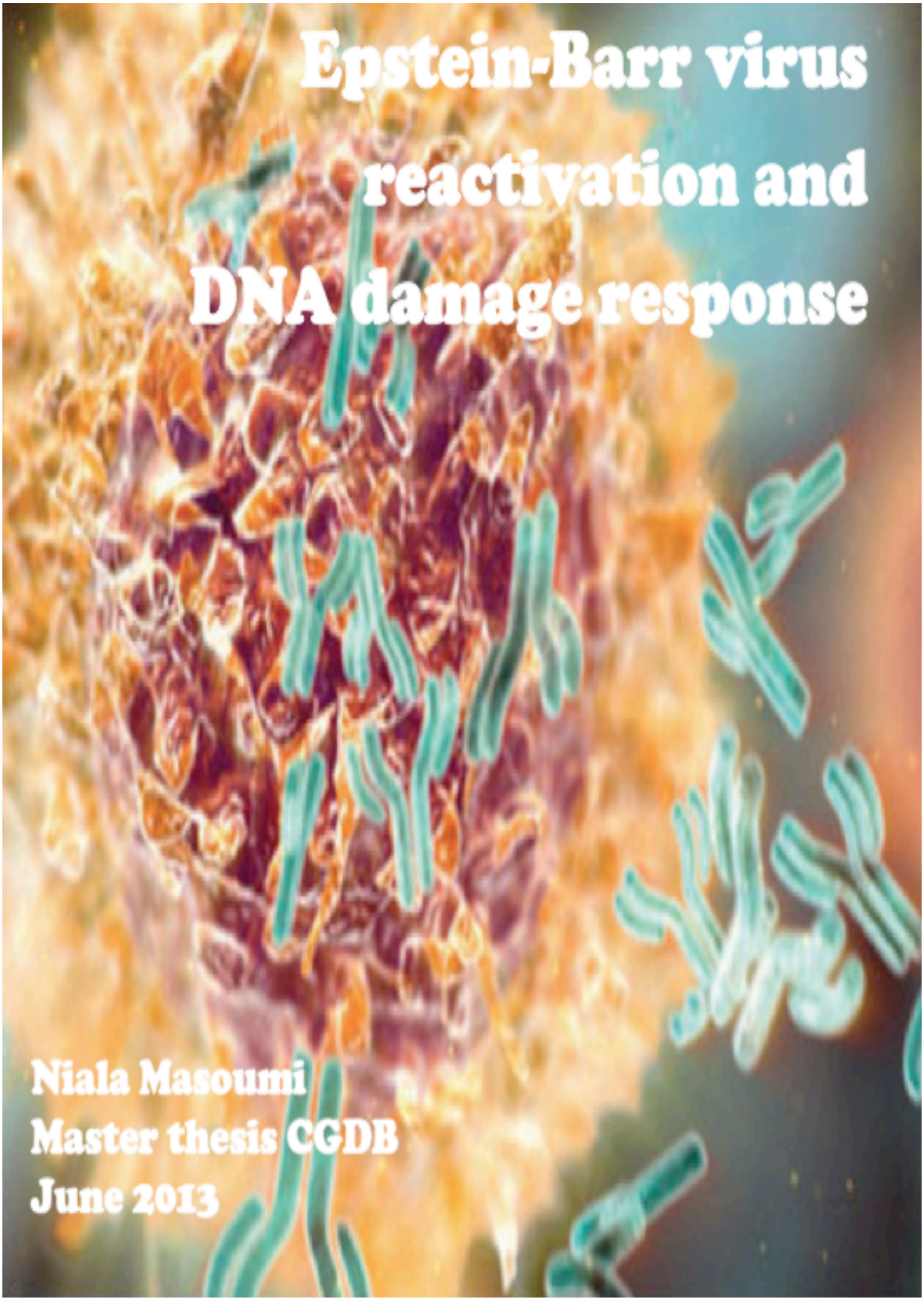
Epstein-Barr virus
reactivation and
DNA damage response
Niala Masoumi
Master thesis CGDB
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Epstein-Barr virus reactivation and DNA damage response

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Master thesis
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Abstract

The latent form of Epstein-Barr virus (EBV) is associated with many malignancies, including nasopharyngeal carcinoma, which makes EBV itself an attractive target of therapy. The reactivation of EBV toward lytic gene expression cascade will lead to expression of proteins involved in viral genome replication and formation of new virions, which will induce a strong immune response. The kinases expressed exclusively during the lytic cycle will also sensitize the tumor cells to antiviral treatment. This concept has recently been incorporated into a combination therapy, referred to as cytolytic virus activation therapy, in which a combination of gemcitabine (a chemoagent), valproic acid (a histone deacetylase inhibitor) and subsequent addition of ganciclovir (an antiviral agent), has been used in clinical studies with promising results. The molecular mechanisms by which these compounds co-operate, in order to trigger DNA damage response with subsequent lytic induction and tumor cytotoxicity, is discussed in this review. These agents are shown to work in multiple ways together. However, other EBV-associated malignancies might benefit form different set of compounds and this needs to be investigated. Further studies are required to analyze the effect of different combinations, in order to create potential stronger responses. These informations will be highly beneficial to treat other EBV-associated malignancies by use of EBV as the main target in a combination therapy.

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Introduction

Epstein–Barr virus (EBV) is a gammaherpes virus associated with multiple malignancies, including undifferentiated nasopharyngeal carcinoma (NPC) (Sun, Tong et al. 2011), which is a leading form of cancer in many countries, including Southeast Asia, the Arctic and the Middle East/North Africa, and southern China (De-The 1981; Parkin and Muir 1992; 2006; Sun, Tong et al. 2011). The consistent presence of EBV genome in many EBV-associated malignancies, including NPC, made EBV itself an attractive target of therapy, for killing of the tumor cells, exclusively. In NPC, among many other EBV-associated diseases, EBV resides in its latent state, in which only a few essential viral proteins necessary for EBV maintenance and tumor growth are expressed, which enable tumor cells to evade the immunsystem (Middeldorp, Brink et al. 2003). Epigenetic modifications, such as methylation and histone modification of the chromatin play major roles in maintaining the latent state of EBV infection (Minarovits 2006). The reactivation of EBV toward its lytic state will lead to expression of proteins involved in viral genome replication and formation of new virions, which will induce a strong immune response. Furthermore, this reactivation step will sensitize the tumor cells to antiviral treatment. Chemoagents alone, or in combination with histone deacetylation inhibitors (HDACi) have been tested in this concept, in order to trigger the lytic induction with promising results (Feng, Israel et al. 2002). These beneficial findings have been incorporated into a combination therapy, named as cytolytic virus activation (CLVA) therapy, in which NPC end stage patients have been treated with a chemoagent, an HDACi, and an antiviral treatment (Wildeman, Novalic et al. 2012). The combination of chemo and HDACi resulted in induction of lytic gene expression of EBV with sinsubsequent tumor cytotoxicity, and an additional antiviral therapy enhanced the cytotoxicity even more. However, it is of high importance to understand the exact molecular mechanism by which these compounds co-operate and mediate a strong synergetic effect. These insights are highly beneficial for designing new combination therapies, in order to treat EBV-associated malignancies. In this review we will discuss the molecular basis of EBV and its associated malignancies, the compounds known to trigger EBV lytic induction and cytolytic effect, and most importantly, how these combinations of compounds co-operate and trigger a potent synergetic effect.

Epstein-Barr virus

Soon after the discovery of Burkitt's lymphoma (BL) (Burkitt 1958; Burkitt 1962) herpesvirus-like particles were discovered in tumor cells from BL biopsies by electron microscopy (Epstein, Achong et al. 1964; Pulvertaft 1964). Subsequently, EBV or human herpesvirus 4 (HHV4) was found to be the virus behind infectious mononucleosis (IM), after a lab technician in Philadelphia experienced an IM and seroconverted in the process (Henle and Henle 1968).

EBV is a virus of the genus Lymphocryptovirus within the subfamily of gammaherpesviruses, which is an enveloped herpesvirus consist of a toroid-shaped protein core wrapped with DNA, a nucleocapsid, a tegument protein, and a linear double stranded DNA molecule of 172 kb (Baer, Bankier et al. 1984; Wedderburn, Edwards et al. 1984; Middeldorp, Brink et al. 2003; Tao, Young et al. 2006; Maeda, Akahane et al. 2009). The EBV genome contains over 100 open reading frames (ORF), located on BamHI restriction fragment, and about 50-60% characterized gene products to date (Brink, Oudejans et al. 1997; Brink, Vervoort et al. 1998; Tempera, Klichinsky et al. 2011). Genomic sequencing of EBV isolates has revealed the existence of two predominant EBV strains; the A-type and B-type. EBV selectively infects two types of target cells, B-lymphocytes and stratified squamous epithelia (Yao, Rowe et al. 1991). The A-type, however, has most been found in EBV-associated diseases and is shown to transform B-cells in vitro more efficiently than the B-type (Yao, Rowe et al. 1991; Khanim, Yao et al. 1996; Fielding, Sandvej et al. 2001).

