Viral Latent Proteins as Targets for Kaposi's Sarcoma and Kaposi's Sarcoma-associated Herpesvirus (KSHV/HHV-8) Induced Lymphoma

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FINAL

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Abstract: Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8) is present in all Kaposi's sarcoma tumor cells as well as in several lymphomas that are linked to this agent. Every tumor cell expresses the viral latent protein LANA, which is required for KSHV latent replication and proper segregation of the viral episome. In certain tumors, other latent KSHV proteins (LANA-2/vIRF3, v-cyclin, v-IL6) are expressed as well. Since all herpesviruses persist for life in infected individuals, only eradication of latent virus can cure infection. The KSHV latent genes serve as bona fide tumor markers, but do they also provide targets for anti-tumor and/or anti-viral drugs? To decide this question we review the known biochemical interactions between KSHV latent proteins and their viral and cellular partners. Recent epidemiological studies show that KSHV lytic replication precedes KSHV associated cancers. Gancilovir has been linked to KS tumor regression, which implicates the KSHV-encoded polymerase as a potential intervention point. Yet, KSHV specific transactivators might represent more specific targets, as they have no cellular homologs. In particular Rta/orf50 is necessary and sufficient for lytic replication and deserves serious consideration as a target for KSHV-specific antivirals.

Key Words: Kaposi sarcoma, KSHV, LANA, orf50

INTRODUCTION

Using representational difference analysis Chang *et al.* [1] demonstrated the presence of a novel human virus in Kaposi's sarcoma biopsies: Kaposi's sarcoma-associated Herpesvirus (KSHV/HHV-8). On the basis of the complete sequence of the 137 Kbp unique region, KSHV is classified as a gamma2-herpesvirus, a member of the lymphotropic subgroup of the herpesviridae. Consistent with this classification KSHV is found primarily in $CD19^+$ B-cells of KS patients, although monocytes, KS tumor endothelial cells and in some instances even CD4 T-cells also harbor KSHV genomes.

KSHV causes Kaposi's sarcoma (KS). Every KS patient develops antibodies against KSHV, every KS tumor harbors the viral genome, and every KS tumor cell expresses at least some viral proteins. Like other human herpesviruses, primary KSHV infection is mild, rapidly cleared, and lifelong latency is subsequently established in the host. KS represents a viral reactivation phenotype, brought about by systemic immuno-suppression. Consequently, lowering the immunosuppressive regime will lead to regression of transplant-associated KS, and restoration of CD4 levels will clear KS lesions in AIDS-associated KS. Often, however, it is not possible to re-establish the normal immune functions, which in the case of transplant KS may result in graft rejection. Here, therapeutics targeted specifically against KSHV will offer a survival benefit.

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In AIDS patients, KSHV is also found in two, rare B-cell lymphoproliferative disorders: primary effusion lymphoma (PEL) and multi-centric Castleman's disease (MCD) (reviewed in [2]). Again, KSHV viral latent proteins are expressed in every lymphoma cell and serve as unique tumor markers, as well as targets for vaccine and drug development. Additional factors, such as local elevation of endogenous cytokines or HIV-1 tat, also influence tumor progression, but only in the presence of KSHV. Like all other herpesviruses, KSHV can enter two modes of replication: lytic or latent. During lytic replication, all viral proteins are expressed and the host cell is destroyed by virion egress, while during latency, the viral episome is replicated once per cell cycle and faithfully segregated into both daughter cells, see Fig. (**1**).

LANA/orf 73

Latent episome maintenance and proper segregation is absolutely dependent on the KSHV latency-associated nuclear antigen (LANA). In fact, LANA is present in every KSHV-infected cell [3], in KS, MCD, and PEL. LANA is a 200 kD nuclear phosphoprotein that is encoded by open reading frame (orf) 73. It binds directly to a consensus DNA sequence in the terminal repeat (TR) units of the KSHV genome and to cellular chromosomes. LANA binds to the KSHV TR *in vitro* with an apparent KD of 1.5 nM [4]. DNA binding and episome replication is abrogated by deletion of C-terminal amino acids [5-7]. Unfortunately, the interactions between LANA and cellular chromosomes are much less defined. They are mediated by several cellular proteins and possibly multiple mechanisms [8-13]. In tissue culture,

