Chapter 6

Review: Biological and Pharmacological Basis of Cytolytic Viral Activation in EBV-Associated Nasopharyngeal Carcinoma

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Abstract

Epstein-Barr virus (EBV) infection contributes to the development of different types of human malignancies, especially nasopharyngeal carcinoma. As a herpesvirus, EBV can establish two major modes of virus-cell interactions: a latent or a lytic infection. Latent infection is prevalent in the vast majority of malignant cells in EBV-related malignancies. Inducing a switch from latent to lytic infection in a substantial fraction of malignant cells has long been considered as a potentially interesting therapeutic approach. Therapeutic benefits are expected from (1) the cytotoxic or cytostatic effects of viral products expressed in the context of the lytic cycle; (2) expression of viral enzymes capable of metabolizing pro-drugs selectively inside these cells and (3) broadening the expression spectrum of antigenic viral proteins. In this chapter, addressing non EBV-specialized readers, we first summarize the main aspects of EBV biology with emphasis on the cellular mechanisms known to control latent and lytic infections. Then, we outline the basic principles and requirements of cytolytic EBV activation performed with a therapeutic intent. Finally, we review the main categories of pharmacological agents reported to be active in the switch from latent to lytic infection, including drugs used for conventional anti-tumour chemotherapy, histone-deacetylase inhibitors and various miscellaneous compounds.

Keywords: nasopharyngeal carcinoma, Epstein-Barr virus, lytic cycle, histone-deacetylase inhibitors, epigenetics, immunotherapy, phenotypic screening, compound library

1. Introduction

Nasopharyngeal carcinomas (NPCs) are consistently associated with the Epstein-Barr virus (EBV) [1] and represent a major public health problem worldwide. In order of frequency, it is the third leading cause of virus-related human malignancies, ranking just behind hepatocellular carcinomas linked to the hepatitis B and C viruses and cervix carcinoma involving human papilloma viruses (HPV) [2]. The incidence of NPC is particularly high in Southern China, especially in the Guangdong province (approximately 25 cases per 100,000 persons per year). In addition, there are areas of intermediate incidence in Southeast Asia and North Africa. Men have been shown to be two to three times more likely to develop NPC than women, the most frequent age of disease occurrence being 50–60 years. Regardless of patient geographical origin, NPCs are constantly associated with EBV (except for a very small number of highly differentiated atypical forms related to tobacco and alcohol and a few cases associated with human papilloma viruses [HPV] mainly observed in Europe and North America) (reviewed in [3]). Like other EBV-associated malignancies, NPC is clearly a multifactorial disease. The non-viral risk factors include germline genetic susceptibility involving alleles of the major histocompatibility complex (MHC) region on chromosome 6 [4]. One example of a susceptibility gene not linked to the MHC region is *MST1R*. It encodes a protein detected in the ciliated epithelial cells in normal nasopharyngeal mucosa, which plays a role in the cilia motility, thus being essential for host defence [5]. The action of diet carcinogens, like salt-preserved fish in South China, probably accounts for multiple acquired cellular genetic and epigenetic alterations detected in malignant cells [4].

Investigations on the mechanisms of NPC oncogenesis and novel therapeutic approaches have long been hampered by a lack of biological resources. It has been proven very difficult to derive tumour lines propagated *in vitro* or even patient-derived xenografts (PDXs), which retain the EBV-genome, using clinical NPC specimens. Currently, there is only one EBV-positive NPC tumour line (C666-1), which is routinely propagated *in vitro*, and a small number of EBV-positive NPC PDXs whose cells are not easily handled *in vitro* [6–9]. There is evidence that malignant NPC cells tend to lose the EBV genome when one attempts their propagation *in vitro*. The resulting EBV-negative cell lines seem to have a phenotype, which does not fit with the NPC cell phenotype *in situ*, especially with regard to immunological characteristics (e.g., HONE1) [10]. They are sometimes artificially reinfected by EBV *in vitro*, but this does not restore a typical NPC cell phenotype.

In addition to the latent EBV-infection, the malignant phenotype of NPC cells is explained by a number of genetic and epigenetic alterations. None of these alterations is constant in all NPC tumours, and few are highly specific of NPCs. In most cases, there are no alterations in the *TP53* gene (it is mutated in less than 10% of the cases; [11]). In contrast, many other tumour suppressor genes (TSGs) are frequently silenced in NPC cells, especially *CDKN2A* (chromosome 9p21.3) and *RASSF1* (chromosome 3p21.3). Their inactivation often results from a combination of hemizygous deletion and promoter hypermethylation on the remaining allele. However, deletions of both alleles and inactivating point mutations have been also reported. Silencing of these TSGs often occurs very early in the carcinogenic process [12]. Many TSGs

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like the gene encoding the Wnt inhibitory factor 1 (*WIF*, 12q14.3) or E-cadherin (*CDH*, 16q22.1) are consistently inactivated by methylation of both alleles [13, 14]. One has to keep in mind that prolonged latent—or even transient EBV infection—favours genome-wide hypermethylation of gene promoters [15]. In addition to the silencing of multiple TSGs, NPC cells exhibit alterations of multiple oncogenes, which are often affected by copy number gains, especially *CCND1* (encoding cyclin D1 on chromosome 11q13.3) and *PIK3CA* (3q26.1) [12, 16]. There are also frequent copy-number gains of the gene encoding the lymphotoxin-β receptor (*LTβR*) on chromosome 12p13.31 [12]. This is a rare – or even unique – example of a genetic alteration which is highly specific of NPC tumours. Two oncogenic fusion genes—*UBR5-ZNF423* and *FGFR-TACC3*—have been identified by high-throughput sequencing of tumour mRNAs (RNAseq), but they are only detected in a minority of NPC specimens, mainly among late stage tumours [17, 18]. Whole exome sequencing of 128 NPC specimens has revealed mutations in genes encoding proteins involved in chromatin remodelling, especially *ARID1a* (about 10% of the specimens) and in the process of DNA methylation (*TET1*, *TET2* and *TET3*, altogether in about 9% of the specimens) [11].

On average, NPCs are more radio-sensitive and chemo-sensitive than other head and neck tumours. However, they still raise serious therapeutic issues: (1) NPCs are often discovered at a late stage whereas lymph node and distant organ metastases occur early in tumour evolution; (2) despite remarkable advances in 3D radiotherapy, irradiation often leads to severe functional sequels (subcutaneous and muscular sclerosis and xerostomia); (3) although metastatic lesions are initially sensitive to chemotherapy, they often escape from treatment control after a few months [19, 20]. In summary, despite the remarkable progress achieved in the recent years, there is still an urgent need for better therapeutic modalities. In this context, the idea of using EBV as a kind of endogenous oncolytic virus has been in the air for a very long time. The proof of principle that pharmacological agents can disrupt latent infection and push at least a fraction of EBV-infected malignant cells towards the reactivation of the viral lytic cycle was presented almost 40 years ago. Sodium butyrate was reported as a potent activator of the lytic cycle in EBV-infected human B-lymphocytes in 1979 [21]. However, the progress of this concept towards therapeutic applications has been very slow, especially regarding NPC. The aim of this review is to assemble key information for a public of non-EBV-specialists who want to understand the cellular, molecular and pharmacological basis of translational research on cytolytic viral activation in nasopharyngeal carcinomas.

