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Development of Drugs for Epstein - Barr virus using High-Throughput *in silico* Virtual Screening

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Abstract

Importance of the field—Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus that is causally associated with endemic forms of Burkitt's lymphoma (BL), nasopharyngeal carcinoma, and lymphoproliferative disease in immunosuppressed individuals. On a global scale, EBV infects over 90% of the adult population and is responsible for ~1% of all human cancers. To date, there is no efficacious drug or therapy for the treatment of EBV infection and EBV-related diseases.

Areas covered in this review—In this review, we discuss the existing anti-EBV inhibitors and those under development. We discuss the value of different molecular targets, including EBV lytic DNA replication enzymes, as well as proteins that are expressed exclusively during latent infection, like EBNA1 and LMP1. Since the atomic structure of the EBNA1 DNA binding domain has been described, it is an attractive target for *in silico* methods of drug design and small molecule screening. We discuss the use of computational methods that can greatly facilitate the development of novel inhibitors and how *in silico* screening methods can be applied to target proteins with known structures, like EBNA1, to treat EBV infection and disease.

What the reader will gain—The reader will be familiarized with the problems in targeting of EBV for inhibition by small molecules and how computational methods can greatly facilitate this process.

Take home message—Despite the impressive efficacy of nucleoside analogues for the treatment of herpesvirus lytic infection, there remain few effective treatments for latent infections. Since EBV-latent infection persists within and contributes to the formation of EBV-associated cancers, targeting EBV latent proteins is an unmet medical need. High throughput *in silico* screening can accelerate the process of drug discovery for novel and selective agents that inhibit EBV latent infection and associated disease.

Keywords

Epstein-Barr virus (EBV); DNA polymerase; LMP1; EBNA1; computational screening

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Declaration of Interest

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1. Introduction

The Epstein–Barr virus (EBV) was discovered 46 years ago in tumor tissue derived from Burkitt's lymphomas, and was classified as the first human tumor virus [1]. Subsequently, EBV has been implicated as a causal cofactor of a variety of human disorders, including infectious mononucleosis, oral hairy leukoplakia, nasopharyngeal carcinoma, 10% of gastric carcinomas, Burkitt's lymphoma and lymphoproliferative diseases occurring in immunocompromised individuals [2–6].

EBV may be considered among the most successful viruses since it readily establishes a lifelong persistent infection in more than 90% of the adult population. EBV has been estimated to be responsible for ~1% of all human cancers worldwide. Despite this significant impact on human health, there are no well-established and regulatory agency-approved treatments for EBV-infection and associated disease. Like all herpesviruses, EBV can infect cells in either the latent or lytic form. Almost all EBV-associated malignancies carry the latent form of the virus. Immunological or pharmacological targeting of latent infection would, in principle, provide a direct treatment for EBV-associated cancers. However, most of the presently available anti-herpesvirus compounds target enzymes associated with lytic replication. Targeting the lytic cycle for EBV has some clear therapeutic benefit. Lytic infection is required for horizontal spread of the virus, and some of the lytic gene products may contribute to pathogenesis and early growth-transforming events. Lytic EBV infection is directly linked to oral hairy leukoplakia in immunosuppressed individuals [7] and to an increased risk of EBV-associated nasopharyngeal carcinoma [8]. Chronic lytic EBV infection caused by co-infection of malaria is suspected of promoting endemic Burkitt's lymphoma [9, 10]. For these reasons, inhibitors of lytic infection may be of therapeutic value for the prevention and treatment of EBV-associated disease.

During the lytic form of viral replication, the EBV genome is amplified 100–1000 fold by viral encoded-replication enzymes, including a viral DNA polymerase and thymidine kinase [11, 12]. Although there are many viral-specific features of herpesvirus lytic replication, including genome packaging and virion assembly, the most successful therapeutic interventions currently used against lytic infection target the viral DNA polymerase. Drugs such as nucleoside and nucleotide analogues act by the incorporating their tri-phosphate forms into herpesvirus DNA resulting in chain termination[13], and in some cases, the functional inactivation of the viral DNA polymerase[14]. These generic anti-herpesvirus compounds can also inhibit EBV DNA polymerase, but have limited efficacy towards most EBV associated disease.

