

Phorbol esters (TPA/PMA) combined with HDACi-s

The most commonly used phorbol ester is 12-O-tradecanoylphorbol-13-acetate (TPA), also known as phorbol-12-myristate-13-acetate (PMA). Zur Hausen et al. first reported TPA-induced EBV lytic cycle gene expression [101]. In B cells, TPA-mediated lytic induction requires activation of cellular protein kinase C (PKC) [102]. TPA treatment induces lytic EBV gene transcription in some cell lines by quick activation of PKC, but this is not an obligatory event suggesting more complex regulation of lytic induction [103,104]. TPA-mediated EBV lytic induction is strongly enhanced by the addition of sodium butyrate, and therefore the TPA/SB combination is widely used. The mechanism of TPA reactivation of EBV has been studied in depth (see later section), and antagonism between some HDACi-s (VPA) was reported [105]. Moreover, the capacity of other HDACi-s including the benzamide derivative Entinostat (MS 275), cyclic tetrapeptide Apicidin, cyclic depsipeptide Largazole (from a marine cyanobacterium), hydroxamic acids Oxamflatin, Scriptaid, Panobinostat (LBH 589) and Belinostat (PXD 101) were studied in the context of EBV lytic induction and cytotoxicity, but available (clinical trial) data are limited [58,105].

Effects of combined HDACi-s and chemotherapeutics on EBV reactivation are summarized in Table 1.

HDACi-s and Susceptibility to Ganciclovir for Induction of EBV Lytic Phase

Antiviral (val)Ganciclovir (GCV) treatment alone is not effective for treating EBV-associated malignancies, since tumor cells harbouring latent EBV do not express viral kinases (PK/TK), required for conversion of this nucleoside analogue into its cytotoxic form. TK/PK enzyme activity requires initiation of the EBV lytic cycle through Zta/Rta expression from the latent viral genome. Park and Faller (2002) demonstrated that EBV reactivation in a number of EBV-positive BL lines is induced by butyrate or other HDACi-s alone [90]. Among several different EBV-positive BL lines studied (Akata,

Daudi, JY, Namalwa), P3HR-1 was the cell line most responsive to HDACi-s. Mentzer (2001) applied this strategy for treating EBV-associated lymphoma/ PTLD using arginine butyrate combined with GCV in clinic, based on their finding on arginine butyrate-induced susceptibility to GCV [64,106]. The promising results of this study led to phase I-II trial demonstrating that this combination regime was well-tolerated and induced significant biologic activity in recurrent EBV-positive lymphoid malignancies [107].

In the case-report study of Stevens et al. a combination of 5-FU/ VPA was administered to a young EBV-positive NPC patient with advanced recurrent disease to induce EBV lytic induction, followed by (val)GCV treatment. This treatment resulted in detectable levels of EBV-DNA load in circulation, due to release of viral genomes from the EBV positive tumor [108]. Jones et al. reported another case-study where the combination of VPA and GCV resulted in detectable EBV-DNA load in plasma in patient with relapsed EBV-positive Diffuse Large B cell Lymphoma (DLBCL) [109]. Increased expression of IE proteins by addition of TSA in combination with GCV was reported by Jung et al. [110]. Ghosh et al. tested different HDACi-s in EBV-positive lymphoma cells and found that with the exception of SAHA and PXD101, all the other tested (butyrate, valproate, Oxamflatin, LBH 589 and Scriptaid) were effective in sensitization the EBV positive tumor cells to GCV at low (nM) concentrations [58].

These observations strongly point to the beneficial effect of combining EBV lytic cycle inducers together with antiviral compounds such as (val)Ganciclovir, to not only prevent full viral replication and spread *in vivo* [98], but more importantly, to kill proliferating EBV-infected cells by DNA chain termination.

DNA Demethylating Agents

During viral latency the ZEBRA promoter (Zp) is in an inactive, hypermethylated state [111,112]. The hypermethylation is probably caused by deregulation of methyltransferases in early tumorigenesis, driven by viral factors such as LMP1 [113-115]. Induction of EBV lytic cycle gene expression is probably only possible after the release of EBV genomic silencing by both promoter demethylation and histone acetylation [79]. Once persistent in the host cell EBV is interfering with cellular pathways and deregulating diverse homeostatic cellular functions [66,116,117]. In EBV-associated cancers viral genome is subject to extensive epigenetic regulation and viral promoters are heavily methylated, but such a virus is still powerful in mimicking the effect of growth, transcription and anti-apoptotic factors. On the other

hand, complex mechanisms in the host cell are employed to suppress the ability of cellular transcription factors to activate the expression of the EBV IE gene promoters, in order to maintain virus in the latent form of infection [74]. The genomes of EBV-infected tumor cells and their normal counterparts have differing epigenetic patterns, due to diverse epigenetic dysregulation. Hypermethylation of several host genes has been described in EBV-associated cancers [118,119]. Methylation, to some extent, might be beneficial for selectivity of BZLF1 activation, since Zta preferably binds to methylated domains of BRLF1 promoter, thereby initiating the lytic cascade [28,33].

Demethylating agents, such as **5-azacytidine** (5-aza-CR) have been studied, in order to enhance the effects of lytic induction. The results showed increased lytic viral gene expression in Burkitt's lymphoma cell lines [120], but not in lymphoblastoid cell lines [62], indicating that other factors besides methylation might be involved in desensitizing of EBV early gene promoters. The effect of **5-aza-2'deoxycytidine** (5-AZA) on lytic induction is investigated in several studies too. Ben-Sasson and Klein (1981) found an 8 times higher EA-gene expression in BL cells treated with this drug, compared to spontaneous levels, evaluated with IF [121]. This was confirmed in another BL cell line by Moore et al. [122]. Feng et al. also showed that 5-AZA caused lytic induction in a BL cell line, but no effect was seen in three different LCL cell lines [62]. Cell viability was not impaired by the drug, except when combined with GCV [86]. In addition, in an epithelial cell line of GC origin, results were similar, and a combination of 5-AZA with GCV even showed an enhanced effect on inducing cell death [110]. Details are summarized in Table 2.