2. Main aspects of EBV biology and regulation of the latent/lytic modes of infection

2.1. General aspects of EBV biology

EBV is one of the eight human Herpesviridae. It belongs to the subfamily of the γ-herpesvirinae and to the genus of lymphocryptoviruses. Like all Herpesviridae, it is an enveloped double-stranded DNA virus, containing about 80 genes. It is the causative agent of infectious mononucleosis, and it has an etiological role in several human malignant diseases mainly of

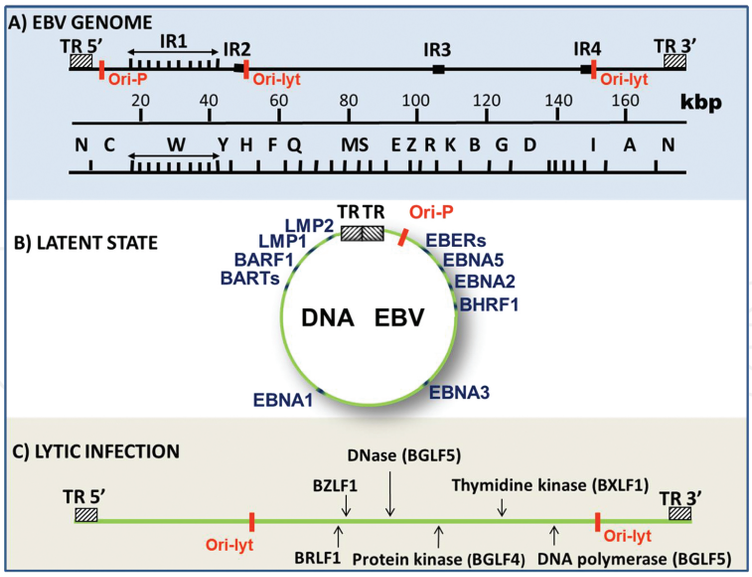

Figure 1. EBV-genome structure and configuration in connection with latent or lytic infection: (A) EBV genome map (linear representation). EBV genome contains about 180 kilobase pairs (kb). Both ends of the viral DNA contain a variable number of repeated non-coding sequences of 500 bp (called TR for terminal repeats). In addition, there are internal repeated sequences called internal repeats (major IR1 and minor IR 2–4). Ori-P is the replication origin of the viral genome used in the latent state of infection. The Ori-lyt are two replication origins used during the lytic infection. The reference restriction map is based on the digestion of the viral DNA by the restriction enzyme BamH1. Restriction fragments are classified from the largest (BamH1A) to the smallest (BamH1Z). Designation of open-reading frames is based on this restriction map. For example, BZLF1 is the first leftward open reading frame in the BamH1 Z fragment (Bam Z leftward open-reading frame 1) (BZLF1 by extension also means the corresponding protein). (B) Configuration of the EBV genome characteristic of the latently infected cells. When the infected cell enters a state of latent infection the viral DNA is circularized probably by recombination of the TRs. This circular form of the genome called episome is contained in the nucleus in combination with cellular chromatin but apart from chromosomes. The episomes are passively replicated by cellular DNA polymerases starting from the Ori-P origin of replication. During latent infection, most viral genes are silent. However, about 10% of them are consistently expressed (in blue). Most of these genes encode final products—viral non-coding RNAs or proteins—with transforming properties. They are called "latent genes" whereas genes expressed only during lytic infection are called "lytic genes". There is no topographical separation between the two categories of genes.(C) Configuration of the EBV-genome characteristic of the lytic infection. As usual in virology, the phase of the lytic cycle which precedes viral DNA replication is called the early phase. As soon as the viral DNA pol starts the autonomous replication of the viral genome, lytically infected cells enter the late phase. Newly synthesized viral genomes are in a linear configuration. Viral proteins expressed at the very beginning of the early phase of the lytic cycle like BZLF1 and BRLF1 are encoded by immediate early genes. Early viral proteins are expressed at a more advanced stage. Many of them are involved in DNA metabolism, for example, the viral thymidine-kinase, DNAse and DNA polymerase. The EBV protein kinase (BGLF4; protein encoded by the Bam G leftward open reading frame 4) can phosphorylate various substrates. It is also one component of the viral particle.

lymphoid and epithelial origin as well as several autoimmune diseases. Examples of EBV-related malignancies are nasopharyngeal carcinomas, several types of lymphomas (endemic Burkitt's Lymphoma (BL), more rarely Hodgkin lymphomas, centro-facial NK-T lymphoma, post-transplant lymphomas and AIDS-associated lymphomas), and approximately 10% of gastric cancers worldwide [22–25]. Examples of autoimmune diseases likely to involve EBV are multiple sclerosis and systemic lupus erythematosus [26, 27]. Like all other herpesviruses, EBV causes lifelong infection following the primo-infection. More than 90% of adult humans are healthy carriers regardless of their geographic origin. Its persistence in healthy carriers is

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the result of three types of viral characteristics: (1) its capacity to continuously induce bursts of productive infections in various tissues, especially in the upper aerodigestive tract and more specifically in the tonsils, the salivary glands and possibly gingiva; (2) its capacity to achieve latent infection mainly in B-lymphocytes, especially memory B cells in blood, bone marrow and lymphoid organs; (3) its sophisticated strategies of immune evasion [28].

The distinction between two major modes of virus-cell interactions is a key to understanding EBV biology [29]. (1) Latent infections are cellular infection modalities characterized by the absence of viral particle production, a restricted expression of viral genes (often less than 10 out of 80) and a circular configuration of the viral genome (**Figure 1**). (2) Lytic/productive infections are characterized by sequential or concomitant expression of most viral genes, abundant synthesis of linear viral genomes and finally extracellular release of viral particles or virions in a context of mandatory cell death.

2.2. Latent EBV infection in LCL and NPC cells

One major common characteristic of most EBV-related malignancies is the predominance of latent modes of virus-cell interactions. As a rule in EBV-associated tumours, no EBV particles are detected by electron microscopy on tumour sections, whereas the viral DNA can be visualized in the nuclei of malignant cells by *in situ* hybridization. Consistently, very few malignant cells exhibit expression of viral proteins characteristic of the lytic/productive cycle. This minimal expression of lytic viral proteins in EBV-infected malignant cells favours immune evasion. In most latently infected cells, the virus-cell interactions are bidirectional: a few viral genes often modify the phenotype of the host cell, whereas the cellular context contributes to the repression of most viral genes (see subsequent section B3). For laboratory investigations, a model of latent EBV infection is easily obtained *in vitro* by infection of peripheral blood mononuclear cells (PBMCs) from normal donors, which results in the oncogenic transformation of resting B cells. These EBV-transformed B-cell lines are often called lymphoblastoid cell lines (LCLs). LCLs are immortalized and tumorigenic in SCID (severe combined immune-deficiency) mice [30]. They represent a privileged model for *in vitro* investigations of the molecular basis of latent infections.