In contrast to lytic infection, EBV more typically establishes a long-term latent infection in B-lymphocytes where the genome resides as a multicopy, chromatin-associated episome[15, 16]. Latent infection is characterized by the expression of only a few viral genes that contribute to viral genome maintenance and host-cell survival. During latent infection, the viral genomes are maintained as multicopy episomes that replicate in synchrony with the cellular genome [17, 18]. The EBV encoded EBNA1 protein is expressed consistently during latent infection and is required for episomal maintenance of the latent genome. EBNA1 binds to the viral origin of plasmid replication (OriP) and can also regulate transcription of other viral genes important for EBV latency and host-cell survival [19, 20]. Several other viral-encoded proteins contribute to pathogenesis, but their expression can be variable in EBV-associated cancers [4].

Some clinically approved anti-neoplastic agents have been tested for their ability to treat latent EBV infection. Hydroxyurea (HU), which can inhibit cellular ribonucleotide

reductase, can eliminate EBV episomes from some cell types in tissue culture [21, 22]. HU has been used in clinical trials for treating EBV-positive central nervous system lymphomas and thymic carcinomas [22, 23]. Although HU has the ability to inhibit ribonucleotide reductase and slow cell cycle progression [24], the mechanism of HU-induced loss of double double minutes and EBV episomes is not known. Since the linear double-stranded genomes characteristic of productive lytic infection and encapsidated in virus particles are not made in latent infection, it is not thought that HU inhibits viral ribonucleotide reductase, but rather that it indirectly inhibits cellular enzymes or mechanisms that control latent genome persistence [25].

After nearly three decades of study, there is still no small molecule inhibitor that specifically targets EBV and prevents EBV-associated disease. This unmet medical need demands that we consider new strategies for EBV drug discovery that will provide more effective methods for preventing disease. In this review, we discuss a few of the approved pharmaceutical reagents used for inhibiting EBV lytic and latent infection, and consider future opportunities to improve these therapies. We also discuss the value of using *in silico* screening approaches to accelerate the drug discovery process, and how this can be applied to the development of treatments for EBV and its associated diseases.

2. Drug targets for EBV infection

2.1 Targeting lytic DNA replication

The lytic form of infection is required for horizontal spread of the virus from cell to cell and from host to host. It has been implicated in nasopharyngeal carcinoma and methotrexate-induced lymphomas, arising in patients treated for rheumatoid arthritis and polymyositis [8, 26]. Spontaneous lytic reactivation occurs during B-cell terminal differentiation into plasma cells but may also be triggered by other stress-related signaling pathways [27]. To date, the most successful therapeutic interventions currently used against EBV infection and associated diseases target the lytic replication of EBV.

2.1.1 EBV-encoded DNA polymerase—DNA polymerase performs a key step in DNA replication, in which the polymerase “reads” an intact DNA strand as a template and uses it to synthesize the new strand. During the lytic phase of the EBV life cycle, EBV DNA polymerase mediates viral DNA replication. The viral DNA polymerases, like many high-fidelity cellular polymerases, possess a 3'-5' exonuclease editing function in addition to its deoxyribonucleotide polymerizing [12]. The EBV DNA polymerase has several regions of amino acid sequence similarity with the catalytic subunits of γ -like DNA polymerases, which include the eukaryotic polymerases α , β , and γ [28]. A few crystal structures of herpesvirus polymerase have been determined [29, 30]. The compounds that target EBV DNA polymerase were used to treat diseases associated with lytic EBV infection, and have a wide usage in various clinical settings. The drugs that might be candidates for targeting viral DNA polymerase fall into two groups: Nucleoside analogues and Non-nucleoside DNA polymerase inhibitors, shown in Figure 1

2.1.1.1 Nucleoside analogues: The discovery in the late 1970s that a nucleoside analogue, 9-(2-hydroxyethoxymethyl) guanine (acyclovir), is a selective inhibitor of herpes simplex virus (HSV) and EBV revolutionized antiviral chemotherapy, and initiated an intensive search for more potent nucleoside drugs [31]. Nucleoside analogues are actually prodrugs that require phosphorylation by viral thymidine kinase to become active. Early generation nucleoside analogues, such as acyclovir, are highly effective against HSV, but have reduced activity towards EBV DNA polymerase [32, 33]. Since these inhibitors function through interactions with viral thymidine kinase and DNA polymerase, it is possible that there reduced activity is a result of reduced interactions with either or both of these viral targets

