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Bortezomib-induced EBV and KSHV lytic gene expression: Oncolytic strategies

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Abstract

Purpose of review—Gamma herpesviruses are responsible for a substantial proportion of virus-associated human cancers, particularly in immunocompromised individuals. Methods that employ lytic activation of viruses latently infecting tumors represent a novel strategy of anti-neoplastic therapy.

Recent Findings—The proteasome inhibitor bortezomib has been shown to be a potent activator of gamma herpesvirus lytic cycle and has demonstrated activity in case reports of gamma herpesvirus-related malignancies. While initial reports implicated inhibition of the NF-kappaB pathway, more recent studies identify alternative pathways responsible for bortezomib-mediated lytic induction of gammaherpes viruses and activity against the malignancies that harbor them.

Summary—Further exploration of proteasome inhibition as an oncolytic strategy is warranted and will require clinical/translational trials to identify whether lytic induction of gamma herpesviruses correlates with clinical response to bortezomib, and, if so, to optimize this oncolytic strategy.

Keywords

bortezomib; Epstein-Barr virus (EBV); Kaposi sarcoma herpes virus (KSHV); gamma herpesvirus (GHV); oncolytic

Introduction

The role of infectious agents in cancer is increasingly recognized with more than 20% of human cancers now known to be associated with viruses.[1] Epstein-Barr virus (EBV) and Kaposi sarcoma herpes virus (KSHV, also known as human herpes virus-8, or HHV-8) are two of the most prominent infectious agents associated with cancers. These closely related members of the gamma herpesviruses (GHV) subfamily are characterized by their ability to establish latency in lymphocytes and typically exist as latent infections when associated with cancer. Strategies that employ lytic activation of the viruses represent a novel strategy of anti-tumor therapy for such viral-associated malignancies. Bortezomib (Velcade, Millennium Pharmaceuticals, Cambridge, MA) – a first in class proteasome inhibitor -- has been identified as a potent inducer of both EBV and KSHV lytic cycle. This review will focus on the preclinical and clinical data supporting therapeutic strategies employing

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bortezomib to induce lytic activation of EBV and KSHV in the treatment of GHV-associated cancers.

Body

Gamma Herpesvirus-associated malignancies—EBV was the first infectious agent associated with cancer after it was cultured in the early 1960s from tumor cells of patients with endemic Burkitt's lymphoma.[2] Since then, EBV has also been identified in other types of non-Hodgkin lymphoma, Hodgkin lymphoma, lymphomas arising in the setting of immunosuppression, nasopharyngeal carcinoma, and gastric carcinoma. During the 1980s, a dramatic increase was noted in the incidence of EBV-associated lymphomas as well as Kaposi sarcoma (KS), heralding the AIDS epidemic.[3] In the mid 1990s, KSHV, a closely related GHV, was identified from KS tissues in an AIDS patient establishing its association with KS. As with EBV, the intervening years have witnessed additional malignant associations with KSHV, including primary effusion lymphoma (PEL), plasmablastic lymphoma and the malignantly behaving oligoclonal lymphoproliferative disorder: multicentric Castleman's disease.[4] Table 1 summarizes the most commonly recognized EBV and KSHV-associated malignancies.

Many of the GHV-associated malignancies are over-represented in the immunocompromised population, particularly in persons with acquired immune deficiency syndrome (AIDS). Prior to the era of highly active anti-retroviral therapy (HAART), Kaposi sarcoma (KS) and non-Hodgkin lymphoma (NHL) comprised 90% of cancers in persons with AIDS.[3] In the large United States HIV/AIDS Cancer Match study, standardized incidence ratios (SIRs) of various cancers in persons with AIDS were compared to the general population.[3] The most dramatic increases involved EBV and KSHV-associated malignancies ranging from an SIR of 16 for Hodgkin lymphoma to approximately 1000 for primary CNS lymphoma and 3600 Kaposi sarcoma (see Table 2) in the HAART era. For most of the cancers, these ratios were even more dramatic prior to the HAART era which has resulted in overall improved survival and quality of life for persons with HIV. A notable exception to this is Hodgkin's lymphoma (HL) where the absolute incidence and SIR has continued to increase in the HAART era. A summary of the SIRs for the reported GHV-related cancers and their GHV association is presented in Table 2.