During latency, the viral genome is conserved under the form of circular copies, called episomes, from one to several tens, which are present in the nuclei apart from the cell chromosomal DNA but also coated with cellular chromatin and replicated by cellular enzymes at each cell division. Most viral genes expressed in EBV-associated malignancies belong to the category of the latent genes, which are expressed in LCLs *in vitro* and contribute to the maintenance of the transformed phenotype. Their oncogenic effects are summarized in **Table 1**. The latent genes encode nuclear proteins like the EBNAs (Epstein-Barr nuclear antigens), membrane proteins like LMP1, LMP-2A and LMP-2B (latent membrane proteins 1, 2A and 2B) and non-coding RNAs. There are two main categories of non-coding viral RNAs: genuine microRNAs and the EBERs (Epstein-Barr virus encoded small RNAs) [31]. Two families of viral microRNAs, called "BHRF1" and "BART" microRNAs, can be transcribed from two distinct regions of the EBV genome, the Bam-H1 H and Bam-H1 A segments, respectively. The BHRF1 (Bam H rightward open reading frame 1) cluster includes at least

four microRNAs which are transcribed in LCLs but not detectable in most NPC specimens. They have a key role in the inhibition of apoptosis [32]. In contrast, the microRNAs of the BART (Bam A rightward transcripts) family are abundant in most NPC cells and not detected in LCLs. About 40 BART microRNAs have been identified so far [33, 34]. They target a great variety of mRNAs, for example, those encoding the pro-apoptotic protein Puma (miR-BART5), the tumour suppressor protein PTEN (miR-BART1) or the viral DNA polymerase (miR-BART2) [35–37]. The EBERs are single-stranded RNAs of about 170 nucleotides with a complex secondary structure containing double-stranded segments which can react with various cellular receptors of double-stranded RNAs such as the PKR (protein-kinase RNA-dependent), RIG1 (retinoic acid-inducible gene 1) and TLR3 (Toll-like receptor 3) [38]. These interactions stimulate resistance to interferon and production of growth factors like IGF-1 (insulin-like growth factor 1) [38].

	Type	EBV product	Examples of functions
Latency product	Non-coding latent transcripts	**EBERs 1 and 2** (non-coding RNAs)	Inhibit the RNA dependent protein kinase (PKR) activate the Toll-like receptor 3 (TLR3) [38]
		miR-BART (microRNAs)	Inhibit expression of some viral lytic and cellular pro-apoptotic genes, promote metastases [35, 37, 42]
	Non-membrane protein	**EBNA 1** (nuclear protein)	Episome maintenance, contributes to disruption of PML bodies [43]
		BARF1 (secreted protein)	Ligand of the m-CSF or CSF1 receptor [44]
	Membrane latent protein	**LMP1**	Activator of Bcl3/p50/P50 NF-kB complex [1]
		LMP2A	Activator of PI3 kinase/Akt pathway [1]
		LMP2B	Accelerates the degradation of interferon receptors [1]
Lytic cycle products	Immediate early proteins (IE)	**BZLF1**	Act as transactivators, enhancing the expression of later lytic genes [45, 46]
		BRLF1	
	Early proteins (E)	**DNA polymerase**	Lytic replication of the viral genome
		EBV-DNase	Alkaline nuclease with endonuclease and exonuclease activities
		EBV-thymidine kinase (TK)	Phosphokinase with a probable role in the phosphorylation of FIAU [47, 48]
		EBV-protein kinase (PK)	Phosphokinase involved in DNA replication and virion production. Phosphorylates ganciclovir [49, 50]
	Late proteins (L)	**VCA** (viral capsid antigen)	A complex of structural proteins assembled in the viral capsid
		Gp350	Viral envelope glycoprotein binding to the cell membrane receptor CD21 (also known as CR2) [51]

Table 1. Examples of EBV latency and lytic cycle products with clues on their functions.

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In comparison to LCLs, latent EBV infection in NPC cells has some specific features. The expression of latent viral proteins is more restricted than in LCLs. Only one latent nuclear protein—EBNA1—is expressed in contrast to six in LCLs. The latent membrane proteins, LMP1 and LMP2, are present in most tumour specimens but often at a low level and with major heterogeneity among tumour cells [39, 40]. Another specific feature of latent EBV-infection in NPCs is the huge abundance of the BART microRNAs which on average account for 20% of total tumour microRNAs [41]).

2.3. The switch from latency to the lytic/productive infection and its regulation

2.3.1. General features of the switch from latent to lytic EBV-infection

Experimental lytic/productive infections by EBV can be achieved in several manners. As explained in the previous section, EBV infection of resting B cells *in vitro* results mostly in transformed cells, which are latently infected. However, EBV-transformed B cells can enter the lytic/productive cycle at a secondary stage triggered by plasmacytic differentiation or treatment with various chemicals (**Table 2**. The experimental systems used for EBV infection of epithelial cells *in vitro* are completely different. First, penetration of viral particles often requires special procedures, as for example, pre-adsorption on B cells [52]. Next, penetration of EBV in epithelial cells often results in direct lytic infection (**Figure 2**) [53, 54]. With most experimental systems, latently infected epithelial cells remain rare, and their isolation usually requires the use of recombinant viruses carrying selectable markers [53]. In some models based on cultured primary epithelial cells, primary lytic infection can be blocked by overexpression of cyclin D1 or inhibition of CDKN2A. So far, most laboratory investigations on EBV lytic/productive infection have been done starting from latently infected cells switching to lytic viral activation (see **Figure 2**). This switch is triggered by external factors and/or modifications of physiological cell conditions, for example, differentiation or senescence. It is the result of a change in the balance of factors either blocking or enhancing the expression of lytic viral genes. As reviewed by Miller et al [29] and more recently by Kenney et al [46] the switch from latent to lytic infection is a sequential process, comprising two main sets of events:

1. Upstream events leading to the expression of the immediate early viral transactivators BZLF1 (also called ZEBRA, Zta or EB-1) and BRLF1 (also called Rta) [29].

2. Downstream events resulting from the interaction of BZLF1 and BRLF1 with a number of viral promoters controlling genes encoding products of the lytic cycle, such as, the EBV DNA-polymerase or EBV-TK (thereafter called lytic promoters) [55].

The genes encoding BZLF1 and BRLF1 are themselves under the control of promoter regions (Zp and Rp), which are regulated by a wide number of cellular factors, either activators or inhibitors. The influence of the inhibitors is predominant in the latently infected cells and vice versa during lytic activation.

Class of drugs	Example
Direct PKC activators	Phorbol-esters [65]
Conventional chemotherapy [30, 60, 74, 88–90]	*cis*-platinum or cisplatin (alkylating agent); paclitaxel (stabilizer of mitotic spindle); 5-fluoro-uracil (pyrimidine analog); gemcitabine (cytidine analog)
Inhibitors of histone deacetylases (HDACi)	Sodium butyrate [21], Trichostatin A [61] Valproic acid [86–88, 104], Panobinostat (LBH-589) [94], Vorinostat (SAHA) [95, 96], Romidepsin [60, 97]
Demethylating agents	5-Azacitidine [100]
Protease inhibitors	Bortezomib [47, 48]
Gamma-secretase inhibitors	Dibenzazepine [101]
Tetrahydrocarbolines and other compounds recently selected by high-throughput assays [103], [102]	
Prodrugs activated by EBV enzymes [59, 80, 105]	Ganciclovir (9-(1,3-dihydroxy-2-propoxyméthyl) guanine or DHPG) FIAU (2′-fluoro-2′-deoxy-β-D-5-iodo-uracil-arabinofuranoside) [47, 48]

Table 2. Summary of the different drugs with reported capacity of lytic EBV activation and prodrugs phosphorylated by EBV-encoded kinases.