[34]. Subsequent to the development of acyclovir, related drugs, such as the nucleoside analogues ganciclovir and penciclovir, and the nucleotide analogues cidofovir [35], and adefovir [36]. Orally available prodrugs valaciclovir [37], valganciclovir [38] and famciclovir [39], were introduced in the mid 1990s (Figure 1). While these compounds have improved efficacy for HSV infection, only ganciclovir has been shown to be more effective in inhibiting EBV lytic infection. Both acyclovir and ganciclovir have been used to prevent post-transplant lymphoproliferative disorder and in treatment of oral hairy leukoplakia [40, 41]. Despite the impressive efficacy of these nucleoside analogues in the treatment of herpes simplex infection, all these compounds suffer from the same drawbacks, including toxic side effects, poor oral bioavailability and potential for mutagenesis. In addition, nearly all clinically effective nucleoside analogues target the same active sites on the viral DNA polymerase molecules, such that mutant viruses resistant to one drug are commonly resistant to others [34].

2.1.1.2 Non-nucleoside inhibitors: Given the success of acyclovir and its analogues, it is not surprising that additional inhibitors of DNA polymerases were identified. For example, foscarnet(phosphonoformic acid)[42] is a structural mimic of the anion pyrophosphate that selectively inhibits the pyrophosphate binding site on viral DNA polymerases that do not affect human DNA polymerases. Because foscarnet is not activated by viral kinases, it is often used as alternative treatment for EBV, and for acyclovir- or ganciclovir-resistant patients. But it is more toxic than acyclovir, has profound metabolic side effects and must be administered intravenously [34]. However, a novel class of nonnucleoside inhibitors against DNA polymerases, 4-oxo-dihydroquinolines (4-oxo-DHQs), as represented by PNU-182171 and PNU-183792 [43, 44], shown in Figure 1, were identified by an automated screen of a proprietary chemical library and a subsequent synthetic chemistry effort. These drugs have broad-spectrum anti-herpesvirus activity. In cell culture assays, these compounds inhibit the DNA polymerase function of HSV-1, HSV-2, HCMV, and EBV [45], without affecting cellular polymerases. Analysis of drug-resistant mutants, together with the results of protein modeling, demonstrate that the 4-oxo-DHQs target a site, which is unique to the herpesvirus DNA polymerases, either overlaps with or resides close to the binding site for nucleoside or nucleotide analogs [46]. Significantly, these compounds did not show signs of cytotoxicity in the concentrations used for achieving antiviral effect and PNU-183792 presented with good oral bioavailability and shows efficacy in an animal model[43, 45].

2.1.2 Potential targets for drug discovery—All human herpesviruses genomes contain genes that encode at least one protein kinase (PK), which have been implicated in viral gene expression, viral DNA replication, and inhibition of apoptosis [47, 48]. The members of this family can be divided into two groups, exemplified by the alphaherpesvirus-encoded PKs[49–51]. The group exemplified by the herpes simplex virus (HSV) UL13 PK is conserved in all herpesviruses[52, 53]. EBV PK encoded by the BGLF4 gene is a homolog of UL13 PK. It has been identified as a Ser/Thr protein kinase PK [52, 53] and is the only potential protein kinase identified in the EBV genome. EBV PK can be expressed to high levels during the EBV lytic program with an early expression kinetics [54]. It is detected mainly in the nuclei of EBV-infected cells [55]. The protein phosphorylates a number of viral and cellular proteins [56], including the EBV BMRF1 gene product(EA-D) [57], the DNA polymerase processivity factor; EBNA2 [56], a regulator of EBV latency gene expression; EBV nuclear antigen leader protein (EBNA-LP) [58]; PK itself [57–59]; and the cellular translation-elongation factor 1 delta (EF-1 δ) [60]. The variety of the EBV PK targets implies a multiplicity of processes and steps in viral replication in which this PK is involved.