In addition to finding a higher incidence of GHV-associated malignancies in the setting of HIV/AIDS, the proportion of many cancer types associated with GHV is noted to be higher as well. For example, approximately 90% of HIV-related HL is associated with EBV compared to 33% in the general population.[5] Between 30 and 80% of cases of AIDS-NHL have latent EBV infection with additional lymphoma types latently infected with KSHV, which are very unusual outside of HIV/AIDS.[6] Focusing on DLBCL, EBV is present in approximately 30%[7] compared to 10-20% in non-AIDS DLBCL.[8] While AIDS-related Burkitt lymphoma has a higher prevalence of EBV compared to non-endemic (30-60 vs < 20%), HIV seronegative Burkitt's lymphoma, endemic Burkitt's NHL is associated with the highest rates (>95%).[9]

A consistent feature of GHV-associated malignancies is presence of the virus in a predominately latent state within the tumor cells. For example, KSHV latent genes products,

including LANA and v-FLIP, are the predominate genes expressed in both KS and PEL.[10] With EBV, latent infections have been classified into 3 types based on EBV gene expression patterns with type I expressing the most limited and type III the most expanded profile. EBNA-1 expression is common to all types of EBV latency states and is poorly immunogenic.[8] By contrast, type III latency is characterized by expression of all 9 latency genes resulting in increased visibility to cytotoxic T cell response.[8]

Oncolytic viral strategies—That lytic viral replication can result in clinical tumor regression is well established in the setting of oncolytic viral therapy. Onyx-015, an adenovirus engineered for tumor- selective replication and viral-mediated lysis of tumor cells has demonstrated lytic activity in a wide variety of tumor cell lines *in vitro* and in mouse xenograft models.[10] Clinical responses have been observed in both metastatic colorectal cancer and head and neck carcinoma and lytic viral replication was documented in each setting.[11] Inflammatory responses as measured by TNF, gamma interferon, IL1, IL2 and IL6 occurred in treated colorectal cancer subjects.[12] The colon cancer study of Onyx-015 with 5-FU demonstrated persistent viremia and both radiographical and biochemical evidence of inflammation centered at the tumor masses.[11], [12] These parameters demonstrate an immune response to viral mediated tumor cell lysis and correlated with improved longer-term clinical response even in the setting of heavily pre-treated patients who were receiving concurrent cytotoxic chemotherapy.[11], [12]

In the setting of GHV-associated malignancies, Lechowicz *et al* reported a pilot AIDS Malignancy Consortium (AMC) trial, exploring a viral lytic activation strategy in KS using valproic acid[13]. Valproic acid has modest activity as a histone deacetylase (HDAC) inhibitor, which in turn have been demonstrated to induce KSHV lytic activation. This pilot trial sought to determine changes in lytic expression of KSHV within the KS lesions after 1 and 4 weeks on valproic acid. This small study found that valproic acid trough levels at day 8 of therapy correlated with lytic KSHV mRNA but no changes in lytic protein levels in biopsies were found. The trial was designed with early stopping rules in the event that the lytic strategy resulted in an inflammatory syndrome or accelerated progression of disease but the treatment was well tolerated. One of the 18 patients treated had a partial response that persisted at last follow up (5 months post treatment).[13]

Proteasome inhibition, Bortezomib and Gamma Herpesviruses—The ubiquitin-proteasome pathway plays an important role in eukaryote cell cycle regulation and has been hijacked by many viruses -- including EBV, KSHV and HIV -- during cellular infection. In addition, it is a crucial regulator of NFkappaB (NF-kB) signaling. Bortezomib (Velcade) is a novel chemotherapeutic agent that has activity against a variety of tumor types through multiple mechanisms related to its inhibition of the human 26S proteasome.[14] Bortezomib has been shown to stabilize cellular proteins involved in suppressing cellular proliferation and promoting apoptosis including p21, p27, p53, and Ikappa-B;[15] it is most widely recognized for stabilization of the latter protein resulting in net inhibition of the NFkappaB pathway.