BZLF1 and BRLF1 activate one another's promoter and their cooperation is required for optimal activation of many lytic viral promoters [56]. BZLF1 preferentially activates these promoters when they are methylated, whereas BRLF1 is more active on unmethylated promoters [45]. Therefore, they cooperate to induce lytic activation, no matter if the viral genome is methylated or unmethylated. BRLF1 ability to transactivate its target promoters is also dependent on another epigenetic mark, which is the 5-hydroxymethylation of cytosine in promoter CpG islands. Hydroxy-methylation induces an open chromatin conformation, which is critical for optimal action of BRLF1 but not BZLF1 [56].

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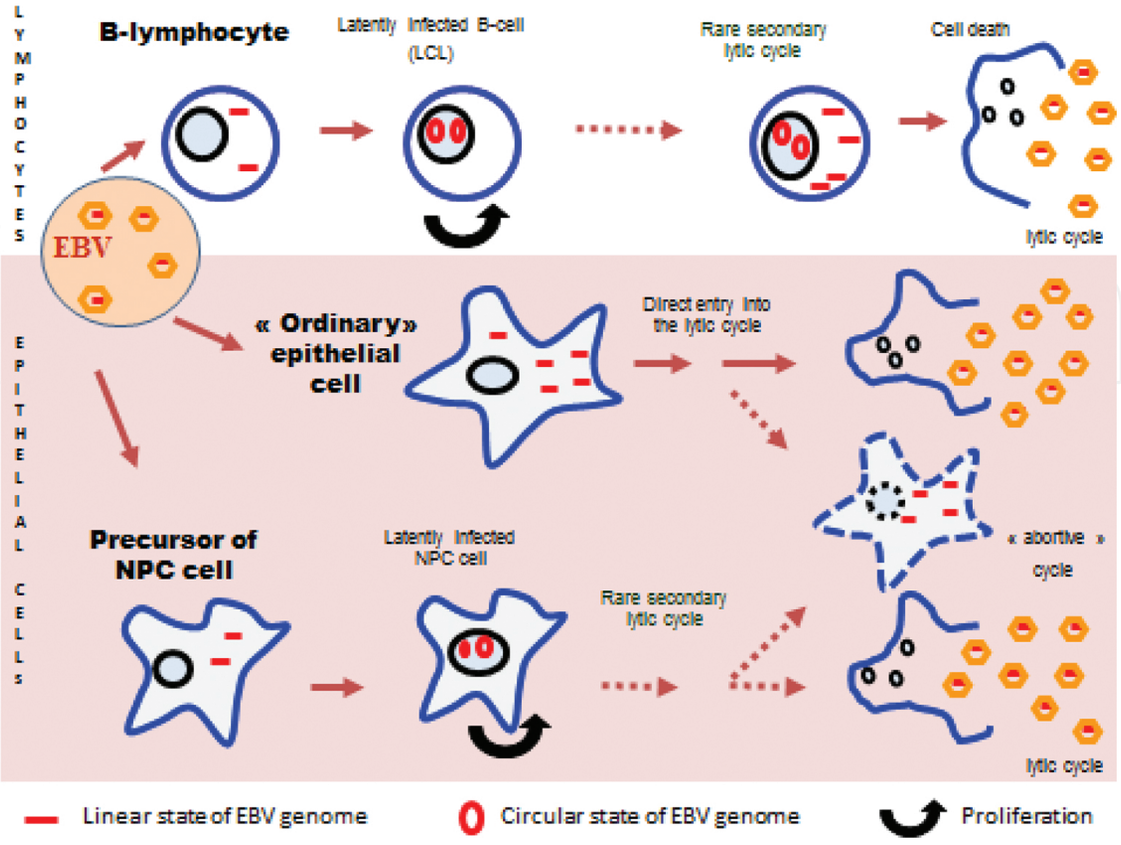

Figure 2. Main modalities of virus-cell interactions in lymphoid and epithelial cells.

– When human resting B-lymphocytes are infected by EBV, a fraction of them enter a state of viral latency requiring the circularization of the EBV genome and the expression of several viral latent genes. Simultaneously, the host cell undergoes an oncogenic transformation driven by the viral latent genes, especially EBNA2, EBNA3A and 3C and LMP1. These transformed B-cells can proliferate indefinitely resulting in LCLs. However, a secondary switch from latency to the lytic cycle can occur in a fraction of them. This fraction is generally very small but it can be increased by some pharmacological agents like HDACi (histone deacetylase inhibitors) or PKC (protein-kinase C) activators.

– EBV infection of epithelial cells *in vitro* requires special experimental systems like co-cultivation with B-cells carrying viral particles . In most of these experiments, penetration of the viral particles results in infections which are directly cytolytic or abortive. There is evidence that the same type of events often occur when epithelial cells are infected *in situ*, for example in the salivary glands or in the tonsil epithelium (normal epithelial cells are depicted by a cell shape named "ordinary epithelial cell") [57, 58].

– However for some reasons, malignant NPC cells undergo a latent infection suggesting a special type of virus-cell interactions at the initial stage of the malignant cell proliferation. There is evidence that the precursor cells have premalignant alterations, especially knock-out of cyclin kinases' inhibitors which make them resistant to virus-induced senescence (cells with pre-malignant lesions are depicted by a cell shape named "precursor of NPC cell"). Obvious-

ly, this is not sufficient for the establishment of latency since it is difficult to establish latency in many epithelial cells which are fully transformed and grown *in vitro*. In latently infected NPC cells secondary lytic or abortive cycle can occur spontaneously in a small fraction of the cells. One important aim of cytolytic activation therapy is to dramatically increase the number of the malignant cells undergoing the lytic cycle.

2.3.2. Overview of the cell physiological conditions and transcription factors involved in the switch from latent to lytic EBV infection

The factors controlling the switch from latent to lytic infection are numerous. They are intricate and highly dependent on the cellular context. **Figure 3** is intended to give a concise overview of these factors. As a first approximation, absence of cellular differentiation, cell proliferation, EMT (epithelial to mesenchymal transition) and inflammation tend to favour the maintenance of a latent infection. In contrast, cell differentiation, senescence, DNA damage response and hypoxia favour the entry into the lytic cycle.