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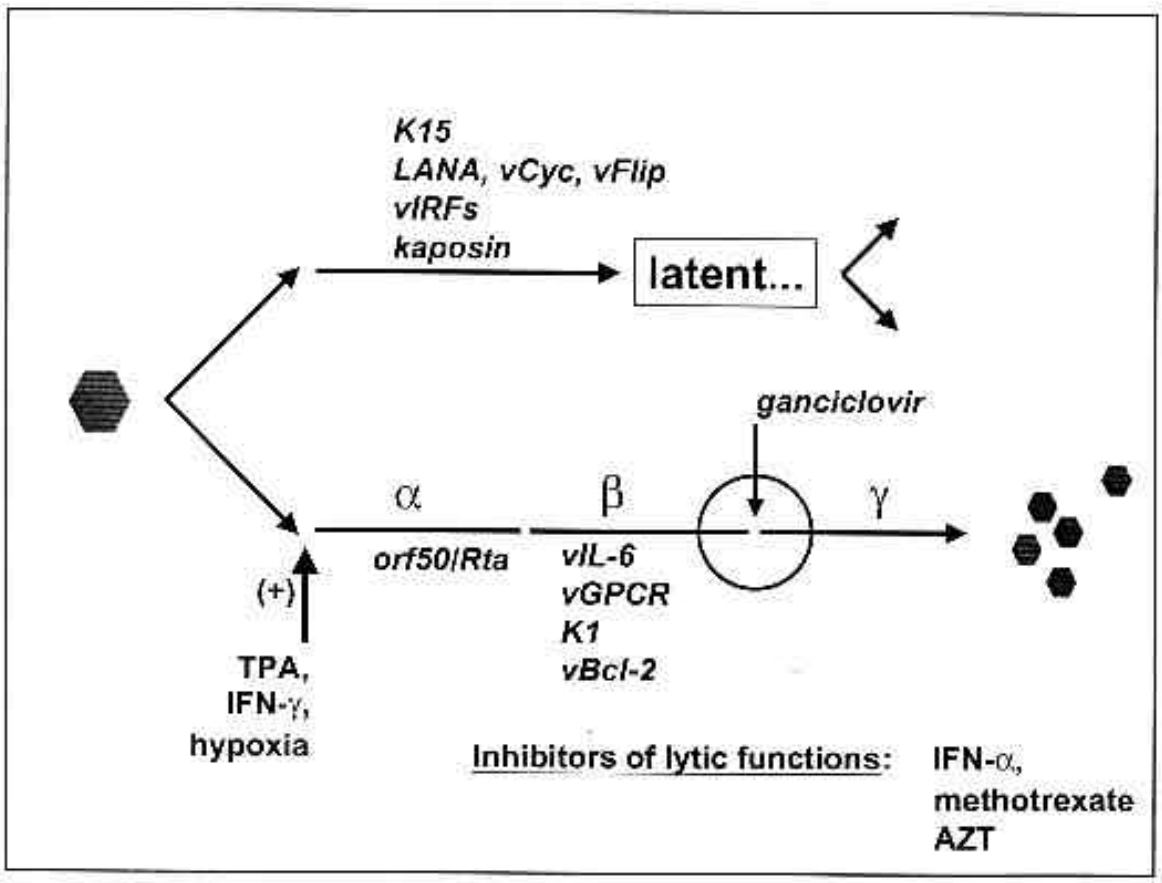

Fig (1). Summary of the KSHV latent (upper arrow) and lytic (lower arrow) life cycle. Indicated are stimuli, which reactivate KSHV from latency (phorbol ester, IFN-gamma, hypoxia) as well as currently known inhibitors of replication (ganciclovir, IFN-alpha, methotrexate, AZT). Also shown are KSHV genes that could be potential targets for intervention.

LANA alone suffices to maintain and replicate the KSHV episome as well as artificial substrates [14-17]. This phenotype is analogous to the function of the Epstein-Barr-Virus (EBV) EBNA-1 protein, but LANA seems to be less efficient in fulfilling its role.