Data is accumulating that indicates bortezomib (Velcade) may have activity against cancers associated with GHV. The KSHV protein LANA has been demonstrated to target p53 and

von Hippel-Lindau (VHL) tumor suppressors for proteasomal degradation via its intrinsic ubiquitin E3 ligase activity encoded by a SOCS-box-like motif.[16] Decreased p53 and VHL activity would be expected to impair apoptosis and result in increased HIF-1alpha levels which in turn activates genes involved in angiogenesis, cell proliferation and survival. Supporting this, bortezomib inhibited cellular proliferation and induced apoptosis in cell lines derived from Primary Effusion Lymphoma (PEL), a subtype of NHL associated with infection by two gamma herpes viruses: KSHV and EBV.[17] In this study, bortezomib demonstrated more cytotoxicity against PEL cells than against cell lines derived from multiple myeloma, a disease for which bortezomib is currently approved for clinical use. Apoptosis induced by bortezomib was associated with inhibition of the classical and alternative NF-kappaB pathways, upregulation of p53, p21 and p27 and activation of the caspase cascade. Finally, treatment of PEL cells with bortezomib exerted a synergistic or additive cytotoxic effect in combination with chemotherapeutic drugs.

Other preclinical data suggest that the inhibitory effect of bortezomib on NF-kB would be expected to facilitate lytic activation of EBV and KSHV.[18] Bortezomib was identified in a screen of 2700 FDA-approved drugs as the most active inducer of the EBV lytic cycle. In this study, nanomolar concentrations of bortezomib led to up-regulation of EBV lytic proteins and increased EBV viral copy number. Similar viral copy increases occurred with transfection of I-kB, suggesting bortezomib's inhibition of NF-kB pathway is at least in part, responsible for bortezomib's lytic activation of EBV.[19] This group went on to demonstrate bortezomib activation of lytic EBV and KSHV both *in vitro* and in a murine xenograft model using human lymphoma lines.[20]

Others have similarly documented bortezomib-mediated EBV and KSHV lytic activation in B lymphocyte cell lines. Bagni et al., reported induction of EBV lytic replication in two latency type III B cell lines, HCL-B and HCL-P exposed to bortezomib. They also assayed samples from a patient with an EBV-related polymorphic B cell lymphoma treated with bortezomib and ganciclovir. EBV viral loads increased after bortezomib/ganciclovir treatment then fell to nearly 10% of baseline values after four total doses of the combination. Clinical response was not reported. [21] Brown, *et al.*, demonstrated expression of early-immediate, early and late KSHV gene expression from two lymphoma cell lines after bortezomib treatment.[22] Kaluza, et al., found bortezomib induces EBV lytic cycle in the human Burkitt's lymphoma cell line HR-1 and the EBV+ Akata cell line. They also demonstrated lytic gene induction *in vivo* using a murine xenograft model growing the Akata A.15 cell line subcutaneously.[23]

Regarding T cell lymphomas, Iwata, et al. found that bortezomib induced lytic infection in EBV-positive T lymphoma cells and NK lymphoma cells, although the presence of EBV did not appear to modulate the *in vitro* cytoxic effect of bortezomib. They did report potent induction of caspase dependent apoptosis by bortezomib which may account for the lack of differential effects. Supporting this hypothesis, the group found that bortezomib had a greater killing effect on EBV-infected cells when they administered bortezomib to peripheral blood mononuclear cells from two patients with EBV-associated lymphoproliferative diseases.[24]

Clinically, activity of bortezomib has been reported in various GHV-associated malignancies. Three separate reports of bortezomib activity in plasmablastic lymphoma in HIV seropositive and negative subjects have been published. In one case of a 19 year old man with stage IVB, EBV and KSHV HIV-associated PEL, had response to bortezomib + dexamethasone after rapidly progressive early relapse post autologous hematopoietic stem cell transplant.[25] The second case involved a newly diagnosed HIV infection with advanced immunosuppression (CD4 count 61/microl) and tumor related hyperbilirubinemia limiting other chemotherapeutic options. The patient experienced a dramatic response within 7 days of single agent bortezomib as documented by PET scan, but died of septic shock 15 days after 2 weeks of biweekly bortezomib treatments.[26] In the third case, bortezomib treatment resulted in such rapid response that clinical tumor lysis syndrome occurred.[27] With respect to PEL, the responses reported have been mixed with Boulanger et al.[28] reporting 3 cases failing to respond to bortezomib in second or fourth line salvage settings but with Siddiqi et al.[29] and Sarosiek et al.[30] both reporting prolonged remissions in one case each with bortezomib-containing regimens.