Many signalling pathways which favour lytic EBV activation involve protein-kinases C (PKCs). PKCs phosphorylate hydroxyl groups of serine and threonine in multiple protein substrates, including MAP kinases and MARCK proteins. They are involved in multiple and diverse biological processes including transcriptional regulations, cell growth and immune responses. Physiological activation of "conventional" PKCs (α, β, γ) requires stimulation by diacylglycerol and calcium, whereas activation of "novel" PKCs (δ, ε, H, Θ) requires only diacylglycerol. There is evidence for a special contribution of PKCδ to the control of the switch from latent to lytic infection. For example, as explained in sections C1, D2 and D3, PKCδ plays a critical role downstream of various pharmacological agents inducing lytic EBV activation [59–61]. On the contrary, the intracellular form of the cytokine IL-32 has been shown to prevent lytic activation by sequestration of PKCδ [62]. Several PKC isoforms activate cellular immediate early proteins, for example, the FOS and EGR1 (early growth response) transcription

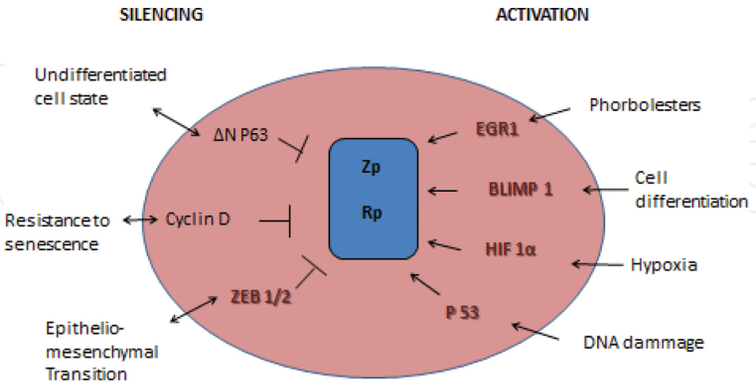

Figure 3. Summary of the physiological cell conditions and transcription factors, which modulate the activity of the *BZLF1* (Zp) and *BRLF1* (Rp) gene promoters in the context of epithelial cells. Both promoters are under the control of inhibitory (left side) and activating (right side) factors. Various physiological cell conditions are enumerated outside the pink circle, whereas the corresponding regulatory proteins are presented inside the pink circle with transcription factors appearing in bold brown letters. Detailed explanations and references are provided in section B3-2.

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factors, which have specific binding motifs on the Zp and Rp promoters, respectively [63, 64]. For information, phorbol-esters, which have long been known to induce lytic EBV activation in lymphoid cells, are direct pharmacological agonists of PKCs [65].

The Blimp-1 transcription factor, which is a key player for lymphoid and epithelial cell differentiation, is a strong inducer of lytic EBV activation, especially in epithelial cells; it stimulates the expression of both BZLF1 and BRLF1 [66]. TGF-β has been reported to induce a partial lytic activation in EBV-positive gastric carcinoma cell lines [67]. Various factors, which antagonize senescence, favour the maintenance of EBV latency. As already mentioned, overexpression of cyclin-D1 or loss of CDKN2A, which enhances G0/G1 transition in the cell cycle, prevents lytic activation in some models of EBV latent infection in epithelial cells [53]. Generally speaking, inflammation—for example, inflammation triggered by TNF-α or interferon-γ—is rather seen as a factor which strengthens EBV latency [68]. Activation of NF-κB has been reported to stabilize latency in various γ-herpesviruses [69]. Stat3 is also reported to stabilize latency in EBV-infected cells [63]. However, the influence of inflammatory factors on EBV latency in NPC cells has not been well documented. The ZEB1/ZEB2 transcription factors, which are known to contribute to the EMT in various cell types, have been proven to antagonize EBV lytic activation [46]. Finally, there is evidence that ΔNp63 can contribute to the maintenance of EBV latency. ΔNp63 is a variant of the p63 transcription factor, which is preferentially expressed in undifferentiated basal epithelial cells [46].

The switch of latent to lytic infection is also triggered by various types of cellular stress and adaptive responses. Genotoxic stress and DNA damage response have long been known to favour EBV lytic activation in various cell backgrounds, especially in NPC cells. BRdU treatment was used in one of the oldest reports on lytic EBV activation achieved in an NPC model [70]. Ionizing radiations have been shown to induce lytic activation in LCLs [71]. As explained in details in section D1, many drugs in the arsenal of conventional cancer chemotherapy are inducers of lytic EBV activation in various cellular backgrounds. The ATM (ataxia-telangiectasia mutated) kinase and to a lesser extent TP53 often play a critical role in the sequence of events leading from DNA damage to BZLF1 expression [72, 73]. In addition, there is evidence that ATM is involved in the lytic cascade downstream of BZLF1 [74]. In other words, ATM is apparently involved in the lytic cascade upstream as well as downstream of BZLF1 expression. Endoplasmic reticulum stress, which results from large-scale protein misfolding, has been reported as a condition leading to lytic activation in LCLs [75]. Finally, the cellular stress resulting from hypoxia was reported to favour the switch from latent to lytic infection with a role of the HIF1 transcription factor in the activation of the Zp promoter [46].

2.3.3. Epigenetic factors control the switch from latent to lytic EBV infection

This point will be addressed briefly with focus on two epigenetic processes, which are critical for the switch from latent to lytic EBV infection: DNA methylation and transcriptional control by microRNAs. We have previously mentioned that BZLF1 preferentially interacts with methylated lytic promoters, whereas it is the opposite for BRLF1. Therefore, the net impact of the methylation or the demethylation of the viral genome is not easily predicted and is highly dependent on the cell background [56]. Recent work has shed some light on the role of cellular

microRNAs in the maintenance of EBV latency. Members of the miR-200 family, like miR-200b, target the transcription factors ZEB1 and ZEB2, thus having the ability to reactivate the EBV lytic gene expression when added to EBV-infected cells [76]. This applies not only to endogenous microRNAs but also to members of the miR-200 family conveyed by extracellular vesicles, which can induce lytic EBV activation in recipient cells [77]. In contrast, other microRNAs of cellular and viral origin have the ability to repress viral reactivation by targeting elements of its machinery [78].

3. Basic principles and requirements of therapies based on viral cytolytic activation in EBV-associated malignancies

Like for all Herpesviridae, production of viral particles is automatically associated with the death of cells infected by EBV. This is the reason why the productive cycle—as opposed to the state of latent infection—is often called the lytic cycle [29]. With this in mind, for a long time —going as far as the end of the seventies—many authors have considered reactivation of the lytic cycle as a possible therapeutic strategy. For a long time, the concept has evolved very slowly. One hurdle was the fact that the state of latent infection is generally very stable. In addition, in most cases it is difficult to go beyond partial reactivation with expression of some proteins of the lytic cycle like the immediate-early BZLF1 transactivator. On the other hand it was recognized that, in many cases, even partial reactivation is sufficient to block cell growth [79]. This intrinsic cytotoxic effect of partial reactivation, the possible induction of the expression of viral enzymes capable to of metabolizing a prodrug and the wish to enlarge the range of viral targets for the immune system are currently the main incentives to investigate the induction of the viral lytic activation as a therapeutic approach [30]. One additional perspective is to use precursors of radio-opaque molecules processed by viral enzymes and selectively retained in the malignant cells for specific medical imaging [73, 48]. Two EBV-encoded enzymes are usually cited as having the potential to metabolize and activate prodrugs: the EBV thymidine kinase (TK) and the EBV protein kinase (PK) encoded by the BXLF1 and BGLF4 genes, respectively. Ganciclovir (GCV) and acyclovir (ACV) are not substrates of EBV-TK. Meng et al. have formally demonstrated that among EBV-encoded kinases, EBV-PK is necessary and sufficient to phosphorylate GCV and ACV [49]. Consistent data had been previously reported by Gustafson et al [80]. The phosphorylated active forms of GCV and ACV inhibits cellular and viral replication. Fialuridine or FIAU (2′-fluoro-2′-deoxy-β-D-5-iodouracil-arabinofuranoside) is also a prodrug activated in EBV-infected cells, presumably by the EBV-TK, but its use is limited by its hepatic and metabolic toxicity [47, 48, 81]. However, it can be used occasionally, at least in animal models, for imaging purpose. Indeed, its phosphorylation by EBV-TK results in its selective retention in malignant cells. Thanks to this property, FIAU can be used for imaging in two ways either as a carrier of iodine atoms, which are opaque to X-ray or when the cold iodine atoms are substituted by radioactive isotopes as a tumour-selective radioactive emitter [47, 48, 73].