EBV is linked to a variety of neoplasms (Thompson and Kurzrock 2004; Miszczak, Slonska et al. 2013), including lymphoid tumors like BL, Hodgkin's disease (HD), lymphoproliferations in solid organ transplant, natural killer (NK) T-cell lymphoma or bone marrow recipients (posttransplantation lymphoproliferative disease, PTLD), AIDS-associated lymphomas, NPC, gastric carcinoma, carcinomas of the salivary glands, some cases of thymic carcinoma, and a mesothelial tumor, leiomyosarcoma. Furthermore, EBV is linked to a panel of autoimmune diseases, like rheumatoid arthritis (RA), and multiple sclerosis (MS), and systemic lupus erythematosus (SLE) (Serafini, Rosicarelli et al. 2007; Niller, Wolf et al. 2008; Niller, Wolf et al. 2009).

EBV and cancer

EBV was the first directly implicated human virus in carcinogenesis. The variations in the prevalence of these cancers have shown that normal host populations might have vastly different susceptibilities to EBV-related tumors (Sixbey, Nedrud et al. 1984; Murray and Young 2002). EBV uses its viral proteins that mimic many transcription factors and growth factors, in order to alter the cellular pathways and deregulate multiple homeostatic cellular functions (Sixbey, Nedrud et al. 1984; Babcock, Decker et al. 1998). In many EBV-associated cancers the EBV genome is subject to extensive epigenetic regulation. The cellular genomes of EBV-infected tumor cells and their normal counterparts carry differing epigenetic marks, due to diverse epigenetic dysregulations. Hypermethylation of many genes, for instance, has been noted in a number of EBV-associated cancers (Hutajulu, Indrasari et al. 2011)

Nasopharyngeal carcinoma

NPC is a specific cancer with ethnic and geographic distributions. NPC is rarely found in most parts of the world, but is a leading form of cancer in some well-defined populations, such as natives of southern China, Southeast Asia, the Arctic and the Middle East/North Africa (De-The 1981; Parkin and Muir 1992; 2006; Sun, Tong et al. 2011). In the generation of NPC carcinogens (in food) play major roles, along with a long-lasting childhood viremia and the patient's genetic background (Feng, Huang et al. 2002; Lo, To et al. 2004; Xiong, Zeng et al. 2004; Zeng, Liu et al. 2004). All NPC tumors are shown to be monoclonally EBV infected (Raab-Traub and Flynn 1986). Furthermore, it is known that NPC tissue carry a high load of chromosomal abnormalities, determined by comparative genomic hybridization (CGH) and loss of heterozygosity (LOH)-analysis (Lo, To et al. 2004; Li, Wang et al. 2006; Tao and Chan 2007). In contrast to severe dysplastic lesions and carcinoma in situ, EBV infection has not been found in in normal epithelia or in low-grade dysplasia to date (Pathmanathan, Prasad et al. 1995). Therefore, one hypothesis is that the EBV infects cells first and this will cause mutations that cause malignancy. The other theory is that cells with genetic or epigenetic defects may survive and clonally expand throughout the nasopharynx. EBV infection may come in second and cause a CGI methylator phenotype (CIMP), (which is a mechanism of gene inactivation in cancer) (Toyota, Ahuja et al. 1999), and pushing the epigenetically disrupted cells towards malignancy, invasion and metastasis (Tao and Chan 2007). Besides the frequent genetic damage, high levels of CGI methylation spread throughout the whole genome were especially observed in EBV+ NPC (Kwong, Lo et al. 2002). The activity of DNA methyltransferases 1, 3a, and 3b that are induced by the latent membrane protein (LMP)1, which is a viral oncoprotein, might have caused this increased methylation event in EBV+ NPC.

LMP1 transfection into carcinoma cells is shown to suppress E-cadherin (CDH1) expression, which correlates with more invasive growth (Tsai, Tsai et

al. 2002). The CDH1 promoter is methylated in about 50% of primary tumors (Zheng, Pan et al. 1999; Tsao, Liu et al. 2003; Krishna, Kattoor et al. 2005). EBV-infection of NPC correlates strongly with the occurrence of CDH1 hypermethylation and therefore EBV has been associated with the rapid metastasis of the disease, especially when LMP1 is expressed (Krishna, Kattoor et al. 2005; Niemhom, Kitazawa et al. 2008).

EBV (mechanism of) infection

EBV is known to infect about 90% of the adult population worldwide and its infection is generally restricted to humans, with exception of some experimentally infected monkeys (Wedderburn, Edwards et al. 1984; Robertson, Ooka et al. 1996; Toussirot and Roudier 2008; Odumade, Hogquist et al. 2011). The virus is shed into the saliva of persistently infected individuals who spread the virus to uninfected individuals. The primary EBV infection is usually asymptomatic and occurs through close contacts between adults and children, generally through saliva, within the first 3 years of life, and the majority of the world population passes through primary infection within their first 10 years of life (Wolf, Haus et al. 1984; Borza and Hutt-Fletcher 2002). EBV infections in adults, however, can result in infectious mononucleosis. Once the infection has occurred, EBV becomes latent and resides in lymphocytes in the peripheral blood, rendering the infected individual a lifelong EBV carrier (Tao, Young et al. 2006; Maeda, Akahane et al. 2009).
EBV is capable of accessing both squamous epithelial cells of the oropharynx and B cells. When transmitted orally, EBV might initially infect or resting B lymphocytes and squamous epithelial cells, which reside at the surface of many lymphoid organs, such as tonsillar epithelia. EBV virions then bind to the complement receptor CD21, which is expressed on primary B cells, through its viral envelope glycoprotein gp350. Subsequently, they partly enter the cells but partly remain on the B cell surface (Shannon-Lowe, Neuhierl et al. 2006) (Wolf, Haus et al. 1984; Borza and Hutt-Fletcher 2002).
In epithelial cells lacking CD21, the EBV BMRF-2 protein is known to interacts with β1 integrins. The EBV gH/gL envelope protein interaction with αvβ6/8 integrins is shown to trigger fusion. EBV binding to CD21 will cause the activation of tyrosine kinase *lck* and mobilization of calcium (Gordon, Walker et al. 1986; Cheung and Dosch 1991). A subsequent incensement of mRNA synthesis, homotypic cell adhesion, surface CD23 expression, which is a characteristic surface marker for activated B cells, and interleukin (IL)-6 production (Tanner, Weis et al. 1987; Alfieri, Birkenbach et al. 1991; Tanner, Alfieri et al. 1996) will occur, in response. nucleocapsid will get released into cytoplasm in response to endocytosis of the virus into vesicles and its fusion with the vesicle membrane. The viral genome will subsequently get uncoated, and delivered to the nucleus, and will immediately circularize (Allday, Crawford et al. 1989).

Upon EBV infection, the EBV nuclear antigen leader protein (EBNA-LP) and EBV nuclear protein (EBNA) 2 proteins are shown to be the first proteins to be detected (Sung, Kenney et al. 1991). At 24 to 48 hours after infection, the promoter will shift between the C promoter (Cp) and the W promoter (Wp) (Rowe 1999), which launches a well-orchestrated series of molecular events starting with the expression of LMP1, the major viral oncogene, and other host genes (see table 1) (Middeldorp and Pegtel 2008). The EBNA transcriptional products are shown to affect the transcriptional control and the expression of the viral LMP-encoding genes, and many other cellular genes. 24 to 48 hours after the infection, cellular S-phase will get initiated, in response to the combined action of viral and cellular proteins (Tugizov, Berline et al. 2003; Xiao, Palefsky et al. 2009).

After the acute phase of primary infection the virus persists lifelong in the organism, residing latently in memory B cells, with their genome present as multicopy circular plasmids (episomes) (Niller, Glaser et al. 1995). The latent and lytic replication cycles of EBV will be discussed in the next chapter.