One possible application of viral lytic induction involves coupling lytic induction to targeted therapy dependent on expression of lytic viral proteins. The thymidine kinases (TK) expressed during lytic cycles of EBV and KSHV are capable of phosphorylating the nucleoside analogues ganciclovir (GCV) and 3′-azido-3′deoxythymidine (AZT) into their active, cytotoxic forms which inhibit cellular as well as viral DNA polymerase. Others have used high dose AZT and gancyclovir in GHV-associated malignancies using alternative methods to induce lytic activation such as radiation. Fu et al. capitalize on bortezomib-mediated induction of TK expression by following bortezomib treatment with a radiolabeled nucleoside analogue to further inhibit growth of naturally infected EBV and KSHV cell lines and xenografts.[20]

Alternative pathways impacted by bortezomib in GHV malignancies—Recent findings have challenged the hypothesis that bortezomib's inhibitory effects on the NFkappa B pathway are responsible for its lytic activation of GHV. O'Farrell et al.[31] studied the promoters of EBV and KSHV immediate early viral protein reporters (EBV Zta and KSHV Rta) and found that IKappaB did not activate these promoter reporters suggesting that other pathways must be important in activation. They looked to C/EBP proteins as previous studies identified C/EBP family members as activators of EBV and KSHV immediate early gene expression, and bortezomib has been noted to lead to increased levels of C/EBPbeta in a variety of tumor cell lines. They found that bortezomib treatment leads to increased binding of C/EBP-beta to the EBV Zta promoter and that knock down of C/EBP-beta inhibits bortezomib activation of EBV lytic gene expression. They further demonstrated that bortezomib induces the unfolded protein response (UPR) involved in endoplasmic reticulum (ER) stress pathways. Using thapsigargin, an inducer of the UPR that does not interfere with proteasome function, they also found induction of EBV lytic gene expression that was similarly inhibited by C/EBP-beta knock down.[32] Their studies support a model where bortezomib-mediated GHV lytic induction is NFkappa B-independent and rather is mediated through ER stress pathways involving C/EBP-beta.

In another recent report, apoptosis induced by bortezomib in a model of PEL did not appear to be associated with either NFkappa B nor UPR. Sarosiek et al.[30] report on an elderly HIV negative patient diagnosed with EBV and KSHV positive PEL who responded to combination therapy with bortezomib and doxorubicin. Cells freshly isolated from the pleural fluid of this patient were transferred into the peritoneal cavities of NOD/SCID mice without *in vitro* cell growth to avoid the changes in viral gene expression that can occur in cultured cells. Using this model they found bortezomib-induced PEL remission and extended overall survival of mice bearing lymphomatous effusions. They demonstrated lytic induction of KSHV with both bortezomib and doxorubicin, with bortezomib inducing higher levels of apoptosis at earlier time points (24 hours vs 1 week) and at clinically achievable concentrations. As is typical of PEL, this model was found to constitutively exhibit activation of NFkappaB and a partially activated UPR. However, the proapoptotic effects of bortezomib noted did not appear to be mediated by inhibition of NFkappaB pathway or by induction of UPR as profiles of these pathways were not significantly changed post bortezomib in these *in vivo* mouse models. An alternative pathway known to be involved in PEL survival - the PI3 kinase/Akt/mammalian target of rapamycin (mTOR) signaling axis – was also assessed but did not appear to be significantly affected either. Transcriptome analysis by genomic arrays revealed that bortezomib down-regulated cell-cycle progression, DNA replication, and Myc-target genes.

Risks of viral lytic activation strategies—Possible risks of lytic viral induction treatment strategies include unintended consequences of increased GHV viremia. Longer-term consequences of lytic induction may lead to infection of additional lymphocytes and/or endothelial reservoirs which may in turn increase the long-term risk of gammaherpes virus-associated syndromes or malignancies including new primary KS tumors within the body in the case of KSHV-infected individuals. An important KSHV-related syndrome characterized by high levels of KSHV lytic gene products is multicentric Castleman's disease (MCD).[33] MCD presents with systemic inflammation marked by elevated IL-6, C-reactive protein, lymphadenopathy, splenomegaly, fevers, hypotension pancytopenia, hypoalbuminemia, and oligo-/mono-clonal gammopathy.[9] MCD can lead to death via respiratory, renal or other organ failure related to systemic inflammation. It follows that in driving lytic activation of KSHV, there is a theoretical risk of precipitating multicentric Castleman's disease or a similar life-threatening systemic inflammatory reaction.