Induction of the lytic cycle can also have potentially deleterious effects. Several publications have shown that lytic activation in a small fraction of malignant cells can enhance malignant

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growth [82]. This is probably due to the increased production of inflammatory cytokines, angiogenic and growth factors in some cells undergoing the lytic cycle [83, 84]. To prevent these deleterious effects, it is important: (a) to ensure that the lytic cycle activation occurs in a large fraction of malignant cells and (b) if possible to combine lytic activation with administration of a cytotoxic prodrug, preferably with a bystander effect (see **Figure 4**).

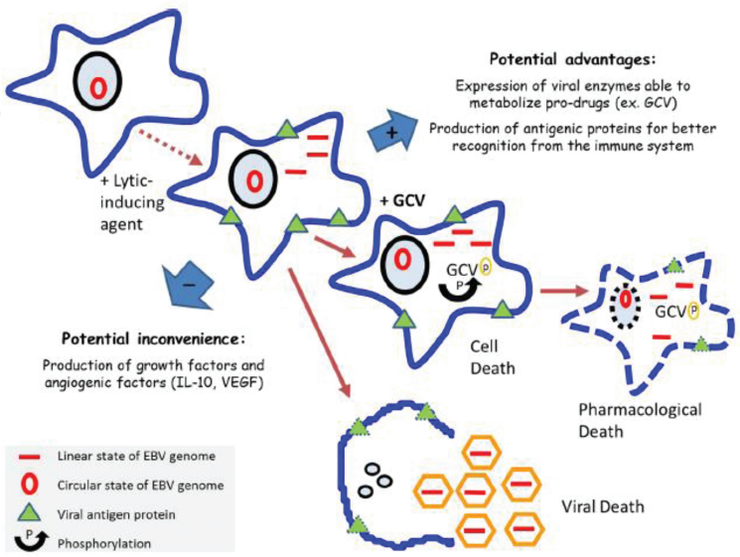

Figure 4. Importance of combining the induction of the lytic cycle with the administration of prodrugs. From a therapeutic point of view, induction of the lytic cycle has both positive and negative consequences. Positive consequences are (1) the expression of viral enzymes, which can activate prodrugs specifically in malignant cells and (2) the broadening of viral antigen expression, which is expected to facilitate specific recognition and destruction of malignant cells by the immune system. One potential drawback is an increase in the production of growth factors and angiogenic factors by malignant cells. Several advantages are expected from the use of a prodrug specifically activated in malignant cells (e.g., ganciclovir or GCV): (1) rapid pharmacological killing of malignant cells including many cells that would not undergo a full lytic cycle and therefore would stay alive for a long time in the absence of the prodrug; (2) reduction in the production of growth and angiogenic factors coming from cells undergoing the lytic cycle; (3) possible bystander killing of cells resistant to lytic cycle induction, for example, by diffusion of phosphorylated GCV through gap junctions.

These experimental approaches will be classified in three categories: (1) one based on drugs used for conventional antitumour chemotherapy; (2) one based on HDAC inhibitors and (3) finally, a miscellaneous category including various agents from PKC activators to demethylating agents.

4. Main categories of pharmacological agents with the potential of cytolytic activation of EBV in the context of NPC cells

4.1. Impact of pharmacological agents used in conventional chemotherapy

A number of drugs used in conventional chemotherapy have the ability to induce the EBV lytic cycle in various lymphoid or epithelial cell backgrounds. The drugs which are the most

active in this process—gemcitabine and to a lesser extent doxorubicin, taxol, *cis*-platinum (CDDP), 5-fluoro-uracil (5-FU) and methotrexate, have been empirically identified (*cis*-platinum and 5-FU are only active in epithelial cells; methotrexate probably only in lymphoid cells) [30, 59, 85]. Regarding NPC cells, initial data on this topic have been published by Shannon Kenney's group in 2002. They showed that CDDP, 5-FU and taxol were able to induce —or to enhance—the expression of the lytic proteins BZLF1, BRLF1 and BMRF1 in a gastric carcinoma cell line artificially infected by EBV (AGS-EBV) [59]. Simultaneously in cytotoxicity assays, synergies were found between GCV and *cis*-platinum or 5-FU. Similar results were obtained using the xenografted NPC tumour line, C18. *De novo* expression of BZLF1 and enhancement of BMRF1 expression were observed after treatment with CDDP or 5-FU. In addition, there was a synergy in tumour growth inhibition when GCV was combined with CDDP or 5-FU in intraperitoneal injections to nude mice bearing C18 xenografts. It is noteworthy that the same agents had no impact on lytic EBV-protein expression in two other NPC xenografts, C15 and C17. One specific feature of C18 shared with AGS-EBV is that, even in the absence of drug treatment, it has a low level of constitutive lytic protein expression. This is not the case for the two other NPC xenografts ([7] and P. Busson, unpublished data).

Subsequently, regardless of the cell background, gemcitabine has been confirmed as the most potent inducer of EBV lytic activation among all drugs routinely used in conventional chemotherapy [30, 73, 86]. Its capacity to contribute to lytic activation in NPC cells has been well documented by Wildeman et al. (2012) and to a lesser extent by Hsu et al. (2015) [86, 87]. In both cases, induction of lytic activation in the C666-1 cell line was stronger when gemcitabine (in the range of 3 μM *in vitro*) was combined with valproic acid (in the range of 300 μM *in vitro*). Valproic acid is a short chain fatty acid typically used as an anti-epileptic drug, which behaves, to some extent, as an HDAC inhibitor. Using the gemcitabine/valproic acid combination *in vitro* more than 80% C666-1 cells were positive for BZLF1 by immunofluorescence [86]. Wildeman's publication also provided substantial clinical data. Objective tumour responses were reported for three end-stage NPC patients subjected to a three-drug regimen combining gemcitabine, valproic acid and GCV [86]. Interestingly, 3 years later, partial tumour responses were again reported by the same group for five out of eight NPC patients refractory to conventional treatments and subjected to the almost same regimen except that GCV was replaced by valganciclovir [88]. The median survival was 9 months (95% confidence interval 7–17 months).