Chemotherapeutics

The ability of particular chemotherapeutic agent to induce lytic EBV gene expression is mediated by variety of signal transduction pathways and their synergistic engagement, importantly, is dependent on cell type [66]. Details are summarized in Table 3.

Multiple studies show that **Gemcitabine** (GCb), a nucleoside analog, is able to switch on lytic induction in a subset of EBV-positive cell lines, including three types of LCL, Raji (BL), AGS-BX1, SNU-719 (GC) and C666.1 (NPC) [62,63,65,67]. In EBVaGC, GCb was found to act as a lytic phase inducer by activating the ataxia telangiectasia-mutated (ATM)/p53 genotoxic stress pathway [123]. Overall, GCb turns out to be a potent EBV-lytic phase inducer and this effect could even be increased by the addition of VPA or the subsequent administration of GCV [62,63,65]. Interestingly, the addition of

Drug	Cell line (tumor origin)	Assays	Detected proteins	+GCV?	Result on lytic induction	(Optimal) drug concentration	(Optimal) exposure time	Ref.
5-AZA	Daudi (BL)	IF	EA, VCA	No	+	25 µmol/L	6 h	[121]
	Rael (BL)	IHC	Zta, VCA	No	+	15 mmol/L	48 h	[122]
	SNU-719 (GC)	WB	IE	Yes (+)	+	0.5 or 5 µmol/L, 10 µg/mL GCV	6 d	[86]
	Raji (BL)	WB	IE	No	+	1 µmol/L	5 d	[62]
	LCLs	WB	IE	No	-	2.5 µmol/L	5 d	[62]
	Mutu-I	WB	Z, R, BMRF1	No	+	1 µmol/L	2 d	[123]

The column "Result" displays the effect of the drug on lytic induction: an increased (+), inhibited (-) or no significant (0) effect. The column "+GCV" indicates whether the drug described is combined with GCV (Ganciclovir) or not. If a "Yes" is noted in this column; the subsequent symbol shows if the addition of GCV has a positive (+), negative (-) or no effect (0) on the amount of cell killing. WB=Western blot; IF=Immunofluorescence.
Table 2: Demethylating agent 5-aza-2'deoxycytidine effect on EBV lytic induction in cell lines of epithelial and lymphoid origin.

Drug	Cell line (tumor origin)	Assays	Detected proteins	+GCV?	Result	(Optimal) drug concentration	(Optimal) exposure time	Ref.
GCb	LCLs, Raji (BL)	WB IF	IE BMRF1	Yes (+) (only LCLs)	+	1 µg/mL, 10 µg/mL GCV	5 d (WB), 3 d (IF)	[62]
	AGS-BX1 (GC)	WB PCR	ZEBRA ZEBRA	Yes (+)	+	3 µmol/L, 7.5-20 mmol/L GCV	2 d	[65]
	C666.1 (NPC)	WB, IF PCR	ZEBRA ZEBRA, TK, PK, VCA-p18	Yes (+)	+	3 µmol/L, GCV concentration not reported	2 d	[65]
	C666.1 (NPC)	WB	BMRF1	No	0	1 µg/mL	2 d	[63]
5-FU	LCLs, Raji (BL)	WB	IE	No	0	5 µg/mL	5 d	[62]
	AGS-EBV (GC), Akata (BL)	WB FACS	IE, E BZLF1	Yes (+) (only AGS-EBV)	+	5 µg/mL, 10 µg/mL GCV	2 d	[126]
	AGS-EBV (GC)	WB	BMRF1	No	+	1 µg/mL	2 d	[63]
	C666.1 (NPC)	WB	BMRF1	No	0	1 µg/mL	2 d	[63]
	SNU-719 (GC)	WB	E	Yes (+)	+	0.1 µg/mL, 2 mg/mL GCV	6 d	[110]
Dox	LCLs, Raji (BL)	WB	IE	Yes (+) (only LCLs)	+	0.2 µmol/L, 10 µg/mL GCV	5 d	[62]
	AGS-EBV (GC)	WB IF	IE, E IE, E	No	+	10-100 nmol/L 30 nmol/L	72 h	[129]
Cis-platinum	LCLs, Raji (BL)	WB	IE	No	0	1 µg/mL	5 d	[62]
	AGS-EBV (GC), Akata (BL)	WB FACS	IE, E BZLF1	Yes (+) (only AGS-EBV)	+	1 µg/mL 10 µg/mL GCV	2 d	[126]
	AGS-EBV (GC)	WB, IF	BMRF1	No	+	0.25 µg/mL	2 d	[63]
	AGS-Akata	WB	IE, E	No	+	10 µmol/L	2 d	[123]
	SNU-719	WB	E	Yes (+)	+	5 µg/mL, 2 mg/mL GCV	6 d	[110]
Paclitaxel	AGS-EBV (GC), Akata (BL)	WB FACS	IE, E BZLF1	No	+	10 nmol/L	2 d	[126]
	EBV-Akata (BL)	WB	IE, E	No	+	5 µg/mL	2 d	[123]
	SNU-719 (GC)	WB	E	Yes (+)	+	0.1 nmol/L, 2 mg/mL GCV	6 d	[110]
Arsenic trioxide (ATO)	A549-EBVBX1 (lung carcinoma)	IF, qRT-PCR	IE	Yes (+)		50 nmol/L , 40 µmol/L GCV	72 h (IF), 24/72	[131]

The column "Result" displays the effect of the drug on lytic induction: an increased (+), inhibited (-) or no significant (0) effect. The column "+GCV" indicates whether the drug described is combined with GCV (Ganciclovir) or not. If a "Yes" is noted in this column; the subsequent symbol shows if the addition of GCV has a positive (+), negative (-) or no effect (0) on the amount of cell killing. WB=Western blot; IF=Immunofluorescence.