In addition to its essential role in KSHV episome segregation and latent replication, LANA has also been implicated directly in tumorigenesis [18]. LANA binds to an increasing number of cellular proteins, many of which are involved in growth regulation such as Ring3, Sin, Rb and p53. LANA also has the ability to regulate cellular transcription [19] as well as its own viral promoter [20]. At present, it is not clear which of these interactions are essential for tumor development. A useful model to rationalize LANA's multiple functionalities is to think of it as a KSHV analog to SV40 large T antigen (Tag), which also combines replication with growth regulation and even enzymatic functions (ATPase, helicase), although KSHV LANA exhibits nowhere near the transforming potential of SV40 Tag.

v-CYCLIN/orf72

Since LANA is translated from a tri-cistronic latent mRNA that also encodes v-cyclin/orf72 and v-FLIP/orf71 [21-23], it is conceivable that all three viral proteins are required to sustain KSHV latency and that these three proteins also cooperate to cause KS and PEL. The KSHV v-cyclin open reading frame is latently expressed and the protein has been found in PEL and KS lesions. v-Cyclin is a homologue to cellular D-type cyclins, which play a critical role in propelling cell cycle progression from G1 to S phase. D-type cyclins bind to and direct target specificity of their kinase counterparts, cyclin-dependent kinases (CDKs) CDK4 and CDK6, which are able to phosphorylate the retinoblastoma (pRb) tumor suppressor protein. Phosphorylation of pRb mediates the release of repression on E2F. Free E2F then transactivates promoters of genes that are required for DNA replication such as other cyclins, PCNA and DNA polymerase. v-Cyclin primarily binds to and activates CDK6 [24] and directs phosphorylation of pRb *in vitro* [24-26]. Unlike cellular D-type cyclins, this activation proceeds independently of CDK-Activating Kinase (CAK) phosphorylation [27].

In contrast to cellular cyclins, KSHV v-cyclin is resistant to inhibition by cyclin-dependent kinase inhibitors (CDKIs) of both the INK and Cip/Kip families due to a point mutation in the CDKI binding site [28]. In fact, v-cyclin/CDK6 phosphorylates $p27^{Kip}$, triggering its subsequent degradation [29,30]. These unique characteristics enhance KSHV v-cyclin activity by rendering it resistant to the negative

regulation of CDKIs. Consequently, v-Cyclin has been shown to initiate nuclear DNA replication *in vitro* [31] and to stimulate entry into S phase in quiescent fibroblasts. The v-cyclin/CDK6 complex has a wide range of phosphorylation targets including pRb, Histone H1, $p27^{Kip}$, Id-2, Cdc25a, Orc1, Cdc6, and Bcl-2, although the significance of these biochemical interactions needs to be verified.

Of note, a delicate balance exists between cell cycle progression and apoptosis. Discordant signals, such as elevated E2F in the presence of wild-type p53 [32] lead to apoptosis. This may explain why expression of KHSV v-cyclin can induce apoptosis in cells expressing high levels of CDK6 [33], presumably by phosphorylating the anti-apoptotic Bcl-2 cellular protein leading to its subsequent degradation ([34]. This phenotype does not exist in a $p53^{-/-}$ background [35] or if v-cyclin is under control of its own promoter (Staudt and Dittmer, unpublished). The latter scenario is consistent with the low level of apoptosis that can be seen in KS and PEL, where LANA, which inhibits p53, and v-FLIP, which inhibits FAS-signaling, curb apoptosis. Inhibiting LANA:p53 and/or v-FLIP:caspase interactions through pharmacological means may therefore cause latently infected KS cells to self-destruct as a result of unbalanced v-cyclin activity.

v-FLIP/orf71

The v-FLIP (orf71) protein has sequence homology to Equine Herpesvirus-2 E8 and Herpesvirus Saimiri (HVS) orf71 [36]. It inhibits CD95/FAS-induced apoptosis *in vitro* by blocking caspase-3, -8 and –9 [37]. Both CD95/Fas-L and TRAIL/TNF-alpha induce apoptosis through a similar mechanism. Clustering of the receptor upon binding of the ligand recruits an adapter molecule (FADD and TRADD, respectively) with a binding domain (DD) for the receptor and a conserved "death-effector-domain" (DED) that binds and triggers the activation of caspase-8. The death signal is then transduced through a number of cellular caspases resulting in the commencement of cellular apoptosis. A possible mechanism for KSHV v-FLIP postulates competition with the adapter molecule for binding to caspase 8 via its DED domain.