More recently, 1-H- β -L-ribofuranoside-2-isopropylamino-5,6-dichloro benzimidazole (maribavir [MBV] [61], also known as benzimidavir, which represents a new class of

antiviral drugs, has been extensively examined [62]. Maribavir (Figure 1) is active against both HCMV [63, 64] and EBV [65, 66] and its effects were thought to be involved with direct or indirect inhibition of viral protein kinases [25]. Indeed, it inhibited the enzymatic activity of human cytomegalovirus (HCMV) UL97 (homologous to EBV PK) [67, 68], but failed to inhibit EBV PK in vitro [69]. However, phosphorylation of the EBV DNA-processivity factor, EA-D, by the EBV protein kinase is inhibited by maribavir in infected cells [65]. Importantly, it has been shown that BGLF4 gene expression is crucial for production of infectious EBV progeny, which makes its product, a unique PK, a potential target for antiviral therapy [70].

In addition, topoisomerases and helicases play essential roles in cellular and viral chromosome metabolism including DNA replication, recombination, and transcription [71, 72], and are considered to be potential targets for antiviral therapies. Previous studies have found that the topoisomerase I (Topo I) inhibitor camptothecin and the Topo II inhibitor ellipticine can selectively inhibit EBV lytic cycle DNA replication at concentrations that were not toxic to the host cell [73]. It has been reported that cellular Topo I and Topo II, as well as the helicase RecQL1, function at the origin of lytic DNA replication for Kaposi's sarcoma-associated herpesvirus (KSHV) [74]. A more recent study has implicated that Topo I activity is required for RecQL1 recruitment to OriLyt as well as for the transcription activation function of Zta and Rta during EBV lytic replication and reactivation from latency. The precise role of Topo I or Topo II in EBV lytic replication is not known but may be important for the future development of lytic cycle inhibitors.

2.2 Targeting latent infections/EBV-encoded latent proteins

Most EBV associated tumors harbor the latent viral genome as a multi-copy episome in the nucleus of the transformed cells. During latent infection, EBV does not produce progeny virions, but does express a limited set of viral gene products that promote host-cell survival and proliferation. Of the nearly 100 viral genes that are expressed during replication, only a limited set of viral gene products are expressed in latently infected B cells in vitro [75], which comprise six nuclear antigens (EBNA-1, -2, -3A, -3B, 3C and -LP), three integral membrane proteins (LMP1, -2A and -2B) and two small nuclear RNAs (EBER-1 and 2). The EBV-encoded proteins involved in latency that have received the most attention are EBNA-1, EBNA-2, EBNA-3A, EBNA-3C, LMP-1 and LMP-2A. LMP1 is the main transforming protein of EBV, and identified as the principal oncoprotein because it can transform rodent fibroblasts and is essential for immortalization of B cells [76, 77]. EBV LMP-2A is thought to promote both the survival of latently infected cells and prevents reactivation of EBV from latently infected cells by blocking tyrosine kinase phosphorylation [78]. EBNA1 — the one viral protein that is expressed in most, if not all, EBV associated malignancies — is required for episomal maintenance of the latent viral genome, and regulation of gene expression. EBNA2, one of the six viral nuclear proteins expressed in latently infected B cells, is essential for immortalization of B cells by the EBV [79]. Genetic analysis using recombinant viruses has shown that EBNA-3A and 3C, but not EBNA-3B, are essential for this B-lymphocyte growth transformation in vitro [80].

2.2.1 LMP1—Among the EBV latent gene products, LMP1 is the well-documented oncoprotein. LMP1 is integral membrane protein, containing a short N-terminal cytoplasmic tail of 17 amino acids, six hydrophobic transmembrane-spanning domains and a large cytoplasmic C-terminal domain of 200 amino acids [81]. The viral protein is essential not only for EBV-induced immortalization of B lymphocytes, but transfection of LMP-1 into immortalized rat fibroblasts induces full phenotypic transformation and, in human epithelial cells, LMP-1 expression inhibits differentiation [77, 82, 83]. In transgenic mice, LMP1 expression induces development of B cell lymphoma or epidermal hyperplasia [84]. LMP1

induces most of the phenotypic changes seen following EBV infection of B cells, including induction of adhesion molecules, homotypic aggregation, increased cell size, and entry into cell cycle [85, 86]. By functioning as a constitutively activated member of the tumour necrosis factor receptor (TNFR) CD40, LMP1 activates multiple downstream signaling pathways, such as NF- κ B and JNK pathways, resulting in the up regulated expression of cellular genes involved in cell proliferation, cytokine secretion, angiogenesis, and tumor metastasis [4, 87, 88]. All in all, LMP1 plays a central role in the overall EBV strategy to cellular immortalization.