Table 1: ***Overview of the major EBV- gene products and their function***

Name	Protein function
EBNA1	Essential for EBV immortalization of cell, replicates EBV genome, segregates viral episomes at mitosis.
EBNA2	Transcriptional coactivator, one of first viral proteins produced during EBV infection, upregulates expression of viral and cellular genes, especially c-myc, essential for EBV immortalization of cell, .
EBNA3	
3A	Essential for EBV immortalization of cell, interacts with CBF1.
3B	Not essential for EBV immortalization of cell, interacts with CBF1, with largely unknown functions.
3C	Essential for EBV immortalization of cell, overcomes retinoblastoma protein (pRB) checkpoint in cell cycle, increases production of LMP1.
EBNA-LP	one of first viral proteins produced during EBV infection, interacts with EBNA2 to inactivate p53 and Rb, interacts with transcription factors in notch signaling, redistributes EBNA3A in nucleus, contributes to EBV immortalization of cell.
LMP1	Mimics CD40 ligand binding signal, acts a constitutively active receptor, essential for EBV immortalization of cell.
LMP2A and B	Drives EBV into latency. May play a role in oncogenesis in Hodgkin's disease and nasopharyngeal carcinomas.
EBER1 and 2	Normalize transcription block, prevent apoptosis, suppress antiviral effects of interferon α and γ, induce IL-10 production, these all by inhibition of PKR.

EBV latent and lytic replication cycles

The initial EBV infection occurs in the oral compartment, which is also named as the tonsillar. The replication of EBV producing infectious virus, is known as EBV lytic replication (Hammerschmidt and Sugden 1988; Faulkner, Krajewski et al. 2000). In normal oral epithelial cells the lytic form of virus is present, whereas, EBV infection of B cells generally results in one of the three forms of latent viral infection, which will be discussed below (Marchini, Longnecker et al. 1992; Geiser, Cahir-McFarland et al. 2011). Lytic replication state differs from the latent amplification state in multiple rounds of replication (Hammerschmidt and Sugden 1988). In this lytic replication state many gene products, such as six nuclear proteins (EBNA-1, 2, 3A, 3B, 3C and EBNA-LP), three membrane proteins (LMP-1, 2A , 2B), EBV-encoded small RNAs (EBER1, 2) are transcribed by a virally encoded DNA polymerase. The host cell may be killed by the release of infectious viral particles in this viral lytic state (Marchini, Longnecker et al. 1992; Feng and Kenney 2006; Geiser, Cahir-McFarland et al. 2011).

The switch between lytic and latent infection is mediated in response to the expression of BZLF1 (or ZEBRA) and BRLF1, which are the two key EBV immediate-early (IE) lytic genes (see figure 1). These genes encode transactivators that in turn activate viral genes and certain cellular promoters and, thereby, activation of early gene expression initiating viral genome replication and late gene expression of structural proteins and virion formation (Chevallier-Greco, Manet et al. 1986; Adamson and Kenney 1998; Darr, Mauser et al. 2001; Tsurumi, Fujita et al. 2005; Dickerson, Xing et al. 2009).

In addition, expression of the BRRF1-encoded early gene product is sufficient for reactivation of the lytic form of EBV infection in some latently infected epithelial cell lines, because of its ability of activating BZLF1-promoter indirectly through cellular factors (Hong, Delecluse et al. 2004; Hagemeier, Barlow et al. 2011) (see figure 1). A number of the major EBV proteins, named in this review, and their function are listed in table 1.

In contrast to the lytic replication, EBV latency program does not produces virions and latent EBV expresses the genes in one of three latency programs. EBV persists latently within B cells and epithelial cells (Amon and Farrell 2005; Odumade, Hogquist et al. 2011).

However, different latency programs, such as latency I, latency II, or latency III, are possibly present in both types of cell. Each of these latency programs leads to expression of a limited, distinct set of viral genes (Delecluse, Feederle et al. 2008; Hutzinger, Feederle et al. 2009). Latency type II, was first detected in NPC, but is also shown to be the prevailing expression pattern in most other EBV-positive tumors that occur in the immunocompetent host, such as Hodgkin's disease and T- and B-cell non-Hodgkin lymphomas (NHLs) (Middeldorp, Brink et al. 2003).

Only a restricted number of the viral genomes get expressed in the latently infected cells. The replication of the virus is mediated by a viral origin of replication, EBNA-1 protein, and the host-cell DNA polymerase (Marchini,

Longnecker et al. 1992; Geiser, Cahir-McFarland et al. 2011). In this latency state, EBV genomic DNA exists as a closed circular plasmid and appears to behave just like host chromosomal DNA, packaged in nucleosomal arrays with cellular histones (Dyson and Farrell 1985; Adams 1987), and replicated once during S phase (Adams 1987). Only one viral cis element, OriP, and one viral protein, EBNA1, appear to be required for viral DNA maintenance in this state. A latent form of EBV infection is not lethal for the host cell. (Yates, Warren et al. 1984; Yates, Warren et al. 1985).

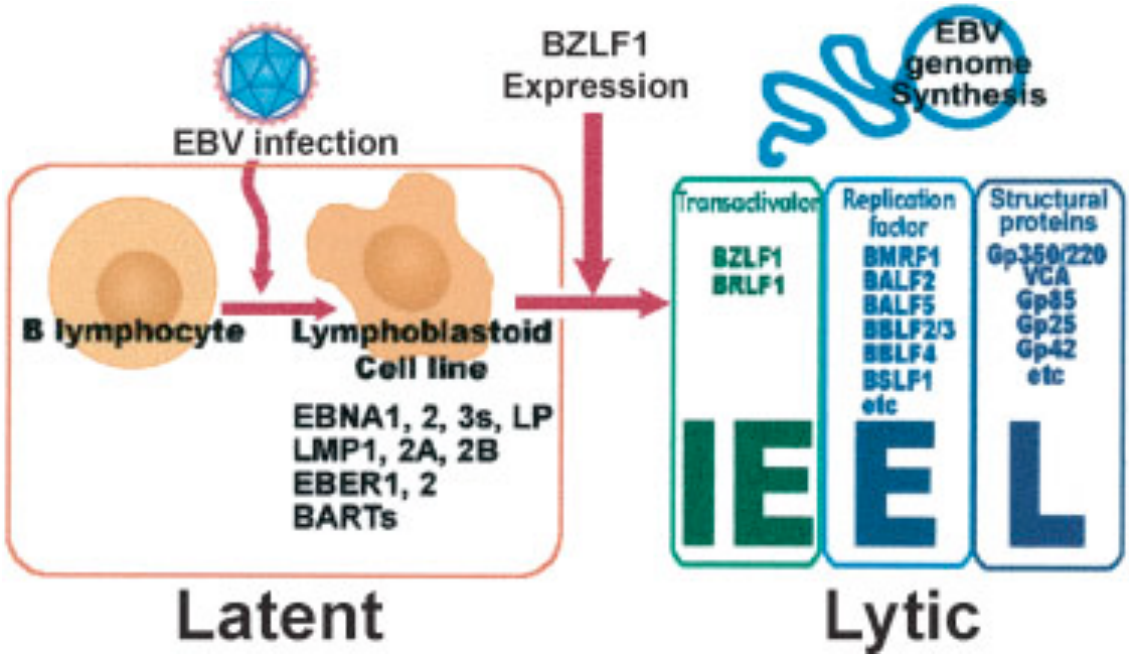

Figure 1: ***The life cycle of the Epstein-Barr virus***
EBV mostly infects resting B lymphocytes, and the resulting lymphoblastoid cell lines (LCLs) express a limited number of EBV gene products, as indicated in the orange box. Upon induction of lytic programme, the two EBV immediate-early (IE) lytic genes, BZLF1 and BRLF1, get expressed and activate viral and certain cellular promoters, thus activation of early gene expression involved in DNA replication and metabolism, followed by expression of late genes and viral structural proteins (Tsurumi, Fujita et al. 2005).

CLVA therapy

Latent state of EBV infection is linked to malignancies, including NPC (Straussberg, Amir et al. 1993). Only the fact that EBV genome is present in all of the EBV-positive tumor cells suggests that using approaches to specifically kill EBV-infected cells could be a useful approach in treatment of malignancies, such as NPC (Straussberg, Amir et al. 1993; Feng, Israel et al. 2002). Several studies have explored the therapeutic benefits of induction of EBV lytic replication in EBV-associated tumors with promising results (Lin, Smith et al. 1984; Lin, Nelson et al. 1986; Rickinson, Lee et al. 1996; Westphal, Mauser et al. 1999). Such an approach is also highly tumor-specific, since it only affects the tumor cells and not the EBV negative neighboring cells.