v-IRFs

KSHV encodes three viral interferon-regulatory factors (IRFs), namely vIRF-1/K9, vIRF-2/K11.1 and vIRF-3/LANA-2 [38-41]. These transcription factors exhibit sequence homology to cellular IRFs. They abrogate interferon signaling in co-transfection experiments, with the aim of blunting the interferon response to viral infection. Both vIRF-1/K9 and vIRF-3/LANA-2 are expressed in KSHV-latently infected cells [42], except that vIRF-3/LANA-2 is present only in KSHV-associated B-lymphoid malignancies, but not KS [39]. Doubtless, the interferon signaling pathways in lymphocytes and endothelial cells differ, and KSHV has evolved tissue-specific response modifiers. All three viral IRFs represent dominant negative alleles that interfere with the transactivation of interferon-responsive element (ISRE) containing promoters. However, the KSHV vIRFs do not bind themselves to the ISRE response element. Rather, they interfere with assembly of the interferon-stimulated gene factor (ISGF) complex (including CBP/p300) as well as STAT signaling. The vIRF-1/K9 and vIRF-3/LANA-2 proteins also bind to the p53 tumor suppressor protein and inhibit its transcriptional as well as pro-apoptotic function [39,40]. The vIRF:p53 interaction may explain why vIRF-1 and vIRF-3, but not vIRF-2, exhibit proliferative potential in fibroblast transformation assays. In contrast, the vIRF-2/K11.1 protein binds to the NFκ-B consensus motif, but not the ISG element and it physically interacts with the double-stranded RNA-activated protein kinase (PKR) [41]. Other interactions between the KSHV vIRFs and interferon signaling pathways are likely to surface soon. It may be prudent to assume that most of the KSHV-induced signaling molecules, which generate an effect in tumorigenesis screens, represent an effort on part of KSHV to escape the cellular immune response. Likewise, virtually all KSHV homologous to cellular proteins (vIL6, vMIPs, vGPCR, vCyclin, vFLIP, vIRFs, vOx2, vBCL2), as well as many of the KSHV-specific proteins (K1, K2, K4, K5, K15) are involved, in one way or another, in combating the host response to viral infection [43].

KSHV SIGNAL TRANSDUCERS: K1, V-GPCR, K15, V-IL6, KAPOSIN

KSHV encodes a number of proteins with cell signaling abilities. The KSHV IL-6 and (three) MIP homologs function as soluble mediators, which induce proliferation and chemotaxis in neighboring lymphocytes. KSHV has "supercharged" the viral homologs relative to their cellular ancestors. For instance, the viral vIL-6 (K2) can bind the IL-6 receptor gp130 subunit, but unlike hu-IL-6 it does not require the gp80 (IL-6Ralpha) subunit for signaling [44]. This allows vIL-6 to bypass one layer of negative regulation that might otherwise be exerted by gp80 resulting in a "net" stimulation of KSHV-infected cells [45]. The K1, K15 and v-GPCR (orf74) KSHV proteins likewise exhibit constitutive, ligand-independent signaling activity and these functions are conserved among the different rhadionoviruses (reviewed in [46]). K1 induces signaling *via* ITAM motifs, K15 *via* SH2/SH3 domains and v-GPCR *via* its seven-transmembrane motif. These signaling activities are constitutive and ligand-independent, although ligand binding may further modulate the activity (e.g. the KSHV v-GPCR still binds human IL-8 with nanomolar affinity). Kaposin/K12 stimulates ARF GTPases *via* cytohesin-1 [47]. Not surprisingly ectopic expression of K1, K15, Kaposin or v-GPCR transforms NIH3T3-fibroblasts in culture through activation of specific cellular signaling cascades. Yet, only K15 and kaposin are expressed during latency. Upon lytic KSHV replication of some cells (<5%) in a KS lesion or PEL, paracrine growth factors are released, which create a milieu that supports hyperplasia of neighboring, even uninfected, endothelial cells, angiogenesis and recruitment of inflammatory leukocytes. Together these three phenotypes define what is called a KS lesion.