Based upon these observations, LMP1 represents a logical target for inhibition of EBV associated cancers. One approach has been to target LMP1 protein expression by using synthetic anti-sense oligonucleotides (ONs) that prevent mRNA translation. Over 50 antisense molecules are currently in clinical trials for the treatment of cancer, cardiovascular diseases and HIV, to name a few, suggesting that this therapeutic approach still has a lot to offer [89]. A number of research groups have used anti-sense oligodeoxynucleotides targeted to LMP-1 as a strategy to suppress LMP-1 expression and thereby inhibit its functions [90, 91]. As expected, silencing LMP-1 expression rendered the EBV-positive lymphoblastoid cell lines susceptible to chemotherapeutic agents. In 2007, Galletti et al. [92] reported that liposomes, dendrimers or transferrin-polylysine-conjugated ON were internalized by the cells at an extent several fold higher than that of the naked oligomers. According to the authors, only the delivery system exploiting the transferrin receptor pathway of internalization, was able to vectorize biologically active anti-sense LMP1-ON. All of these studies suggest that ONs targeting LMP-1 can have therapeutic benefit in EBV-associated malignancies, and might potentiate the effects of other antitumour agents.

An alternative approach that was recently developed to achieve selective knock-out of viral or cellular onco-proteins is the use of intracellular single-chain antibodies. A single-chain antibody (sFv) is the smallest domain region of an antibody that retains the binding specificity of the parental antibody. Intracellular sFvs have been previously used to inhibit various steps of HIV replication and to inactivate cytosolic onco-proteins. In 1998, Piché et al. [93] reported that a sFv was targeted against LMP1 to achieve selective and functional inhibition of LMP1 in EBV-transformed cells. According to the authors, knockout of LMP1 induces chemosensitization of EBV-transformed B lymphocytes to conventional chemotherapeutic agents. While LMP1 is an attractive target for inhibition of many EBV tumors, it is unfortunately the case that LMP1 is not expressed in most EBV-associated Burkitt lymphomas and nasopharyngeal carcinomas. Thus, LMP1 inhibition may not prevent all or most forms of EBV latent infection associated with malignant transformation.

2.2.2 EBNA1—EBNA1 is an attractive candidate for targeting inhibition of EBV latent infection. EBNA1 is consistently expressed in most, if not all, EBV associated malignancies [16] EBNA1 is essential for viral genome maintenance and contributes to infected-cell survival [94, 95]. Most importantly, EBNA1 is a viral-encoded protein that has well-defined biochemical and structural properties. EBNA1 consists of several functional domains, including a well-defined carboxyl-terminal DNA binding domain [96, 97]. The DNA binding domain is essential for interaction with the viral origin of plasmid replication (OriP) [98]. OriP consists of a series of 30 bp repeats to which EBNA1 binds an 18 bp palindromic-sequence as a homodimer [99, 100] The DNA binding and dimerization interface have been solved by high resolution X-ray crystallography in the apo- and DNA-bound forms [101, 102]. These observations suggest that EBNA1 is a member of a family of viral origin binding proteins that have no apparent structural similarity to host-cellular proteins, and therefore may represent attractive targets for inhibitors of viral latent replication and persistence. The treatment of EBV-transformed B cells with antisense oligonucleotides or

adenovirus vector-delivered ribozymes that target EBNA-1 mRNA inhibits EBNA-1 expression and cell proliferation [103–105].