Table 3: Summary of chemotherapeutical agents used for EBV lytic induction in cell lines of epithelial and lymphoid origin.

DNA demethylating agent 5-AZA did not increase the expression of the early protein BMRF1 by GCb [63]. Ferreira et al. confirmed data from Richter-Larrea et al. showing that combined treatment of chemotherapy with NaB resulted in a synergistic effect on apoptosis in BL cell lines, i.e., etoposide or cisplatin treatment together with NaB resulted in up-regulation of Bim, Bax, and Caspase-9 activation, while down-regulating Mcl-1 [124,125].

Similar to GCb, **5-fluoruracil** (5-FU) is an anti-metabolite that interferes with DNA synthesis. This chemotherapeutic agent triggers lytic induction in gastric cell lines AGS-EBV and SNU-719, but also in the BL cell line Akata [63,86,110,126]. Interestingly, Feng et al. did not detect lytic induction by 5-FU in another BL cell line (Raji), nor C666.1 [62,63]. On the other hand, they did observe lytic induction in C666.1 when 5-FU was combined with VPA and found that addition of VPA increased the effect of 5-FU in AGS-EBV cells. The addition of antiviral medication was described in two studies, showing that GCV enhanced toxicity of 5-FU in AGS-EBV and SNU-719 [110,126].

Doxorubicin (Dox) is an anthracycline antibiotic with oncolytic effects exerted through topoisomerase II inhibition and disruption of protein-DNA complexes (histone eviction) causing double-strand DNA breaks during mitosis. In a genome-wide gene knockout study, three independent genes were associated with Dox resistance, suggesting that these factors are important for DNA damage induced by Dox [127]. Knock-out of Keap1 or the SWI/SNF complex inhibited formation of

DNA double-strand breaks topoisomerase IIα inhibition, while loss of C9orf82 (CAAP1) enhanced repair of DNA double-strand breaks. Besides generating DNA double-strand breaks, Dox decreases DNA damage response by evicting histone variant H2AX, thereby impairing DNA repair [128]. Only two studies described on the influence of Dox on lytic induction. Both report a strong lytic inducing effect of Dox, either in LCLs or Raji or AGS-EBV cells [62,129]. Similar as with GCb, the potency of Dox is increased when it is combined with VPA or GCV, but not when combined with 5-AZA [62,63].

Cis-platinum inhibits DNA synthesis by the formation of platinum cross-links in and between DNA strands. Interestingly, this agent seems to be effective in similar cell lines as 5-FU and appears to be a potent lytic inducer in AGS-EBV, SNU-719 and Akata cell lines, but not in Raji and LCLs [62,63,110,123,126]. Similar to 5-FU, the effect on EBV reactivation of *Cis*-platinum is further increased by the addition of VPA (in AGS-EBV) or GCV (in AGS-EBV and SNU-719) [63,110,126].

Paclitaxel (Pacl) is a taxane that disturbs normal microtubule functioning thus interfering with correct chromosomal distribution during mitosis. Feng et al. found that Pacl caused lytic induction in both AGS-EBV (GC) and Akata (BL) cells [123]. Another study confirmed this lytic inducing effect of Pacl in the more natural SNU-719 (GC) cells and showed that, similar to 5-FU, the addition of GCV increased the toxicity of Pacl [110].

Arsenic trioxide (ATO) is a chemotherapeutic drug used to treat acute promyelocytic leukemia, and several other hematological and solid cancers. This drug affects multiple cellular pathways, thereby causing induction of apoptosis, inhibition of growth and angiogenesis, and promotion of differentiation [130]. It has been showed that ATO is able to induce EBV lytic cycle in 100% of treated 549-EBVBX1 (lung adenocarcinoma) cells. Subsequently, these cells became more susceptible to the cell-killing effects of GCV. ATO is able to disrupt promyelocytic leukemia protein nuclear bodies (PML NBs), important for maintenance of EBV latency, thereby activating EBV lytic gene expression and inducing GCV susceptibility in NPC cells [131]. Several clinical trials combining HDACi-s with classical chemotherapeutic and/or target based therapeutics are currently ongoing for solid tumors (including NPC) and haematological malignancies in particular in cutaneous T cell lymphoma (CTCL), peripheral T cell lymphoma (PTCL) and Hodgkin lymphoma (HL) [132-134].