In some epithelial cell backgrounds, the combination of gemcitabine with an HDAC inhibitor is not mandatory for induction of the lytic cycle. Using a naturally EBV-infected gastric carcinoma cell-line, SNU-719, Lee et al. have observed induction of BZLF1 expression by gemcitabine alone at low concentrations (in the 10 nM range *in vitro*) [73]. Consistently, they have observed a synergy of gemcitabine with GCV in the treatment of SCID mice xenografted with SNU-719 gastric carcinoma cells. As pointed by the authors, a synergy between GCV and gemcitabine has also been reported in treatment of non-EBV-related malignant cells but much higher concentrations of gemcitabine were required (about 10 μM) [73, 89].

The mechanisms of lytic activation induced by drugs of conventional chemotherapy are complex and probably diverse. In the case of AGS-EBV cells, Kenney's group has highlight-

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ed several signalling pathways suspected to link cell response to *cis*-platinum and 5-FU with the expression of immediate-early viral proteins: the MAPK/ERK, p38 MAPK and PKCδ pathways. However, all these data resulted from experiments based on chemical inhibitors. Therefore, they would probably need verifications by other approaches. As previously mentioned, endoplasmic reticulum (ER) stress is one possible cell alteration leading to lytic activation [75]. However, ER stress markers are not modified in gastric carcinoma cells subjected to lytic activation driven by gemcitabine [73]. Signalling pathways involved in the response to genotoxic stress seem to contribute more consistently to viral lytic activation, especially in epithelial cells. In SNU-719 cells, knocking-down expression of ATM and TP53 by RNA-interference antagonizes BZLF1 induction by gemcitabine [73].

4.2. Impact of HDAC inhibitors

Histone deacetylases (HDAC) belong to a class of enzymes that remove acetyl groups from histones, allowing them to wrap the DNA more tightly and therefore to decrease gene expression in the corresponding part of the genome. Therefore, depending on the cell context, HDAC inhibitors (HDACi) lead to transcriptional activation of a fraction of cellular genes, which are epigenetically silenced. Many HDACi have anti-oncogenic properties. They inhibit cell proliferation and favour cell cycle arrest, differentiation or apoptosis. These effects are at least partially related to re-expression of tumour suppressor genes such as *CDKN2A* or *ATM*. Their use for viral lytic induction is based on the assumption that histone acetylation is one epigenetic mechanism contributing to the silencing of most viral genes in latently infected cells. In addition, they can have less direct effects by increasing the expression of cellular proteins involved in the lytic cycle like the ATM kinase.

The first generation of HDACi includes different categories of compounds, for example, trichostatin A (one member of the hydroxamic acid family) or short chain fatty acids such as butyrate and valproic acid. As mentioned in the introduction of this chapter, as early as 1979, sodium butyrate was shown to induce lytic EBV activation in lymphoid cells [21]. At this date, the capacity of butyrate to inhibit histone deacetylases was not known, and the exact mechanism of the lytic induction remained raveled. It is noteworthy that, much later, in the years 2000, arginine butyrate was used in combination with GCV by Faller et al. in the first clinical trial aiming at lytic EBV activation in a group of patients bearing EBV-associated malignancies. Fifteen patients with refractory EBV-associated lymphoid malignancies were included in this phase I/II trial. Significant clinical responses were obtained for 10 of them including some complete clinical and pathological responses, although with important secondary effects [90].

The impact of valproic acid on lytic EBV gene expression has been mentioned in the previous part (D1) of this chapter. In contrast with more recent HDACi, very high concentrations of valproic acid are required for lytic gene induction, often in the range of 300 μM *in vitro*. In addition, valproic acid often has limited effects on lytic gene expression when it is not combined with another drug acting by a distinct mechanism, like gemcitabine. Recent studies published by G. Miller's group have shown that valproic acid has more complex biological effects than other HDACi. In some circumstances, it behaves as an antagonist of other HDACi and as an inhibitor of EBV lytic reactivation [63, 91]. Trichostatin A has been reported to induce

EBV lytic activation in an epithelial cell line artificially infected by EBV through pathways involving activation of the PKCδ and its phosphorylation of the Sp1 transcription factor (specificity protein 1) [61]. It is noteworthy that trichostatin has also been reported to induce activation of chromosomally integrated genomes of human herpesvirus 6 (HHV6), at least *in vitro* [92, 93].

HDACi of the second generation are compounds, which have been specifically designed to inhibit HDAC enzymes. They include the hydroxamic acids like vorinostat (suberoyl anilide hydroxamic acid or SAHA) and panobinostat (LBH589) and benzamides like entinostat (MS275). Most of them have the ability to inhibit HDAC enzymes of the class I (HDAC-1, HDAC-2, HDAC-3 and HDAC-8) and class II (HDAC-4, HDAC-5, HDAC-6, HDAC-7, HDAC-9 and HDAC-10). In 2012, Faller's group has reported an interesting comparative investigation of a series of HDACi mainly of the second generation, with an assessment of their relative strength in the induction of lytic EBV activation in lymphoid cells [94]. The most potent agent was panobinostat. Entinostat and apicidin, although less effective, were active in the nanomolar range. Vorinostat, which is active in epithelial cells as mentioned below, was not active in the panel of lymphoid cells used by the authors. Panobinostat has obtained approval from the European Medicines Agency and the Food and Drug Administration (USA) for the treatment of multiple myeloma.

Information on the impact of HDACi on epithelial cells, especially NPC cells, is available in a series of reports published by A.K.S. Chiang and collaborators from Hong Kong [95, 96]. Hui et al. reported in 2010 that in AGS-EBV cells, expression of BZLF1, BMRF1 and gp350 and even production of infectious viral particles were induced by Vorinostat used at μM concentrations [96]. As in previous studies, it was observed that the AGS-EBV cells had a certain level of spontaneous lytic activation in basal conditions prior to any drug treatment [59]. It was also confirmed that Vorinostat was a poor inducer of lytic EBV activation in lymphoid cells in contrast with data obtained using epithelial cells. In 2012, the same group has reported *in vitro* induction by Vorinostat (5 μM) of BZLF1 and BMRF1 but not gp350 in the NPC cell line C666-1. The induction of BZLF1 in C666-1 cells needed higher concentration of SAHA and a longer incubation time than for the artificial EBV(+) epithelial cell lines like HK1-EBV and HONE1-EBV. Nevertheless, induction of BZLF1 was achieved using Vorinostat *in vivo* by systemic treatment of C666-1 cells xenografted into nude mice (50 mg/kg, 5 days a week) [95]. Finally, in a report of 2016, Hui et al. have used a more selective HDACi, a bacterial product called romidepsin derived from *Chromobacterium violaceum* [60]. Romidepsin has selective inhibitory action against class I HDAC enzymes (mainly HDAC-1, HDAC-2 and HDAC-3). Romidepsin was shown to induce BZLF1, BRLF1 and BMRF1 in artificially EBV-infected epithelial cells using concentrations ranging from 0.5 to 5 nM. In mice xenografted with C666-1 cells, combined systemic administration of romidepsin (0.375 mg/kg, 2 days a week) and GCV (50 mg/kg, 5 days a week) resulted in a substantial tumour growth reduction [60]. The impact of romidepsin on lytic protein expression was abrogated by a chemical inhibitor of PKCδ but not inhibitors of PI3K, MEK, p38 MAPK, JNK. Interestingly lytic activation was not impaired by an ATM kinase inhibitor in contrast with observations made on SNU-719 gastric carcinoma cells treated with gemcitabine (see part D1 of this chapter and Lee et al.) [73]. Romidepsin is

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approved by the FDA (Food and Drug Administration) but not the EMA (European Medicines Agency) for the treatment of cutaneous T-cell lymphomas. Adverse effects are nausea, vomiting, thrombocytopenia and leucopenia [97]. For future research, data on romidepsin underline the potential of novel more selective HDACi whose design is based on now-solved HDAC structures (third generation HDACi, for more information see Stahl et al. [98]).