Recently, Sun et al. demonstrate that Hsp90 inhibitors can be used to inhibit expression and translation of EBNA1 in cells with various types of latent EBV infection, and that this effect requires the EBNA1 Gly-Ala repeat domain [106]. Furthermore, Hsp90 inhibitors prevent EBV transformation of primary B cells, and strongly inhibit the growth of EBV-induced lymphoproliferative disease in SCID mice. It is believed that Hsp90 inhibitors might be particularly effective for treating a variety of different EBV-induced diseases, provided that the continued presence of the viral genome is required for these EBV-associated illnesses. Also, Li et al. have identified a new class of small molecule inhibitors of EBV latent infection based on their ability to inhibit the DNA binding function of EBNA1 [107]. The molecules were discovered using computational screening and then validated by biochemical and cell-based assays. Four compounds were found to have biochemical activity and two of which have activity in cell-based assays (see Figure 1). To date, no structure based design of EBNA1 inhibitors have been presented. According to the authors, the data indicates that this approach is promising for EBNA1, and is likely to be a promising approach for other viral proteins with known X-ray crystal structures.

2.2.3 EBNA2 and EBNA3—The Epstein-Barr virus (EBV) nuclear antigen 2 (EBNA2) is one of the first EBV-encoded gene products expressed after infection of primary B lymphocytes. EBNA2 is also known to promote transcriptional transactivation of viral and cellular genes by acting as an adapter molecule that binds to cellular sequence-specific DNA-binding proteins, Jk-recombination-binding protein (RBP-Jk/CBF1), to transactivates cellular genes such as CD23 and in EBV genes the LMP1, LMP2A, LMP2B genes [108, 109]. EBNA3A and EBNA3C can repress EBNA2-mediated transactivation of the EBV genes by binding to a DNA-binding protein RBP-Jk [110, 111], and are also known to influence cell-cycle progression [112].

A 10-aa peptide was identified that was an effective competitor for EBNA2 binding to CBF1, was synthesized as a fusion with the protein transduction domain of HIV-1 TAT for efficient delivery [113]. Farrell et al. demonstrated that treatment of an EBV-immortalized lymphoblastoid cell line with the EBNA2-TAT peptide stopped cell growth and reduced cell viability by down-regulating the EBNA2-responsive viral genes and while up-regulating expression of the cyclin-dependent kinase inhibitor p21. Furthermore, given the role of EBNA3c in modulating cell cycle regulatory complexes including cyclin A/cdk2, SCFSkp2, and Rb [114, 115], Knight et al. generated a HIV TAT-tagged EBNA3C-derived peptide to disrupt EBNA3C-driven cell cycle pathways [116]. The results showed that the peptide specifically inhibited the proliferation of established EBV-infected cell lines and reduced viral transformation of purified primary B lymphocytes.

Considering the known toxicity of the TAT peptide, additional mechanisms may be desirable for the delivery of EBNA2 and EBNA3C inhibitory molecules into cells. Anyway, these experiments suggest that the inhibitors targeting against EBNA2 and EBNA3C may have potential as novel anti-EBV therapeutics.

3. Computational technique for developing EBV inhibitors

Because of the cost-inefficient and time-consuming process of conventional drug discovery over the past decade, computer-aided drug design approaches have emerged as an attractive and complementary approach to traditional high throughput screening (HTS). Virtual screening has been applied to the successful identifications of biologically active molecules against targets such as HIV-1 protease, thymidylate synthase, influenza hemagglutinin, and

parasitic proteases [105, 117]. Virtual screening may also provide a rapid and rational approach towards identification of novel inhibitors of EBV infection. As noted above, none of known and approved drugs are effective and selective therapies for treatment of EBV infection in the clinic. Since EBNA1 is known to be essential and ubiquitously expressed in EBV-infected tumors, it has emerged as a lead target for antiviral therapies. Since EBNA1 is also well-characterized at the functional, molecular and structural levels, it is an attractive target for drug discovery that could take full advantage of the power of virtual *in silico* screening methods. .

Virtual screening (VS) is the use of high-performance computing to analyze large databases of chemical compounds to identify possible drug candidates. VS can utilize a ligand-based drug design (LBDD) approach, which uses filters derived from properties of known active ligands, or it can utilize a structure-based drug design (SBDD) approach, where filters are based on the 3D structure of the protein [118–121]. A depiction of these different VS methods is shown in Figure 2. In the design cycle, the information extracted from the 3D structure of the target protein or, even better, from ligand-protein complexes, is used to design new ligands with improved binding affinities. Recently, Okimoto et al. used massive molecular dynamics (MD) simulations of protein-ligand conformations obtained by molecular docking in order to improve the enrichment performance of virtual screening [122]. The results indicated that the application of MD simulations to virtual screening for lead discovery is effective and practical, but that further optimization of the MD simulation protocols is required for the screening of various target proteins.