Regarding to NPC tumors, it is known that these tumor cells harbor EBV in its latent state (Middeldorp, Brink et al. 2003), which enables them to evade the immune system, even when an strong immune response to multiple viral antigens is created (Hislop, Taylor et al. 2007; Middeldorp and Pegtel 2008). Many studies aimed for activating the EBV lytic phase for therapeutic purposes, since inducing the viral lytic phase makes tumor cells susceptible for immune recognition and antiviral therapy (Moore, Cannon et al. 2001; Hislop, Taylor et al. 2007). A number of chemotherapeutic agents are capable of inducing the switch from the latent to lytic EBV infection (Feng, Israel et al. 2002). The addition of an HDACi to chemotherapeutic agents, in order to achieve a stronger induction of the lytic cycle, is proved to be more successful than chemotherapy alone. The reason behind this enhancement effect is not understood yet, but is hypothetically linked to increasement of DNA-Damage Responses (DDR) sensitivity, caused by HDACi's (Feng and Kenney 2006). An antiviral component was first added to this combination therapy in 1998, in order to block virus replication and kill proliferating infected cells, and was administered to a patient with EBV-positive lymphoma, which further decreased the tumor development (Mentzer, Fingeroth et al. 1998).

In 2012, a novel combination therapy was developed by Wildeman and colleagues, in which the chemotherapeutic agent gemcitabine (GCb) was combined with a HDACi, valproic acid (VPA), as lytic inducer. An antiviral treatment with ganciclovir (GCV) was subsequently added to this combination, which increased specific cytolysis. Sensitizing tumor cells to GCV by HDACi's occur due to induction of TK and BGLF4 expression that are kinases exclusively expressed during the lytic EBV cycle. These kinases phosphorylate GCV, resulting in its conversion into its active cytotoxic form. Phosphorylated GCV gets incorporated into a replicating DNA strand, which is not only able to inhibit the virally encoded and cellular DNA polymerase. This will terminate the nascent DNA, which leads to subsequent cell death (Connors 1995). However, in some cases it also moves to adjacent cells, and creates "bystander" killing (Freeman, Abboud et al. 1993; Tao, Young et al. 2006; Meng, Hagemeier et al. 2010). The combination treatment, named as CLVA therapy, was optimized and validated first in NPC cell lines and, subsequently, tested in 3 Dutch patients with NPC. The results showed a clinical response and improvement in quality of

life during and after therapy, with transient and only moderate side effects (Wildeman, Novalic et al. 2012). The positive outcome of this therapy opened a generic approach for treatment of multiple EBV-associated malignancies.
The main steps of this therapy are first induction of EBV lytic gene expression, by a combined treatment with a chemotherapeutic agent and an HDACi, and the subsequent addition of an antiviral treatment. In this report we will discuss the mechanism of virus-reactivation and DDR by chemotherapy, and the role of HDACi's, such as VPA in this process.

The DNA damage response

Cells have evolved several systems to detect DNA damage, in order to counter the threat of constant assaults by endogenous and environmental agents to the DNA. Such responses, which are referred to as DNA damage response (DDR), have an impact on a wide range of cellular events and are recognized as an important innate tumor suppressor pathway (Lindahl and Barnes 2000; Jackson and Bartek 2009). The wide diversity of DNA-lesion types compels largely distinct DNA-repair mechanisms, such as mismatch repair, base excision repair, nucleotide excision repair (NER), Non-homologous end-joining (NHEJ), Homologous recombination (HR), ataxiatelangiectasia mutated kinase (ATM)-mediated DDR signaling, and ataxia-telangiectasia and RAD3-related kinase (ATR)-mediated DDR signaling (Jackson and Bartek 2009).
In mammalian cells ATR and ATM are the key DDR signaling components. The ATR signaling pathway is activated due to ssDNA exposure following replication fork collapse, while processing of this damage to dsDNA breaks (DSBs) leads to activation of ATM pathway (Khanna and Jackson 2001; Lee, Ghirlando et al. 2003; Zou and Elledge 2003). Protein kinases CHK1 and CHK2, together with ATM and ATR, act to reduce cyclin-dependent kinase (CDK) activity by various mechanisms, such as activation of the p53 transcription factor (Kastan and Bartek 2004; Bartek and Lukas 2007) (see figure 2). ATM/ATR signaling also enhances repair by modulating the phosphorylation, acetylation, ubiquitylation or SUMOylation of DNA repair proteins, leading to their activation (Huen and Chen 2008). The above-mentioned events allow the resumption of normal cell functioning, thus causing a cell-cycle arrest, in order to initiate a DNA repair mechanism. However, in case of failure in removing the damage, chronic DDR signalling triggers cell death by apoptosis or cellular senescence (Campisi and d'Adda di Fagagna 2007; Halazonetis, Gorgoulis et al. 2008). In response to DNA damage chromatin structures get "relaxed" due to demethylation, phosphorylation and acetylation, which in turn activates different pathways. (Bartek and Lukas 2007; Misteli and Soutoglou 2009). A well-characterized example of this is ATM/ATR/DNA-PK mediated phosphorylation of serine 139 of the histone H2A variant, H2AX, on chromatin flanking DSB sites (see figure 2). The phosphorylation of H2AX leads to the recruitment of Mre11-Rad50-Nbs1 (MRN)-complex and 53BP1, at the site of the DSB, and this in turn will lead to

amplification and propagation of DDR, due to its function as a transcriptional coactivator of the p53 tumor suppressor protein (Stewart, Wang et al. 2003; Xiao, Li et al. 2009).

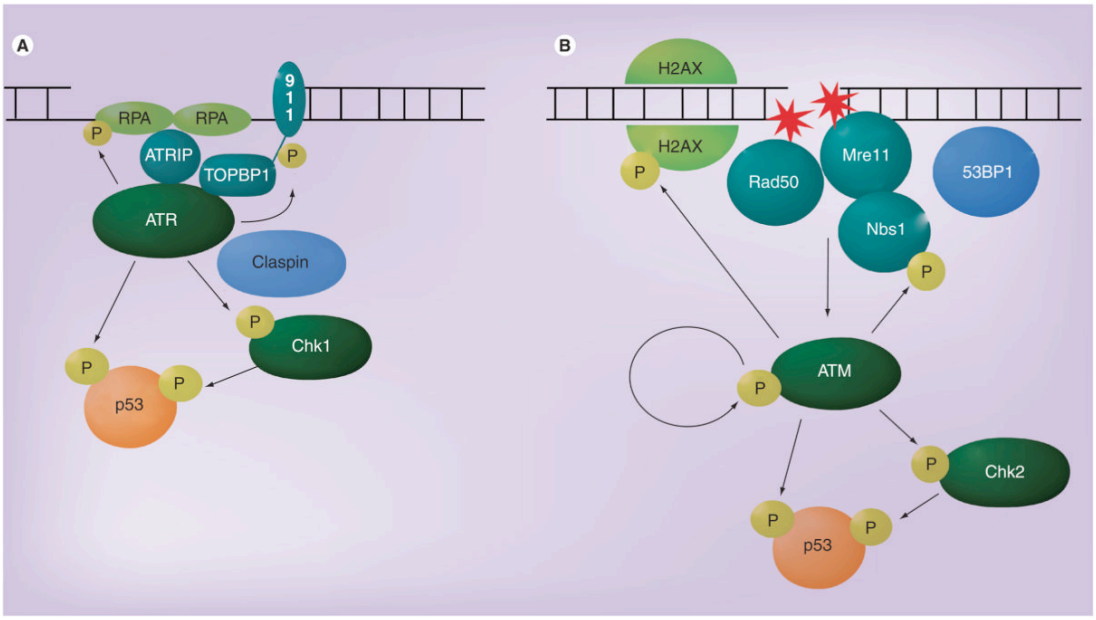

Figure 2: ***DNA damage response to single and double-stranded breaks.***
A: PRPA coats ssDNA , which subsequently gets recognized by an ATR–ATRIP complex. The 9–1–1 complex recruits the adaptor TOPBP1 that activates ATR in the presence of ATRIP. This will lead to phosphorylation of Chk1 and p53, among other ATR-mediated downstream targets.
B: DNA double-stranded breaks are first detected by the MRN-complex, which promotes autophosphorylation of the ATM. Activated ATM initiates a downstream cascade of events, including phosphorylation and activation of its downstream effector Chk2 kinase, p53, H2AX tail (γ H2AX) and Nbs1, among many other targets. The phosphorylation of H2AX serves as a scaffold for the recruitment of 53BP1 at the site of the DSBs, which mainly function as a transcriptional coactivator of the p53 tumor suppressor (Nikitin and Luftig 2012).