Novel Compounds

Recently, Tikhmyanova et al. have reported the effects of a new class **of small molecules with tetrahydrocarboline structure** on EBV reactivation in several cell lines with different EBV latency types (including C666.1; Table 4) [135]. 66840 compounds were screened and C09, C50, C53, C60 and C67 were proven to be more potent lytic inducers compared to NaB or TPA, being effective in nanomolar concentrations and inducing lytic EBV reactivation in nearly 50% of Mutu I BL cells and over 50% of LCLs. The mechanism of induction remains to be elucidated, but thusfar these compounds are proven to affect transcription control of viral IE promoters and Zebra transactivator. NaB caused histone acetylation in Mutu I, Mutu-LCL 352, but none of newly identified lytic inducers stimulated H3 acetylation. To test whether the newly identified compounds share the mechanism of EBV reactivation with TPA, phosphorylation of p38 MAPK, S6, p90RSK and p53 was checked, and the compounds failed to induce detectable levels of phosphorylation of these targets,

suggesting that the mechanisms of EBV activation of these compounds are distinct from TPA/NaB mode of action. A similar approach to find new, stronger EBV lytic inducers was used by Choi et al. [136]. They performed a large phenotypic screening on 50240 chemicals, from which two potent compounds were identified; both structurally and biologically distinct from other lytic inducers. The first compound (C7) is structurally similar to iron chelators and the other (E11) is a potent activator of MAPK pathways. The effect of both newly identified lytic inducers was enhanced when combined with the HDAC inhibitor romidepsin.

Reactive oxygen inducing species (ROS) such as hydrogen peroxide (H_2O_2), Iron (II) sulfate ($FeSO_4$) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) contribute to the reactivation of EBV lytic cycle [137,138]. Hydrogen peroxide is also able to cause lytic induction in AGS-Akata cells [38]. Hagemeier et al. showed that ATM kinase and p53 activation are required for virus reactivation by hydrogen peroxide. On the other hand, they show that EBV-encoded protein kinase (EBV-PK) is not required for ATM activation and subsequently lytic induction by this agent. EBV-PK can activate ATM by upregulating the expression of DNA damage response proteins and TIP60 histone acetyltransferase, thereby promoting lytic induction [139].

TCDD or dioxin (2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin), a halogenated aromatic hydrocarbon, is a chemical carcinogen. One study shows that this compound is able to reactivate EBV in salivary epithelial cells, but not in BL or B cells [140]. In contrast, dioxin did induce EBV lytic cycle when the BL or B cells were simultaneously treated with TPA.

The **nitric oxide synthase** (NOS) **inhibitor NG-monomethyl-L-arginine** (L-NMMA) caused lytic induction in cell lines derived from gastric and Burkitt lymphoma and enhanced the lytic effect of TPA in gastric cells. This effect was greatly inhibited by the NO donor compounds S-nitroso-penicillamine (SNOP) and S-nitroso-acetylpenicillamine (SNAP) [141,142]. NF-κB is involved in the

Drug	Cell line (tumor origin)	Assays	Detected proteins	+GCV?	Result	(Optimal) drug concentration	(Optimal) exposure time	Ref.
Tetrahydrocarboline derivatives	Mutu I & Akata (BL), C666.1 (NPC), LCLs	WB, RT-PCR IF (Mutu-I−Hp-GFP)	BMRF1, BZLF1 VCA	Yes (+) (only Mutu-I)	+	1 μmol/L, 10 μg/mL GCV	72 h (WB), 48 h (RT-PCR)	[135]
C7, E11	AGS-BX1 (GC), YCCEL1 (GC), HONE1-EBV (NPC), C666.1 (NPC, only C7), SNU719 (GC, only C7)	WB	IE, E (E11 also L)	No	+	10-20 μmol/L	24-96 h (IE, E), 48-96 h (L)	[136]
	AGS-BX1	IF, flow cytometry	Zta	No	+	10 μmol/L C7, 20 μmol/L E11	72 h	
FeSO₄	Raji (BL)	RT-PCR	BZLF1	No	+	0.1 mmol/L	30 min	[137]
H₂O₂	Raji (BL)	RT-PCR	BZLF1	No	+	0.2 mmol/L	30 min	[137]
	AGS-Akata	WB	IE, E	No	+	300 μmol/L	2 d	[38]
	TW01, NA	WB	IE, E	No	+	500 μmol/L	1 d	[138]
MNNG	C666.1, NA, HA, H1299A (NPC)	WB Flow cytometry IF	IE, E EAD EAD/Zta	No	+	1 μg/mL	72 h	[138]
TCDD/ dioxin	B95-8 (B cell), P3HR1 (BL)	RT-PCR	BZLF1,BRLF transcripts	No	0	100 nmol/L	24/48 h	[140]
TCDD/ dioxin + TPA	B-95 (B cell), P3HR1 (BL)	RT-PCR	BZLF1,BRLF transcripts	No	+	100 nmol/L dioxin, 1 ng/mL TPA	24 h	[140]
Parthenolide	Raji (BL)	RT-PCR	IE	Yes (+)	+	4 or 6 μmol/L, 500 μg/mL GCV	24 h	[87
SNAP	GT38, GT39 (GC)	WB, NB	IE	No	- (inhibits effect of TPA)	1 mmol/L	9 h	[142]