When the molecular structure of the target protein is available, the SBDD approach can be used with two principle operations: “docking” and “scoring.” Typically, the first is performed by docking a library of small molecules into a receptor active site and determining their optimal orientations by conformational, translational, and rotational movement. Subsequent scoring of these complexes is undertaken to assess the correct binding modes of the complexes, allowing ranking by affinity. This ranking allows prioritization for hit identification and lead optimization [119]. With respect to EBV related drug discovery, SBDD is limited to only those targets with known 3D structures. To date, EBNA1 is the only latency associated protein with a well-defined 3D structure amenable to structure-based virtual screening.

In LBDD process, the most effective biologically active lead molecule is detected using structural or topological similarity or a pharmacophoric similarity search [118]. The leads generated are ranked based on their similarity score, obtained using different methods or algorithms. QSAR(quantitative structure-activity relationship) relies on the definition of chemical descriptors that characterize a large variety of pharmaco-chemical properties of chemical compounds. QSAR correlate these descriptors based on the affinity of a compound for a given target [123]. The pharmacophore modeling is used to define the essential features of one or more molecules with the same biological activity. Pharmacophore-based virtual screening is involved in two steps: identification of pharmacophore model and 3-D search based on the specific constraints. These similarity search approaches offer three distinct access modes to identify potential hits from small molecule databases, thus being very useful in drug discovery [124].

Recently, one promising drug discovery approach named fragment-based drug design (FBDD) is gaining momentum in both large pharmaceutical companies and biotechnology laboratories as a complementary approach to high throughput screening or *in silico* screening. It samples chemical space more efficiently because the number of fragments needed to cover all reasonable chemotypes is many orders of magnitude smaller than that for most drug-like compounds, where multiple chemotypes are combined [125]. The strategy of

FBDD is shown in Figure 3. The first step is to identify key locations in the binding pocket and then bind small fragments to these locations. Once the seed fragments/functional groups are positioned, the next step is to link and combine, and finally to optimize combinations to generate lead compounds. This approach offers an alternative, complementary strategy to high throughput virtual screening (HTVS). It emphasizes efficiency and design, and lead compounds emanating from fragment-based drug discovery have a better chance of being successful. Recent data from Astex Technology indicate that, in a typical pharmaceutical company, 70% of initial hits ultimately fail, whereas 80% of fragment hits prove useful [126]. Therefore, the use of the FBDD method to identify EBV-related disease inhibitors should be a promising and feasible approach.

Despite that the application of virtual screening is still on its way, only Li et al. reported a new class of inhibitors against EBNA1 by *in silico* screening based on the crystal structure of EBNA1/DNA. A first round of screening yielded a set of structurally related compounds with 1.0–20 μM IC_{50} in biochemical inhibition assays for EBNA1 DNA binding (Figure. 1D). While the inhibitory activity of the identified compounds may not be as potent as conventional high-throughput physical screening, they did confirm that the EBNA1 can be a target to be applied in the inhibitor identification against EBV. Combining *in silico* screening with high-resolution X-ray crystallography to solve the EBNA1-inhibitor co-crystal will prove highly valuable for validation and inhibitor optimization through organic synthetic methods. Future application of fragment-based drug design in conjunction with other LBDD and SBDD approaches should also prove to be successful in designing a highly selective small molecule inhibitor of EBNA1.