DDR and EBV

The DDR is implicated as a sensor of oncogenic virus infection. The replication of tumor viruses is linked to their ability to drive cell proliferation, in order to promote an S-phase environment to replicate their nucleic acid during infection, which leads to replicative stress and activation of the DDR. The viral proteins and structures expressed during the lytic and latent cycles of infection are also capable of directly interacting with and engaging the function of DDR components, which generally leads to cell-cycle arrest and DNA repair. Persistent DDR has also been linked to the aberrant secretion of pathogenetically important inflammatory mediators from infected cells, such as interleukin (IL)-6 and tumor necrosis factor α (Freund, Orjalo et al. 2010). Moreover, given the growth-suppressive or deleterious consequences of the viral genome caused by activated DDR components, tumor viruses have developed mechanisms to attenuate the DDR pathways (Leidal, Pringle et al. 2012; Nikitin and Luftig 2012).

Regarding to EBV the DDR has been implicated in regulation of both latent and lytic cycles of EBV infection. The primary infection of human B cells by EBV activates an ATM-dependent DDR, including phosphorylation of H2AX, the formation of nuclear foci of the DDR scaffold 53BP1, autophosphorylation and activating of ATM and Chk2. The increased EBNA-LP protein, modest EBNA3 family protein expression, and high EBNA2-driven output, generally characterize the EBV-infected hyperproliferating cells (see figure 3) (Nikitin, Yan et al. 2010). EBNA3 family proteins accumulate, due to the transition of viral Wp promoter to Cp, leading to attenuation of EBNA2/LP-driven gene expression, including c-Myc transcripts and genome-wide c-Myc targets (see figure 3) (Woisetschlaeger, Yandava et al. 1990; Lacoste, Wiechec et al. 2010; Nikitin, Yan et al. 2010). EBNA3C, in fact, is required for attenuation of the EBV-driven DDR, through repressing the cyclin-dependent kinase inhibitors p14ARF and p16INK4A, among other downstream effectors of the DDR (Yi, Saha et al. 2009; Skalska, White et al. 2010; Maruo, Zhao et al. 2011), as well as association with Chk2 and impinging on its function (Choudhuri, Verma et al. 2007). Of note, also other EBV latent proteins such as EBNA1 and LMP1 have been shown to inhibit the DDR, through increased levels of reactive oxygen species (ROS), and repression of ATM, respectively. Therefore, DDR is no longer activated in EBV-immortalized cells grown out in vitro. (Gruhne, Sompallae et al. 2009; Cai, Guo et al. 2011). The diverse mechanisms by which EBV latency proteins activate or perturb the DDR might affect the pathogenesis of EBV-associated tumors. For an overview of EBV oncoproteins affecting the DDR see table 2.

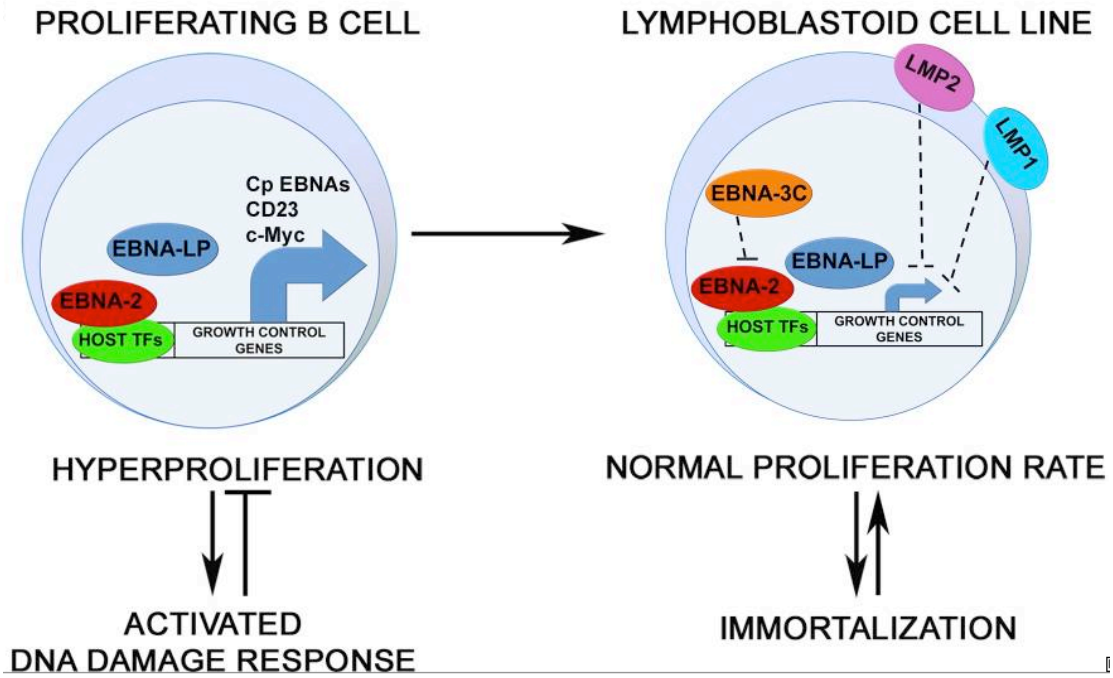

Figure 3: ***the EBV-induced DDR***
EBNA2 and EBNA-LP are associated with cellular transcription factors (TF) to potently upregulate the expression of Cp-driven EBNAs, c-Myc and CD23 and, thereby, activating the host DDR (left). Later in infection, the activity of the EBNA3 proteins, in particular EBNA3C, downregulates EBNA2 function, while LMP1 and LMP2 are upregulated and work together in the constitutive expression of host growth control genes and enhanced cell survival (right) (Nikitin, Yan et al. 2010).

DDR and EBV lytic replication cycle

During the lytic EBV replication cycle the ATM-dependent DNA damage checkpoint signaling, including phosphorylated ATM, H2AX, Chk2 and p53 are activated (Kudoh, Fujita et al. 2005; Kudoh, Iwahori et al. 2009). BPLF1 is a structural tegument protein playing a role in DDR as trans-activator of viral immediate-early genes (Schmaus, Wolf et al. 2004). 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 (Gastaldello, Hildebrand et al. 2010). 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 (Mauser, Saito et al. 2002), along with facilitating the viral DNA replication through engaging the 53BP1, a DNA repair protein (Bailey, Verrall et al. 2009).

Table 2: ***overview of EBV oncoproteins affecting the DDR***

EBV oncoproteins	References
EBNA2/LP ➔DDR	Nikitin et al, 2010
EBNA1➔ROS ➔DDR	Gruhne et al, 2009
EBNA3C ✖ early DDR	Nikitin et al, 2010
EBNA3C ✖ Chk2 ✖p53	Choudhuri et al, 2007; Yi et al, 2009
EBNA3C ✖ G2/M checkpoint	Gruhne et al, 2009b; Parker et al, 2000
LMP1 ✖ ATM	Gruhne et al, 2009

✖ Inhibition
➔ Activation

Chemotherapeutic agents as EBV lytic-cycle inducers

Many different treatments, including sodium butyrate, phorbol ester, transforming growth factor β, and activation of the B-cell receptor, have aimed the activation of BZLF1 and BRLF1 transcription factors, in order to induce the EBV lytic infection (Feng and Kenney 2006). A variety of other disparate agents with different mechanisms of inducing EBV lytic gene expression have also been used to induce EBV lytic gene expression in tumor cells, including butyrate, VPA, rituximab, bortezomib, and cis-platinum (Mentzer, Fingeroth et al. 1998; Westphal, Blackstock et al. 2000; Moore, Cannon et al. 2001; Feng, Hong et al. 2004; Daibata, Bandobashi et al. 2005; Fu, Tanhehco et al. 2008). Some of these compounds are chemotherapeutic agents, such as GCb, or a member of histone deacetylase (HDAC) inhibitors (HDACi's), such as VPA and butyrate.

Different chemotherapeutic agents have been used to treat EBV-associated carcinomas, such as NPC, with pros and cons (Bensouda, Kaikani et al. 2011). A number of chemoagents, such as GCb, doxorubicin, cis-platinum, 5-fluorouracil (5-FU), and methotrexate, are also capable of inducing EBV lytic viral gene expression in EBV-positive epithelial and B-cell tumors (Zheng, Pan et al. 1999; Feng, Israel et al. 2002; Tsai, Tsai et al. 2002), with GCb being one of the most effective chemotherapy treatments for NPC. (Bensouda, Kaikani et al. 2011).

GCb is a nucleoside anti-metabolite that gained worldwide interest in the oncology community for its broad spectrum of activity against various solid tumors, including head and neck, non-small-cell lung, ovarian and pancreatic cancers, and NPC (Catimel, Vermorken et al. 1994; Barton-Burke 1999). Its ease of administration and its favorable toxicity profile has made it attractive for use in the palliative setting (Foo, Tan et al. 2002; Ma, Tannock et al. 2002; Bhende, Seaman et al. 2004; Zhang, Zhang et al. 2008; Bensouda, Kaikani et al.