				+GCV	Result			
SNOP	Akata (BL)	WB	IE, E	No	- (inhibits effect of L-NMMA)	1 mmol/L	7 d	[141]
L-NMMA	Akata, P3HR1 (BL)	IF WB	IE IE, E	No	+	5 mmol/L	7 d	[141]
L-NMMA	GT38, GT39 (GC)	WB	IE	No	+	5 mmol/L	6 d	[142]
Bay11-7082	BL-8 (BL)	qRT-PCR	IE	No	+	2.5 µmol/L	1 h	[91]
	5-8F (NPC)	WB Gardella gel	IE, E, L	Yes (+)	+	30 µmol/L 20 µmol/L	24 h	[146]
Z-LLF-CHO	5-8F (NPC)	WB Gardella gel	IE, E, L	Yes (+)	+	10 µmol/L 5 µmol/L, 100 mg/ml GCV	24 h	[146]
Ca-ionophore A23187 + TPA	B95-8 (LCL)	RT-PCR	IE	No	+	1 mmol/L Ca-ionophore, 20 ng/mL TPA	12 h	[152]
Azidothymidine (AZT)	BL-8, BL-5 (BL)	qRT-PCR (only BL-8 cells) WB	IE E	No	+	10 µg/mL	16 h (real-time qPCR), 32 h (WB)	[91]
	BL-8 (BL), IBL-4 (immunoblastic lymphoma)	qRT-PCR IF	IE	No	+ (BL) 0 (IBL)	1.25-2.5 µg/mL	48 h	[153]
AZT + hydroxyurea (HU)	BL-8, IBL-4	qRT-PCR IF	IE	No	+	1.25-2.5 µg/mL AZT, 25 µmol/L HU	48 h	[153]
HU	BL-8, IBL-4	qRT-PCR IF	IE	No	+ (BL) 0 (IBL)	25 µmol/L	48 h	[153]
	B95-8 (LCL)	qRT-PCR		No	-	1 mmol/L	48 h	[154]
Nocodazole, colchicine, vinblastine	NA	WB	Zta, EA-D	No	+	2 µg/ml, 0.1 µg/ml, 2 g/ml	24 h	[155]
Aspirin	B95-8, Raji	WB	IE, E, L	Yes (+)	+	5 mmol/L, 200 µg/ml GCV	24 h	[148]
Hydrocortisone Dexamethasone	Daudi (BL) Daudi, C666.1 (NPC), B98/HR1 (LCL)	RT-PCR RT-PCR	BZLF1 BZLF1	No No	+ +	10 µmol/L 100 nmol/L	72 h 72 h (Daudi) 24 h (C666.1, B98/HR1)	[158] [156]

The column "Result" displays the effect of the drug on lytic induction: an increased (+), inhibited (-) or no significant (0) effect. The column "+GCV" indicates whether the drug described is combined with GCV (Ganciclovir) or not. If a "Yes" is noted in this column; the subsequent symbol shows if the addition of GCV has a positive (+), negative (-) or no effect (0) on the amount of cell killing. WB=Western blot; IF=Immunofluorescence.
Table 4: Effects on EBV lytic induction in cell lines of epithelial and lymphoid origin by novel compounds.

modulation of EBV lytic cycle. The EBV latency oncogenic protein LMP1 inhibits virus reactivation via NF-κB activation [18-20,143-145]. Liu et al. showed that NF-κB inhibitors Bay11-7082 and Z-LLF-CHO were able to reactivate EBV in NPC cells [146]. Besides, they showed that GCV further amplified the cytotoxicity of these compounds. Bay11-7082 also caused lytic induction in a BL cell line [91].

Aspirin is a non-steroidal anti-inflammatory drug (NSAID) with NF-kB inhibiting properties [147]. Liu et al. confirmed that aspirin depleted NF-kB activity in a dose- and time-dependent manner and furthermore showed that aspirin reactivated EBV in B95-8 (EBV-positive marmoset B-lymphoblastoid cell line) and Raji BL-cells [148]. When GCV was used in combination with aspirin, a synergistic effect on cytotoxicity was observed.

Parthenolide is a sesquiterpene lactone found in medicinal plants. It has antitumor properties including inhibition of DNA synthesis, inhibition of cell proliferation and inhibition of NF-κB [149,150]. Furthermore, Li et al. showed that Parthenolide caused lytic induction in Raji cells. It cytotoxic effect was amplified by the addition of GCV [87].

Faggioni et al. showed that **calcium ionophores** caused EBV antigens expression in LCLs [151]. This effect was enhanced when protein kinase C was activated suggesting that protein kinase C is required for lytic induction [151]. A more recent study described that the same calcium ionophore, in combination with TPA, is able to induce EBV lytic cycle in B95-8 cells [152].

Zidovudine or azidothymidine (AZT) is a nucleoside analog reverse-transcriptase inhibitor (NRTI), an antiviral used to treat HIV infection. It is also an effective lytic inducer in BL cells, but not in immunoblastic lymphoma (IBL) cells [91,153]. On the other hand, in both cell lines the combination of AZT with the cytostatic agent hydroxyurea (HU) resulted in synergistic EBV reactivation. Furthermore, Bayraktar et al. observed promising results of AZT based chemotherapeutic treatment in patients with aggressive EBV-positive non-Hodgkin lymphoma. HU alone was, similar to AZT, only effective in BL cells but not in IBL cells [153]. Another study even shows a suppression of lytic genes expression after HU-treatment in B95-8 cells [154].

Nocodazole is an anti-neoplastic agent that interferes with microtubule polymerization and is able to cause lytic induction in NPC cells. Two other microtubule inhibitors, **Colchicine** and **Vinblastine**, also show induction of ZEBRA and EA-D antigen expression. In contrast, Paclitaxel, which inhibits microtubule depolymerization, and cytochalasin D, which blocks actin polymerization, did not show any lytic induction in these cells. These findings suggest that microtubule depolymerization induces EBV lytic cycle. Furthermore, this study showed that EBV reactivation by Nocodazole depends on PKC and the downstream p38 MAPK and JNK pathways [155].

Yang et al. reported BZLF1-mediated reactivation of EBV by **glucocorticoids** (hydrocortisone and dexamethasone) in Daudi (BL cells naturally expressing glucocorticoid receptors) and GR – transfected NPC cells (C666.1) but not in marmoset lymphoblastoid

B95-8 cell line [156]. Generally, physical and mental stress conditions are considered to induce EBV replication [157]. Daibata et al. described that combination therapy of dexamethasone with rituximab potentates EBV positive lymphoma for GCV-mediated antiviral effects *in vitro* and *in vivo* [158].