4. Expert Opinion

Epstein-Barr Virus (EBV) is a ubiquitous human herpesvirus that can efficiently immortalize primary B-cells *in vitro* and can promote lymphoproliferative disease and lymphomagenesis in immunosuppressed individuals. EBV is also associated with 100% of the endemic forms of Burkitt's lymphoma and nasopharyngeal carcinoma, as well as large percentage of Hodgkin's disease lymphoma. After nearly three decades of study, there is still no drug approved for use in Epstein-Barr virus (EBV) infection despite the fact that many drugs inhibit the lytic replication of this virus in cell culture. Most of these efforts devoted to EBV drug discovery may be categorized as the chance discovery rather than a rational approach. In recognition of this problem, a full-blown effort to search for more effective EBV inhibitors has been initiated, yielding some promising lead molecules. *in silico* screening and modeling will be an essential component of the EBV drug discovery campaign. The success of *in silico* screening is dependent on advances in the computational methods and protein structural biology, especially crystallography. Regarding developing therapy for EBV-associated disease, the discovery of the inhibitors against EBNA1 from computational screening represents a first step in the structure-based design for pharmacological inhibition of EBV infection.

To date, only a few crystal structures of EBV-related targets are now available for us, which restrict the rapid development and wide use of structure-based virtual screening in anti-EBV therapeutics. Structural information may also be generated through the use of homology modeling methods. However, the accuracy of a homology model is related to the percentage sequence identity between the known and unknown structures. For closely related protein sequences with identities over 40%, the alignment is most often close to optimal. When the sequence identity decreases to less than 30%, the alignment becomes more difficult and the model is not reliable for SBDD. On the other hand, LBDD allows the construction of predictive models by learning to discriminate known ligands from non-ligands when the structure of target is unknown. However, the accuracy of ligand-based models quickly

degrades when the number of known ligands decreases. Given the limited number of known structures with high sequence similarity to EBV-related targets, and the few identified ligands with known EBV-related targets, we conclude that none of the classical computational approaches can be effectively utilized for additional EBV-drug discovery.

Recently, new methods have been described for *in silico* strategies which do not require ligand and receptor information. Jacob et al. proposed a general method to combine the chemical and the biological space to predict interactions between any small molecule and any target even in the absence of 3D structure and few or no known ligands [127]. In addition, Geppert et al. reported a different approach that combines ligand and target sequence information through a linear regression algorithm that enables learning [128]. These new techniques may be promising and helpful for the development of EBV inhibitors, especial in combination with conventional physical screening methods.

We conclude that an effective interplay between the experimental and computational approaches is crucial to guide the experimentalists in the synthesis and screening of compounds in a more rational way [129]. As more and more *in silico* approaches are developed, there application to drug discovery should provide novel and effective inhibitors for EBV latent infection and associated diseases.

Article Highlights

- Epstein-Barr Virus (EBV) is a ubiquitous human herpesvirus that can drive B-cell proliferation, and persists as a latent infection in several B-cell lymphomas and epithelial cell malignancies.
- No presently existing drug directly treats the EBV proteins responsible for the persistent latent infection and their associated oncogenic properties.
- EBNA1 is an EBV-encoded protein that is required for viral latency, and is consistently detected in all known EBV-associated tumors. EBNA1 is therefore an attractive candidate for therapeutic intervention.
- High-throughput *in silico* screening methods have been used to identify a class of small molecules that bind to the EBNA1-DNA binding domain and disrupt the essential DNA binding function.
- Several complementary methods can be used for *in silico* screening, including structure based drug design (SBDD), ligand based drug design (LBDD), and fragment based drug design (FBDD).
- All *in silico* approaches require complementary physical screening and structural information, although more advanced computational methods may be applicable to targets with unknown three-dimensional structures.
- Many of these methods can be used to further optimize the first generation of EBNA1 selective inhibitors.

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A. Nucleoside analogues serve as chain terminators for EBV DNA polymerase

B. Non-nucleoside inhibitors target viral DNA polymerase

C. EBV inhibitors with unknown targets

D. EBV inhibitors target EBNA1

Figure 1.

Known inhibitors against various enzymes of EBV. A: Nucleoside analogues serve as chain terminators for EBV DNA polymerase; B: Non-nucleoside inhibitors target viral DNA polymerase; C: EBV inhibitors with unknown targets; D: New compounds recently identified by *in silico* drug discovery targeting EBNA1.

Figure 2.

The categories of *in silico* screening approaches. Ligand based drug design (LBDD), and structure based drug design (SBDD) are considered as separate and complementary approaches for *in silico* screening approaches.

Figure 3.
The general strategy for the fragment-based drug design approach.