2011). But besides its tumor cell apoptosis abilities, it is also known as an EBV lytic-cycle inducer inside the tumor cells, *in vitro* and *in vivo*. (Feng, Israel et al. 2002; Hsu, Hergenhahn et al. 2002; Stevens, Zwaan et al. 2006).
BRLF1 and BZLF1 activation will also result in induction of lytic infection by GCb. This activation requires specific transcription factor binding motifs, such as the EGR-1 site in the BRLF1 promoter and the ZI and ZII sites in the BZLF1 promoter, and the activated p38 stress mitogen-activated protein kinases (MAPK) signaling pathway (Adamson, Darr et al. 2000; Feng, Cohen et al. 2004; Feng, Hong et al. 2004).
However, in general chemotherapy agents alone, induce lytic EBV protein expression in a small portion of the treated cells (Feng and Kenney 2006). A number of different epigenetic mechanisms, such as genome DNA methylation and histone modifications, affect the repression of EBV lytic genes. Therefore different combinations of chemoagents with HDACi's were tested *in vivo* and *in vitro* (Feng and Kenney 2006; Wildeman, Novalic et al. 2012). The role of HDACi's in in this process will be discussed next.

HDACi's

Histone acetylases (HAT) is known to contribute to control of the coiling and uncoiling of DNA surrounding histones. HAT acetylates the lysine residues, which results in a less compact and more transcriptionally active chromatin. The deletion of the acetyl groups by HDAC will lead to the formation of a condensed and transcriptionally silenced gene. The major epigenetic mechanism for remodeling chromatin structure, such as deacetylation, comprises reversible alteration of the terminal tails of core histones. HDACi's are known to inhibit this action and, thereby, hyperacetylate histones, and affecting gene expression (Marks, Richon et al. 2000; Thiagalingam, Cheng et al. 2003; Dokmanovic, Clarke et al. 2007). HDACs are classified into four groups, I to IV, regarding to their homology of accessory domains to yeast histone deacetylases. The classical HDACi's act on class I and II, by binding to the catalytic domain of the HDACs containing zink (Drummond, Noble et al. 2005). The inhibition of HDAC will accumulate hyperacetylated nucleosome core histones, but only alters the expression of some of genes, which leads to transcriptional activation of some genes and repression of other genes. The activity of some transcription factors, including p21 that is a regulator of p53 tumor suppressor gene, are generally enhanced by acetylation and, therefore, benefit from HDACi's. However, the activity of other transcription factors, such as T cell factor is repressed by acetylation. HDACi's have also other functions, such as inhibiting the proliferation of tumor cells, through induction of cell cycle arrest, differentiation or even apoptosis (Wang, Zhou et al. 2012; Bellucci, Dalvai et al. 2013; Sachweh, Drummond et al. 2013).
HDACi's have potent anticancer activities, with remarkable tumor specificity and some toxicity in animal models (Marks, Rifkind et al. 2001; Richon and O'Brien 2002), and some with therapeutic potential. HDACi's have been used

alone or together with DNA demethylating agents or other anticancer chemotherapies to treat leukemias and lymphomas, such as cutaneous T cell lymphoma, myelodysplastic syndrome, and diffuse B cell lymphoma (Bolden, Peart et al. 2006; Iwata, Saito et al. 2012). The mechanism by which HDACi's affect tumor cell-death is not yet completely understood, but it includes induction of cell cycle arrest at the G_1 to S stage, suppressing angiogenesis, enhancing tumor cell antigenicity, and affecting the expression of key cytokines, including tumor necrosis factor-α, interleukin-1, and interferon-γ (Vigushin and Coombes 2004; Palmieri, Coombes et al. 2005; Bolden, Peart et al. 2006).

Regardless of the tumor cell-death capabilities of HDACi's some of these compounds, such as butyrate and VPA, are shown to enhance the gene expression in latent EBV and sensitizing tumor cells to antiviral drugs, including GCV (Ghosh, Perrine et al. 2012; Iwata, Saito et al. 2012). However, efficient lytic EBV-cycle induction activities are only obtained when used in combination with chemoagents and/or antiviral agents (Feng and Kenney 2006).

Enhancement of the efficiency of chemotherapy in EBV-positive tumors by HDACi's is generally correlated to their basic role as inhibtors of deacetylase and, thereby, causing accumulation of acetylated chromatin around the BZLF1 promoter, and in some cases including VPA, also demethylating activities, thus altering the epigenetic modifications of latent EBV genome (Jenkins, Binne et al. 2000; Murphy, Fischle et al. 2002; Feng and Kenney 2006). Butyrate in combination with GCV is shown to affect killing of EBV-positive tumor cells *in vitro* and clinical studies, it also has pharmacokinetic limitation (Ghosh, Perrine et al. 2012). VPA, on the other hand, is shown to induce cell death in human leukemia cell lines (Kawagoe, Kawagoe et al. 2002), endometrial tumor cells (Takai, Desmond et al. 2004), and to enhance the efficacy of chemotherapy in EBV-positive tumor cells (Feng and Kenney 2006), with modest limitations and promising results.

VPA

VPA is a short chain fatty acid, which belongs to class I HDACi's, and is mainly used as a well-tolerated anti-epileptic drug. This activity, however, is independent of its therapeutically exploited anti-epileptic activity (Tunnicliff 1999; Johannessen 2000; Gottlicher, Minucci et al. 2001). VPA has been used for over twenty years in clinical studies, and the pharmacology and side effects of this drug have been extensively studied. VPA triggers proteasome-mediated degradation of HDAC2 and, thereby, acts as an isoenzyme-selective downmodulator of HDAC2 by inducing specific degradation of HDAC2 , and inhibition of HDAC1 catalytic activity (Phiel, Zhang et al. 2001; Kramer, Zhu et al. 2003). Besides its HDACi activity, it also triggers replication-independent active demethylation of DNA. VPA has been demonstrated to reverse DNA methylation patterns in non-dividing cells (Detich, Bovenzi et al. 2003).
VPA induces cell-death in human leukemia cell lines and endometrial tumor cells, and to enhance the efficiency of chemo in EBV-positive tumors (Iwata, Saito et al. 2012), but also to initiate the lytic gene expression in EBV-infected gastric carcinoma and B-cell lines (Feng and Kenney 2006; Hui and Chiang 2010). The killing effects of VPA in EBV-positive and EBV-negative T and NK lymphoma/leukemia cell lines have been studied by Iwata and colleagues (Iwata, Saito et al. 2012), which showed modest decrease in viability of cells independent of EBV presence. Apoptosis was reported in some of these cell lines, but mostly cell cycle arrest, indicating the limited anticancer activities of VPA regardless of its lytic-inducer abilities. Bortezomib, which is an HDACi, was also tested in this study and induced the EBV lytic cycle in EBV-positive T cell lines. However, the combination treatment of VPA and bortezomib showed strong synergetic killing effects in EBV-positive T and NK lymphoma cell lines, which demonstrates the potential ability of VPA to enhance the cytotoxicity effects of anti-cancer drugs, in an EBV-dependent manner. Similar results have been obtained in another study by Feng and colleagues (Feng and Kenney 2006), in which they studied lytic EBV gene expression in EBV-positive epithelial and lymphoid cell lines, mediated by a combination therapy of VPA and other chemotherapeutic drugs, such as GCb. VPA on its own induced only a small increase of lytic viral genes, while when combined with other chemoagents, it significantly enhanced the induction of lytic viral gene expression by this compounds, but also significantly enhanced the cytotoxicity of chemoagents in EBV-positive cells. These results were in line with other studies in EBV-associated T and NK lymphoma cell lines (Iwata, Saito et al. 2012), EBV-positive lymphoma cell lines (Ghosh, Perrine et al. 2012), and were further confirmed in EBV-positive NPC cell lines, studied by Wildeman and colleagues, in which they used a combination of VPA, GCb and an additional antiviral therapy by GCV to treat NPC end-stage patients (Wildeman, Novalic et al. 2012).