Icaritin is a compound with anti-tumor properties, derived from traditional Chinese herbal medicine Epimedium. It was shown to induce lytic cytotoxicity in EBV-positive extranodal NK/T-cell lymphoma (ENKL) cell lines by activation of pro-apoptotic pathways and this effect was amplified by the addition of GCV. The anti-proliferative and pro-apoptotic effects are likely mediated by inhibition of Stat3 and Akt pathways and LMP1 downregulation [159].

Host cell factors influencing susceptibility to EBV lytic reactivation and interference of lytic inducers on signaling pathways

Complex regulation of cellular and viral genomes including epigenetic modifications of histones and DNA, chromatin organization and transcription factors binding determine EBV gene expression programs and latency types in normal and cancer cells [160,161]. Recently viral microRNAs (miRNAs) have been reported to control IE genes and directly suppress lytic induction [162,163]. EBV latency program(s) are tightly regulated by epigenetic control mechanisms [112,115]. EBV lytic induction by chemicals is a cell type specific and generally achieved easier in lymphoma than carcinoma cell lines. Viral latency gene products can also affect (epigenetic) regulation of the host genome. Latent membrane protein 1 (LMP1) is the main EBV oncogene, inhibiting apoptosis and stimulating proliferation, by constitutive activating NF-kB, which is important for maintenance of viral latency [164,165]. On the other hand, canonical NF-kB activation is crucial for regulation of LMP1 [166]. Besides NF-kB, the major cellular signaling pathways triggered by LMP1 (in NPC cells) are AP-1 (JUN, FOS, ATF, MAF), JAK/STAT and PKC [167]. LMP1 C-terminus interaction with JAK3 activates STAT signaling pathway in B cells, which is in line with well-known LMP1 anti-apoptotic properties [21]. Recently Hill et al. reported that lower STAT3 levels promote susceptibility to lytic inducing agents [168]. Sorted HH514-16 latent BL cells (latency type I BL) appeared to have elevated levels of KRAB-ZFP family (transcriptional repressors recruiting histone deacetylases) and histone methyltransferase transcripts compared to untreated or lytic cells. Also low STAT3 levels correlated with high lytic gene expression in circulating B cells in early infected IM patients. On the other hand, high levels of STAT3 in latently infected epithelial cells up-regulate LMP1, which is known to block the induction of the lytic cycle [143,169].

The expression of EBV transactivator BZLF1 is stringently controlled. BZLF1 promoter (Zp) generally exhibits low basal activity. Stimulation by chemical (TPA) and biological inducers is mediated via transcriptional factors such as MEF-2D and SP1/3 [170]. Myocyte enhancer binding factor 2 (MEF-2) family proteins are important host factors for reactivation of EBV from latency [66,171]. When bound to class II HDACs, MEF-2 transcriptional activity is repressed [172-174]. Activation is mediated by several kinases such as ERK5 and CaMK, and transcriptional activity (by disrupting MEF2/HDAC complexes) is allowing the recruitment of histone acetyl-transferases (HATs) [173]. MEF-2D is one of the main (positive) regulators of BZLF1 transcription and phosphorylation by ERK5-kinase enhances its ability to positively regulate the transcription of Zp. In latency, MEF-2D binds to ZI element and interacts with class II HDACi-s, suppressing the Zp transcription [175]. Activation of the host gene *egr1* is essential for the lytic replication of EBV as Zta interacts with methylated ZRE in the promoter of *egr1* [176].

The activation of the lytic cycle by TPA is attributed to activation of the ERK signal transduction pathway [103,177]. Knowing that also VPA is shown to activate the ERK pathway, it is possible VPA might stimulate EBV lytic gene expression via ERK, especially when combined with chemo agents [178].

In BL (Raji) cells, HDACis (sodium butyrate, NaB) are targeting PI3K/Akt pathway and upregulating miR-143, miR-145 and miR-101, preventing cell growth [179].

Daigle et al. compared different HDACi-s for their EBV lytic induction properties and VPA was shown to selectively up-regulate certain cellular genes (including MEF-2D, YY1 and ZEB1) that could repress the EBV lytic cycle [105]. The authors demonstrated that VPA is able to antagonize the capacity of other HDACi-s to activate EBV lytic cycle in lymphoid cells. Murata et al. reported that expression of KLF4 a member of the SP1/KLF family and MEF-2B enhanced Zp activity and demonstrated that MEF-2 enhances the expression of not only BZLF1 but also BRLF1 gene by binding to Zp [180]. Liu et al. have demonstrated involvement of MEF-2D in the activation of the Zp, but in the reporter system used by Murata et al. MEF-2D did not enhance Zp transcriptional activity [170]. HIF-2α was also identified as a strong inducer of the Zp, linking oxygen deprivation to virus lytic induction [180].

Promoters of butyrate-responsive genes have butyrate response elements, and the action of butyrate is often mediated through Sp1/Sp3. These Sp1/Sp3-binding sites (ZIA, ZIC and ZID domains; see Figure 2) are responsive elements critical for TPA-induced Zp activation [41]. Inhibition of Sp1/Sp3–associated HDAC-activity leads to histone hyperacetylation and transcriptional activation of the cell cycle kinase inhibitor p21^{CIP-1} gene, restricted Cdk-2 activity and cell cycle arrest. Depending of the cell background, the non-proliferating cells may enter differentiation or apoptotic pathways [83,181].