Conclusion

GHV are among the most common viruses currently known to be associated with malignancies, particularly in the setting of HIV infection where they are involved in more than half of cancer cases. Recent reports validate earlier reports of GHV lytic induction by bortezomib and suggest its clinical activity, while some studies challenge the pre-existing paradigm of NF-kB as the primary regulator of GHV lytic induction and apoptosis in GHV malignancies. Most significantly, these studies illustrate the pleiotropic effects of proteasome inhibition, highlight the complexity in predicting outcomes in laboratory models when modulating a myriad of pathways, and underscore the need for clinical research that integrates correlative bench studies. Given the relative rarity of each GHV cancer subtype,

novel trial designs and collaborative research methods will be critical to ensure progress in this area. Clinical-translational research trials are currently ongoing through the AIDS Malignancy Consortium (AMC) using bortezomib in AIDS-related Kaposi sarcoma and GHV-associated NHL with objectives including correlating evidence of lytic induction of GHV with clinical response.

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Key Points

- Gamma herpesviruses are commonly associated with malignancies particularly in the setting of HIV infection.
- The proteosome inhibitor, bortezomib, is a potent inducer of gamma herpesviruses activation.
- Lytic activation of EBV/KSHV latently infecting cancer cells is expected to be beneficial both for direct tumor lytic effects as well as increased cytotoxic immune response against cancer cells expressing lytic viral antigens.
- Recent evidence suggests pathways other than NFkappaB as responsible for viral lytic induction and cytotoxicity by bortezomib in gamma herpesviruses-related malignancies.
- Clinical trials are needed to determine whether lytic induction of gamma herpesviruses correlates with clinical response to bortezomib in gamma herpesviruses-related malignancies.

Table 1
EBV and KSHV-associated malignancies. (original)

Epstein-Barr Virus (EBV)	**Kaposis Sarcoma Herpesvirus (KSHV/HHV-8)**
Lymphomas	Lymphomas
Burkitt's lymphoma	Primary effusion lymphoma*
Hodgkin's lymphoma	MCD-associated plasmablastic lymphoma
NK-T cell lymphoma	KSHV-assoicated germinotropic lymphoproliferative disorder*
Immunodeficiency associated lymphomas	
AIDS-related lymphomas	
Immunoblastic lymphoma	
Post-transplant lymphoproliferative disease	
Primary central nervous system lymphoma	
Plasmablastic lymphoma*	
Carcinomas	Kaposi sarcoma
Nasopharyngeal carcinoma	
Gastric carcinoma	

*These diseases may at times have dual KSHV/EBV infection. AIDS = acquired immune deficiency syndrome MCD= Multicentric Castleman's disease. KSHV = Kaposi sarcoma herpes virus

Table 2

Standardized Incidence Ratios (SIRs) of cancers in persons with AIDS in the HAART era (1996-2002) (original – references embedded)

Cancer	Standardized Incidence Ratio (95% CI)[3]	Viral association (%)
Kaposi sarcoma	3640 (3330–3980)	KSHV (100%)
CNS NHL	1020 (838-1220)	EBV (100%)
Non-Hodgkin lymphoma	22.6 (20.8–24.6)	EBV (varies by subtype) KSHV (varies by subtype)
Diffuse large B-cell NHL	29.6 (26.1-33.3)	EBV (30% ABC[7], 90% IB[4])
Hodgkin Lymphoma	13.6 (10.6-17.1)	EBV (∼90% in HIV)[5]

The SIRs represent the ratio of observed to expected new cases of cancer; the expected number is based on the age-specific rates for all of US population. Note that this is limited to AIDS cases and not inclusive of persons with non-AIDS HIV and cancer. Rates of tumor association with virus presented in the third column. AIDS = acquired immune deficiency syndrome. CI = confidence interval; EBV = Epstein Barr virus; KSHV = Kaposi sarcoma herpes virus; NHL = non-Hodgkin lymphoma.