4.3. Impact of miscellaneous pharmacological agents

In this section, we will briefly introduce other agents that have been used for lytic EBV activation but less consistently than HDAC inhibitors or conventional anti-cancer agents like gemcitabine. Phorbol-esters (like 12-*O*-tetradecanoylphorbol-13-acetate) are compounds, which activate proteins of the PKC family. In the history of EBV research, they have been among the first pharmacological agents along with sodium butyrate and BRdU used for induction of EBV lytic activation. However, to our knowledge, phorbol-esters have not been used in experiments involving NPC cells [65]. Nevertheless, as previously mentioned, one protein of the PKC family, PKCδ was reported to contribute to lytic EBV activation induced in epithelial cells by *cis*-platinum and 5-FU on the one hand and by romidepsin, an inhibitor of class I HDAC enzymes, on the other hand (see sections D1 and D2, respectively) [60, 59].

Bortezomib requires a special mention because it has been selected by a systematic screening of an FDA library of hundreds of chemical compounds to identify novel inducers of the lytic cycle (not published data but quoted by [48]). In Burkit lymphoma cells, the effect of Bortezomib on lytic activation has been reported to be dependent on the b-ZIP transcription factor C-EBP β (CCAAT/enhancer-binding protein β [99]). Bortezomib has proven to induce BZLF1 and EBV-TK expression in EBV-positive Burkitt lymphoma or gastric carcinoma cells xenografted on SCID mice [47, 48]. This process has been exploited for selective accumulation of FIAU in tumour cells expressing EBV-TK. Either for imaging purpose using this iodinated compound labelled with [^{125}I] or with therapeutic intent after labelling FIAU with [^{131}I]. To our knowledge, this approach has not been extended to NPC models. As explained in section C of this chapter, the use of FIAU in patients is compromised by the risk of acute metabolic or hepatic toxicity [81]. In more recent publication, the same group was involved in a new systematic screening of another compound library (John Hopkins Drug Library), which has identified gemcitabine as the best candidate for combination treatment with GCV [73].

Demethylating agents, especially 5-azacytidine, have been considered as potential inducers of EBV lytic activation in NPC cells. As reported by Chan et al., eight NPC patients were treated with 5-aza for one to six cycles: only mild lytic activation was recorded by RT-PCR and immunohistochemistry of lytic EBV products [100]. This relative weakness of 5-azacytidine might be explained by some distinct characteristics of BZLF1 and BRLF1 transactivation, which have been observed in the context of epithelial cells. BZLF1 transactivation of lytic gene promoters is enhanced by DNA methylation whereas it is the opposite for BRLF1 [45]. This is bad news for the impact of demethylating agents since cooperation of BZLF1 and BRLF1 is crucial for the optimal progression of lytic EBV activation.

A Notch2 inhibitor—dibenzazepine—has been shown to induce lytic EBV activation in Burkit and LCL cells by Giunco et al [101]. According to the authors, this effect depends on the

downregulation of the BATF transcription factor, which modulates BZLF1 expression. To our knowledge, lytic induction based on Notch2 inhibitors has not been investigated in NPC cells.

Finally, two groups have recently selected novel inducers of the lytic activation by high-throughput phenotypic screening from large chemical libraries containing several ten thousands of compounds [102, 103]. Tikhmyanova et al. have reported five structurally related tetrahydrocarboline derivatives, which are active in the range of 150–170 nM in various EBV-positive cell-lines including the C666-1 NPC cell line [103]. Choi et al. have published data on five novel compounds, which are distinct from PKC agonists and HDACi, one of which activates the MAPK pathways and bears structural resemblance to iron chelators [102].

5. Conclusions

The idea of inducing EBV lytic activation in malignant NPC cells with a therapeutic intent has been in the air for more than 30 years [21]. However, this therapeutic approach is still facing major obstacles. First, there is evidence that EBV latency is heavily locked in NPC cells, and several locking mechanisms are probably intimately connected with the oncogenic alterations at the levels of genome, epigenome and cell phenotype. For example, there is evidence that the wild-type ATM kinase plays a critical role at several steps of lytic EBV activation (although not under treatment by romidepsin) [60, 72, 74]. In fact, ATM is frequently downregulated in NPC cells [106]. There is also evidence that Stat3 activation prevents EBV lytic activation. Again, constitutive Stat3 activation is a common feature of malignant NPC cells. Hydroxy-methylation marks often disappear from the DNA of NPC cells [107, 108]. In a fraction of tumours, this alteration is a consequence of inactivating mutations in the *TET1*, *TET2* or *TET3* genes [11]. Regardless of its mechanism, the loss of DNA hydroxyl-methylation promotes malignant transformation and simultaneously makes the lytic promoters less sensitive to the action of BRLF1 [56].

Another difficulty is the absence of biomarkers for early identification of NPC tumours that will be responsive to various agents expected to induce the lytic cycle. We suggest that, in general, the most sensitive tumours will be those where there is a spontaneous expression of lytic genes prior to any treatment, a point that would deserve a specific investigation. In spite of all these difficulties, significant progress has been accomplished. Several objective tumour responses have been obtained in NPC patients treated with a combination of gemcitabine, valproic acid and GCV [86, 88]. Since HDACi more effective than valproic acid are now available, there is room for progress using similar patterns of treatment. At a time when the success of immunotherapies based on checkpoint inhibitors has a dramatic impact on our approach of cancer biology and therapy, several authors rightly emphasize the importance of EBV lytic activation as a mean to increase the antigenicity of malignant cells [109]. This trend is even stronger since major changes are taking place at the confluence of epigenetics, virology and tumour immunology: recent publications have shown that demethylating agents can increase tumour cell antigenicity by removing the inhibition of endogenous retroviruses [110].

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However, combining the inducers of EBV lytic activation with prodrugs specifically activated by viral enzymes remains a valuable goal. It is striking that while remarkable progress is being made concerning lytic inducers, almost nothing seems to happen regarding the prodrug candidates. So far, most investigators rely on GCV (ganciclovir), which was not designed for EBV-infected cells but is used by default. Nevertheless, a change seems to be in the air in this field too. A recent systematic proteomic analysis of EBV-PK substrates has identified hundreds of cellular proteins involved in DNA damage response, mitosis and cell cycle. More importantly, in terms of pharmacology, the analysis of the phosphosites of these substrates reveals a proline-rich motif signature, which will probably be helpful for the design of artificial substrates [50].

Competing interests

The authors declare no financial or non-financial competing interests.

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