In experiments with TPA/NaB mediated lytic induction in epithelial cells, Huang et al. showed that Zebra expression is regulated, among others by CCAAT-enhancer binding proteins (C/EBP; regulators of differentiation) and proposed model of C/EBP activity in epithelial cells, suggesting that limited expression of C/EBP proteins in epithelial cells (AGS-BX1) may contribute to the tendency to exhibit constitutive low-level Zp activity [182]. Interaction of C/EBP with Zebra is facilitating G1 cell cycle arrest and accumulation of p21^{CIP-1} during EBV lytic cycle [42].

ZEB1 (zinc finger E-box-binding protein 1) was identified as the major cellular factor in B-lymphocytes which can repress BZLF1 gene expression by directly binding to its promoter, Zp [183]. Yu et al. further confirmed that ZEB1 plays a central role in negatively regulating the latent-lytic switch in EBV-infected epithelial and B cells (Figure 2), and suggested to use agents that lower the repressor activity of ZEB1 to treat EBV-positive malignancies [184]. Studies of Ellis et al. suggested that either ZEB1 or ZEB2/SIP1 may play a central role in regulating EBV-reactivation in a cell type specific manner, and proposed a model of BZLF1 gene expression by both ZEBs [185]. ZEB1 and ZEB2 levels vary in different EBV-infected cell lines and both bind to ZV element of Zp. Authors also found evidence that EBV latency-lytic switch is positively regulated by cellular microRNAs (200b and 429) and suggested a role in differential ZEB1 and ZEB2 expression [186]. ZEB1, ZEB2/SIP1 are negative regulators of Zp. Sp1 binds to and activates Zp, counteracting repressing effects of ZEBs. Interestingly, AGS cells contain little or no ZEB1 (and high c-JUN and ATF-2) which may explain why among transformed epithelial cell lines, AGS cells are uniquely susceptible to maintaining EBV infection in a highly lytic form [187]. Recently Zhao et al. reported that ZEB1 is a key mediator in lytic induction of EBV-positive gastric cancer cell lines, therefore considered as a potential

therapeutically target [188].

The protein kinase C (PKC) **pathway** has been considered to be essential for rendering latent EBV into the lytic cycle by anti-Ig treatment and a PKC agonist TPA [103,189]. Lytic induction by HDACi-s is facilitated by a PKC-independent mechanism [104]. The essential role of PKC delta in HDACi-induced EBV reactivation in NPC cells (by TSA/NaB) was deciphered by Lee at al. [190].

Chang et al. found that post-translational modification of Rta by SUMO-1 in P3HR1 cells increases Rta transactivation activity upon lytic induction by TPA/NaB or TSA and identified Ubc9 and PIAS (enzymes involved in sumoylation) as binding partners of Rta by yeast two-hybrid screen [191]. The same authors reported that Sp1–MCAF1–Rta complex is crucial in transcription regulation of Rta and takes part in activation of EBV lytic cycle (in P3HR1 cells treated with TPA and sodium butyrate) [192]. TPA with NaB activates PKC theta-p38 MAPK axis in EBV infected B cells, that promotes the viral lytic cycle and cell survival and dephosphorylates AKT, balancing cell life and cell death [193].

DNA damage response (DDR) and EBV lytic replication cycle

Previous studies have shown that lytic reactivation of EBV in latently-infected cells induces ATM-dependent DNA damage response (DDR). Suppression of ATM inhibits replication of viral DNA. During the lytic EBV replication cycle the ATM-dependent DNA damage checkpoint signaling, including phosphorylated ATM, H2AX, Chk2 and p53 are activated [194,195]. BPLF1 is a structural tegument protein playing a role in DDR as trans-activator of viral immediate-early genes [196]. This protein is sufficient to induce ATM-dependent pathway through stabilization of Cdt1 and induction of EBV lytic cascade in response to activation of BZLF1, thereby, causing a DNA hyper-replication that in turn activates the DDR [197]. During the EBV lytic induction the key lytic switch protein BZLF1 interacts with p53 and inhibits its function through multiple mechanisms, which results in prevention of the cell cycle arrest and apoptosis, along with facilitating the viral DNA replication through engaging the 53BP1, a DNA repair protein [198,199]. A recent study indicates that Zta expression alone is sufficient to trigger ATM-phosphorylation and DDR-pathway activation independent of EBV-DNA replication requiring Zta-DNA complex formation co-associating with HP1β, a heterochromatin binding protein, thus creating a nuclear microenvironment for lytic gene expression and viral DNA replication [200].

Promyelocytic leukemia protein nuclear bodies (PML NBs) are proteinaceous structures present in the nuclei of cells. They are able to affect several nuclear processes, such as regulating gene transcription. Bell et al. observed a connection between EBV replication domains and PML bodies, indicating that replication of EBV occurs in association with PML [201]. EBNA1 is known to affect proper assembly of PML bodies and dysfunctional PML bodies affect DNA damage repair and trigger lytic replication [47]. Furthermore, other researchers discovered that LMP1 (important for the maintenance of EBV latency) increases the IF intensity of PML bodies [131]. This suggests that LMP1 is maintaining EBV latency by stabilizing these bodies. Subsequently to initiate virus replication, IE ZEBRA protein is able to disrupt the PML bodies, implying that these NBs are important to keep EBV in the latent state [202]. Moreover, Sides et al. observed that arsenic trioxide can interrupt these nuclear bodies, inducing EBV lytic phase and additionally accomplishing GCV susceptibility in nasopharyngeal carcinoma cells [131].