The ability of VPA to enhance the cytotoxicity and/or lytic-induction effects of chemoagents in EBV-positive tumors, might be due to its basic role as an

HDACi to enhance HAT activity and thereby increase in the acetylation of viral genes, and also its demethylating abilities. VPA likely contributes to relaxing the chromatin encompassing the episomal viral genome by shifting the histone code toward acetylation. Acetylation of histone in chromatin around the BZLF1 promoter region, for instance, is proved to be involved in activation of its expression (Jenkins, Binne et al. 2000). It is possible that demethylating abilities of VPA contribute to enhance the effects of chemotherapy, but since other demethylating agents alone, such as as aza-CR, were not capable of enhancing the lytic gene expression, it is an indication that demethylation of the viral genome is not enough to increase the killing effect of chemotherapy. Methylation, to some extend, might be beneficial for lytic induction through BZLF1, since BZLF1 is shown to preferably bind to methylated domains of BRLF1 promoter, and thereby initiating lytic cascade (Bhende, Seaman et al. 2004).

Nevertheless, the enhancement of cytotoxicity and lytic induction by VPA is only partially related to its HDAC inhibitory role, since VPA increased the activity of BZLF1 promoter in an experiment carried out by Feng and colleagues, in which they used "naked" DNA. In the same study it has been shown that the ability of VPA to enhance the killing effects of chemotherapy requires the EBV genome together with the lytic viral proteins (Feng and Kenney 2006), suggesting that VPA might interact directly in the lytic induction pathway of EBV, at least partially independent of its HDAC inhibitory activity. Notably, this hypothetic interaction is significantly stronger when combined with chemoagents, such as GCb, as indicated by Feng and colleagues (Feng and Kenney 2006). A possible mechanism by which VPA influence the lytic-induction, regardless of its HDACi activity, might be through direct interaction with cellular transcription factors that bind and activate the viral immediate early gene promoters. This hypothesis was supported by an experiment demonstrating that, even in the absence of chromatinized template, VPA significantly enhanced the luciferase activity derived from the Zp-luciferase construct, carried out by Feng and colleagues (Feng and Kenney 2006). MEF-2D is one of the main regulators of Zp transcription, and its phosphorylation by ERK5-kinase enhances its ability to regulate the transcription of Zp, in a positive manner (Yuan, Huang et al. 2001). Since VPA is shown to activate the ERK-pathway (Gregoire and Yang 2005), this might be another mechanism by which VPA directly influences the lytic gene expression, regardless of its HDACi activity.

Furthermore, VPA does not only enhance the effects of chemo, but it is also known as a sensitizer to antivirals for the treatment of EBV-associated malignancies, since the combination of VPA and GCV induced efficient cell killing of EBV-positive lymphomas (Ghosh, Perrine et al. 2012), and end-stage NPC patients (Wildeman, Novalic et al. 2012). As explained above, TK and BGLF4 phosphorylate GCV and turn it into its active form. Many HDACi's have therefore been tested for their abilities to overexpress TK and BGLF4, which correlated with levels of tumor cytotoxicity (Ghosh, Perrine et al. 2012). Nevertheless, this correlation was not unique in all of these HDACi's. VPA in combination with GCV, for instance, did demonstrate tumor cytotoxicity,

although its TK inducing abilities were modest. This lack of correlation might suggest that other kinases than TK might be involved in conversion of GCV, and VPA might have a positive effect on expression and/or effect of these kinases (Ghosh, Perrine et al. 2012). In a study carried out by Wildeman and colleagues the combination of VPA and GCV showed a clear synergetic action on the expression of BZLF1, TK, PK, which are only expressed during the EBV lytic replication cycle. VCA-p18, a viral capsid protein, was blocked, indicating the inhibition of viral replication mediated by GCV. The combination of VPA and GCb strongly induced the lytic induction, and this in turn sensitized the EBV-positive cell lines to GCV (Wildeman, Novalic et al. 2012). In addition, the EBV lytic gene expression is shown in another study to increase the apoptosis in EBV-positive gastric cancer cell lines (Jung, Lee et al. 2007). Since this effect was only seen when a combination of GCV and HDACi's were used, it has been concluded that GCV-activating viral kinase rather than the cancer-related genes, might be responsible for the apoptosis. VPA, however, was not included in their test-panel and its (indirect) role in apoptosis, when combined with GCV, needs to be investigated. But since VPA is already known to induce EBV lytic gene expression in combination with chemoagents, the combination of VPA and GCV would have the same apoptotic effects, as observed in study by Jung *et al*.

Discussion

EBV is linked to multiple cancers, including NPC, and its genome is found in all tumor cells in its latent form. Therefore, a novel combination therapy referred to as CLVA therapy based on reactivating EBV in these tumor cells is developed by Wildeman and colleagues, in which they make use of a combination therapy consisting of a chemoagent GCb, a HDACi VPA, and an antiviral drug GCV, in order to trigger the immune response and increase the susceptibility of these tumors to antiviral therapy (Wildeman, Novalic et al. 2012). The basic principle of induction of EBV-lytic gene expression in tumor cells with a combined treatment of chemotherapy and an HDACi was first tested in EBV-positive cells of a lymphoma model, and caused significant reduction in tumor volume. An additional antiviral therapy was further shown to enhance this effect (Mentzer, Fingeroth et al. 1998; Feng and Kenney 2006). CLVA therapy tested on three NPC end-stage patients and showed a significant biological effect with moderate toxicity (Wildeman, Novalic et al. 2012), and may open a way to develop oral therapies, which is highly useful for developing countries where complex and expensive therapy is impossible. The use of EBV as a target in these NPC cells and the consistent presence of EBV genome in many other EBV-associated malignancies could be incorporated into new approaches, in order to treat other EBV-associated carcinomas. Therefore, it is important to understand the mechanism of which these combinations of compounds work together to reactivate EBV and cause a cytotoxic effect in these tumor cells.

EBV genome is present in EBV associated carcinomas in its latent form, in which only a small portion of viral genome is expressed (Feng and Kenney 2006). In this latent form of expression, a variety of epigenetic mechanisms, including viral DNA methylation and histone modification of the chromatin contribute to suppressing the ability of transcription factors to enhance the activation of EBV immediate early gene promoters, in order to maintain the latent form of infection (Ernberg, Falk et al. 1989; Robertson and Ambinder 1997). The switch from latent to lytic is mediated by BZLF1 and BRLF1, immediate-early proteins that initiate the expression of EBV lytic gene expression, in which many additional viral gene products are transcribed. As a result, the host cell might be killed by release of viral particles. Overexpression of either BZLF1 or BRLF1 is sufficient to reactivate the lytic form of EBV gene expression (Adamson and Kenney 1998; Dickerson, Xing et al. 2009).
Studies have revealed the role of DNA-damaging agents, including chemo, some HDACi's, and radiation, in reactivation of EBV genome, mainly initiated by ATM-pathway signaling (Countryman, Jenson et al. 1987; Nikitin, Yan et al. 2010; Hagemeier, Barlow et al. 2012). However, even in the absence of DNA damage response ATM kinase gets activated in response to reactive oxygen species (ROS), and ATM is shown cause an to induction of EBV lytic gene expression (Guo, Deshpande et al. 2010; Hagemeier, Barlow et al. 2012) (see figure 4). The mechanisms by which chemoagents and HDACi's activate the lytic induction of EBV are not yet completely understood. It is likely that

activation of p53, which is a downstream target of ATM, which occurs subsequent of ATM-phosphorylation at ser-1981 in response to different stimuli, is one way by which DNA-damaging agents potentially induce EBV-reactivation (Chang, Lo et al. 2008; Hagemeier, Barlow et al. 2011; Chua, Chiu et al. 2012).

p53 is recently shown to be required for HDACi-mediated lytic reactivation in EBV-positive NPC cell lines and lymphoblastoid B cell lines, which further supports the previous findings about the critical role of p53 and ATM in EBV-reactivation (Hagemeier, Barlow et al. 2011; Chua, Chiu et al. 2012). 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 (Ellis, Wang et al. 2010; Hagemeier, Barlow et al. 2011; Chua, Chiu et al. 2012). BGLF4 phosphorylates and activates TIP60 (Li, Zhu et al. 2011), and acetylation of ATM by TIP60, which is a HAT, contributes to ATM activation. (see figure 4). Several EBV latent proteins, such as EBNA1, EBNA3C, LMP1, and LMP2A, have been shown to inhibit the DDR, with LMP1 and LMPA2 in particular inhibiting the ATM kinase activity (Gruhne, Sompallae et al. 2009; Cai, Guo et al. 2011). 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, we speculate that ATM plays a significant role in promoting lytic EBV reactivation via both p53-dependent and p53-independent pathways (Hagemeier, Barlow et al. 2011). 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 (Matsuoka, Ballif et al. 2007).