Several studies revealed the role of DNA-damaging agents, including chemotherapeutics, some HDACi-s and radiation, in reactivation of

EBV genome initiated by ATM-pathway signaling [123,203,204]. In the absence of DNA damage, ATM kinase can also be activated in response to reactive oxygen species (ROS). ATM activity contributes to the induction of EBV lytic gene expression [123,205]. The mechanisms by which chemoagents and HDACi-s activate the lytic induction of EBV are not fully understood. It is likely that activation of p53, which is a downstream target of ATM and requires ATM phosphorylation at ser-1981 in response to different stimuli, is an essential factor by which DNA-damaging agents induce EBV reactivation [38,206,207].

Tumor suppressor p53 plays a central part in DNA damage responses (DDR) mediating cell cycle arrest/apoptosis. The regulation of Zp activity by p53 is achieved through interaction with Sp1 (recruitment to Sp1-binding site located in the ZID domain of Zp) and new studies showed involvement of PKC delta [207,208]. EBV inhibits p53 by facilitating its degradation via ubiquitin-proteasome pathway [209]. The cell cycle regulator p53 is required for HDACi-mediated lytic reactivation both in EBV-positive NPC cell lines and lymphoblastoid B cell lines, which further supports the critical role of p53 and ATM in EBV reactivation [123,207]. The p53 has been shown to induce the lytic gene expression in many cell lines by different mechanisms, including the overexpression of BRRF1 and BRLF1, activating BZLF1 promoter by forming a complex with SP1, which binds and activates BZLF1 promoter, and inhibiting two potent repressors of BZLF1, ZEB1 and ZEB2 [38,185,207]. BGLF4 phosphorylates and activates TIP60 and acetylation of ATM by TIP60, which is a HAT, contributes to ATM activation [139]. Several EBV latent proteins, such as EBNA1, EBNA3C, LMP1, and LMP2A, have been shown to inhibit the DDR, with LMP1 and LMP2A in particular inhibiting the ATM kinase activity [210,211]. Besides the p53-dependent activation of lytic-cycle by ATM, this kinase is also known to enhance the activation of BZLF1 promoter by a different set of cellular and viral proteins. Since ATM inhibition was shown to block EBV reactivation in p53-negative cells, it is proposed that ATM plays a significant role in promoting lytic EBV reactivation via both p53-dependent and p53-independent pathways [123]. It has been speculated that multiple different targets of ATM, such as H2AX and Chk2, are involved in EBV viral gene expression, since ATM phosphorylates over 700 cellular proteins. However, the exact mechanism of this is not yet completely understood [212]. In recent study of Hau et al. ATM-dependent DDR involvement in EBV lytic reactivation in nasopharyngeal epithelial cells was investigated ATM-mediated activation of Sp1 (Serine -101 residue phosphorylation) was identified as an essential process in viral DNA replication [208].

Conclusions

EBV has evolved a complex interaction with its human host, resulting in a lifelong balanced latency where the virus limits host damage by staying undercover in memory B-cells and replicating only in mucosal lymphoid tissues and epithelia. Although generally harmless, under specific conditions EBV plays a causal role as (co-)factor in triggering defined malignancies, all harboring EBV in a state of latency, without overt lytic replication. In these tumors the oncogenic character of EBV latency proteins is clearly visible and contributes to the malignant process, stimulating cell growth and metastasis, as well as apoptosis resistance and immune evasion [1,6,15-17]. Reactivating EBV from latency into early-stage lytic replication induces expression of viral genes that can be targeted by immunological and antiviral strategies to eliminate virus carrying tumor cells [4,60,62,64,69].

Over the years, significant progress has been made towards identifying and testing novel highly specific and efficient agents (drugs) for targeted reactivation of the latent viral genome and inducing cytocidal effects in various EBV-driven tumor cells. This strategy is

referred to as viral cytolytic virus activation (CLVA) therapy which has recently entered the clinical testing stage in Phase-I/-II trials [64,65,68,107]. One of the complications we faced when comparing the various lytic inducers, was the heterogeneity of research models and methods used. To begin with, the different studies used multi-variable drug concentrations and treatment durations and many compounds were tested in different cell lines, often derived from different tissues (i.e., gastric carcinoma, nasopharyngeal carcinoma and lymphoma cell lines). Furthermore, effectiveness of lytic inducers was demonstrated by different techniques (presented in Tables 1-4) including detection of different EBV (immediate) early or late lytic proteins and mRNA expression, as well as functional characteristics such as apoptosis induction and sensitivity to antiviral treatment. Active compounds were classified based on their mechanism(s) of action and efficiency in different tumor cell systems. Some of the major signalling pathways are highlighted in response to different stimuli and susceptibility to EBV reactivation in different cell backgrounds is discussed. The antiviral nucleoside analogue (val)Ganciclovir combined with lytic EBV inducers is promising for treating EBV-positive cancers creating DNA chain termination in dividing cells expressing the early lytic stage TK and PK proteins. New agents and combinations remain to be tested in (pre-) clinical situations. A particularly interesting category are novel HDACI's [213]. Recently, novel compounds were identified from screening a library of small organic compounds and 2 of them demonstrated to be especially promising in inducing rapid EBV reactivation from latency in GC and NPC cells [136]. Relevant "targetable" pathways in EBV-associated GC tumors and nasopharyngeal carcinoma, including overviews of (current) clinical trials were recently summarized [69,70,214]. Currently efforts are ongoing to identify specific natural edible fito-compounds and derivatives potent enough to induce EBV reactivation [159]. Additional work is necessary to study the effect of the lytic inducers in a more uniform way and to bring consensus in crucial pathways leading to virus reactivation in different (tumor) cell backgrounds. Recent CLVA trials show promising results in patients with either EBV-driven lymphoma or carcinoma [65,68,107]. This virus-targeted therapy might initiate a general treatment approach for various EBV-associated malignancies.

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