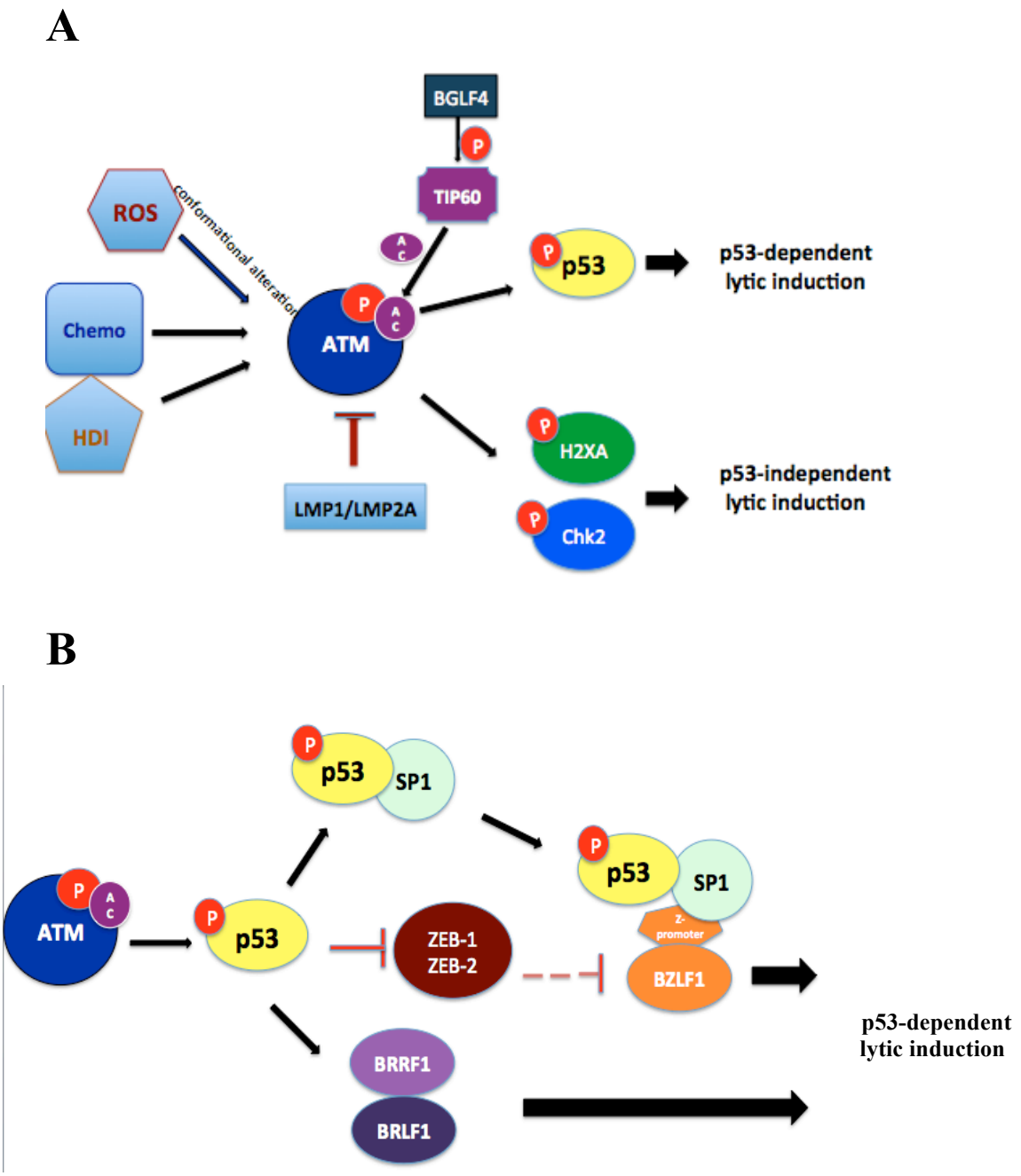

Figure 4: ***Suggested model of ATM-induced EBV lytic induction***

A: ATM gets phosphorylated and acetylated in response to different (DDR) stimuli, such as chemo, HDACi's and ROS, and phosphorylates in turn many downstream targets, including p53, H2Xa and Chk2. This initiates a p53-dependent and p53-independent lytic induction. LMP1 and LMP2a are the repressors of this pathway, by inhibiting ATM kinase.

B: p53 gets activated/phosphorylated by ATM. This will initiate lytic induction by many ways including, complex formation with SP1 and Z-promoter and thereby activating BZLF1, inhibition of ZEB1-ZEB2, which are the repressors of Z-promoter, and overexpression of BRRF1 and BRLF1.

- **P: Phosphorylation**
- **Ac: Acetylation**

ATM-pathway is, however, not the only pathway required for EBV-lytic induction. The ability of some chemoagents, including GCb, to improve the induction of EBV lytic gene expression requires multiple signal transduction pathways, such as cellular stress mitogen-activated protein kinase (MAPK) p38, phosphatidylinositol 3-kinase (PI3 kinase), and MAPK/ERK kinase (MEK) pathways (Feng, Hong et al. 2004). Stress-inducing chemotherapeutic agents induce BZLF1 and BRLF1 transcription through activation of several different signal transduction pathways and their downstream targets, which possibly bind to cyclic AMP-responsive element (CRE), EGR-1, and MEF2D binding motifs (Borras, Strominger et al. 1996; Liu, Liu et al. 1997; Wang, Huang et al. 1997). GCb, for instance, has been shown to induce low levels of lytic viral protein expression (Feng and Kenney 2006). The combination of chemotherapy and GCV produces severe killing of EBV-positive tumors. Thus, the lytically infected and not latently infected EBV-positive tumors benefit form the cytotoxic effects of GCV (Feng, Hong et al. 2004). A major advantage of inducing expression of the lytic EBV proteins by chemotherapy prior to treatment with GCV is the exclusive killing of EBV-positive cells. Although the combined treatment of GCb and GCV creates tumor cytotoxicity, this effect is created due to activation of GVC by kinases expressed during the lytic replication cycle in response to chemo, and GCV does not contribute to enhancement of EBV lytic induction by chemo.

Nevertheless, only chemotherapy induces lytic viral gene expression in a limited portion of EBV-positive tumor cells, likely due to the highly epigenetic modified state of latent EBV genome (Feng and Kenney 2006). The latent form of EBV is highly methylated in variety of EBV-positive tumors (Ernberg, Falk et al. 1989). Methylation initiates transcriptional repression by different mechanisms, such as altering the histone acetylation state and prevention of transcription factors to bind to DNA and is, therefore, generally associated with an inactive form of chromatin (Bednarik, Cook et al. 1990; Boyes and Bird 1992). Demethylating agents, such as 5-aza-cytidine have been studied, in order to enhance the effects of lytic induction. The results showed increased amounts of lytic viral gene expression in Burkitt's lymphoma cell-lines (Ben-Sasson and Klein 1981; Nonkwelo and Long 1993), but not in lymphoblastoid cell-lines (Feng, Hong et al. 2004), indicating that other factors beside methylation might be involved in desensitizing of EBV early gene promoters to chemoagents. Reduced accumulation of acetylated chromatins is thought to be one of the epigenetic modifications involved in inactivation of viral genome. HDACi's, in general, have shown potent anticancer activities beside their basic role as HDAC inhibitory activities, via different pathways, including reversing aberrant epigenetic changes associated with cancer (Bolden, Peart et al. 2006), induction of cell cycle kinase inhibitor $p21^{WAF1}$ expression that is a regulator of p53's tumor suppressor activity, and alteration of pathways in which the retinoblastoma protein (pRb) suppresses cell proliferation (Richon, Sandhoff et al. 2000). Some of them, including VPA, have also demethylating activities with therapeutic potentials (Detich, Bovenzi et al. 2003; Yeow, Ziauddin et al. 2006). In CLVA therapy studied by Wildeman and colleagues, VPA has been used in combination with chemoagents and antiviral agent, *in vitro* and *in vivo,* and has

shown significant synergetic effects on activating the lytic EBV gene expression and higher cytotoxicity effects, compared with chemotherapy alone (Wildeman, Novalic et al. 2012).
In conclusion, the combination of HDACi's, chemoagents and antiviral therapy is shown to be useful to treat EBV-positive malignancies, such as NPC. These compounds work in multiple ways together, in order to induce the lytic replication cycle of EBV and tumor cytotoxicity. However, only small portions of HDACi's and chemoagents have been studied and different combinations might create stronger and/or more effective responses. Addition of other compounds, such as demethylating agents might further enhance this effect. Although the mechanism of this co-operation is not always completely understood, the promising results might open up new avenues to treat other EBV-associated malignancies by use of EBV as a target in a combination therapy. Other EBV-associated malignancies might benefit form different set of compounds, and these possibilities need to be further investigated.

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