Orf50/Rta

Herpesvirus lytic replication follows an ordered cascade of gene expression. Immediate early (alpha) proteins are

expressed first, followed by early (beta) and finally late (gamma) proteins. Rta/orf50 is a KSHV immediate-early transactivator. It alone is sufficient to initiate the entire lytic cascade [48-50]. More important, a dominant negative orf50 allele abolishes KSHV replication [51] and in the related mouse rhadinovirus MHV-68 Rta/orf50 fulfills the same reactivation function [52]. Rta/orf50 is the homolog of EBV Rta/BRLF-1. It transactivates a number of KSHV early (beta) promoters, but by several different mechanisms: (i) Purified Rta/orf50 binds directly to its consensus sequence in the KSHV KbZIP, orf57, nut-1/PAN, K12 and IL-6 promoters [53-55]; (ii) it can interact with RBP-jk and this interaction positively regulates the orf57, ssB and TK promoter [56]; (iii) it autoregulates its own promoter via Oct-1 and may also aid in Sp-1 mediated transcription [57] [58].

KSHV encodes several other immediate early proteins (orf57/Mta, orkK8/KbZIP, orf45) that like Rta/orf50 contribute to lytic replication. Unlike Rta/orf50, none of these proteins alone is able to induce KSHV lytic reactivation from latently infected PEL. Recent evidence suggest that these players are primarily concerned with the regulation of cellular pathways to pave the way for full-blown viral lytic replication in more restrictive cell types [59,60].

EXPERIMENTAL MODELS OF KSHV PATHO-GENESIS

How could potential drugs against KSHV genes be evaluated? Two effects need to be considered: inhibition of viral replication and direct anti-tumor toxicity, which should be specific for KSHV infected cells. Inhibitors of KSHV replication can be screened rapidly in the BCBL-1 culture model of TPA-induced, viral reactivation [61-63] as well as in humanized SCID mice, which are susceptible to primary KSHV infection [64]. There is a caveat, however, as illustrated by methotrexate [65]. Methotrexate inhibits KSHV by interfering with Rta/orf50-induced reactivation, but its effect on viral replication per se is unclear.

Nucleoside analogs, such as gancilovir, inhibit the KSHV polymerase and have a beneficial effect on KS, since they seem to lower tumor burden and spread in patients [66]. More targeted clinical studies are needed, but it is conceivable that limiting primary viremia lowers the proportion of KSHV latently infected cells, and the subsequent probability of reactivation. Furthermore, KSHV lytic proteins (such as the vGPCR, vIL-6 and vMIPs) contribute to KS development through paracrine effects. Therefore, eliminating or limiting the number of KS cells that undergo KSHV lytic replication will shutdown any such paracrine loops.

Conventional cytotoxic cancer therapy against KS and KSHV-associated lymphoma should be augmented by targeting viral anti-apoptosis genes (vFLIP, vBCL-2) or viral pro-proliferative genes (vCyclin, LANA, vGPCR). Targeting viral IRFs should synergize with IFN-alpha, which is FDA-approved as a treatment against KS. The various PEL-derived cell lines, which harbor latent KSHV, provide a first line screen for agents against KSHV-associated lymphomas. Harrington and colleagues [67] used AZT together with interferon alpha to induce apoptosis in PEL and EBV-positive Burkitt's lymphoma. In contrast EBV- and KSHV-negative non-Hodgkin's lymphoma were resistant to treatment. The exact mechanism of action remains to be elucidated, but interfering with the nucleotide metabolism in KSHV-infected lymphoma proved a rational choice: (a) all lymphocytes are inherently sensitive to changes in nucleotide metabolism, since the precursor pools are limited and (b) lymphotropic herpesviruses, such as KSHV, encode a number of enzymes which affect nucleotide synthesis, such as thymidine kinase, thymidilate synthase, dehydrofolate reductase and ribonucleotide reductase.

The SCID-hu Thy/Liv mouse model supports KSHV replication [64], as do other SCID-human implant models for KSHV (skin, cord-blood). In SCID-hu Thy/Liv mice fetal thymus and liver are implanted under the murine kidney capsule and human hematopoietic and lymphoid precursor cells reconstitute an organ that faithfully reproduces human multi lineage hematopoiesis, including thymopoiesis. T-lymphocytes in various stages of development comprise the bulk of cells in the implant, but cells of all hematopoietic lineages (including monocytes and B cells) as well as stromal endothelial cells are present. Replication of many of human viruses is observed in the lymphoid (e.g. HIV-1, HTLV-1, HHV-6, VZV) and stromal (e.g. HIV-1, HCMV, measles virus) compartment of the graft. Depending on the biology of the particular virus, the resulting infection may be non-cytopathic, or may induce severe target T-cell depletion. KSHV infection of SCID-hu mice proceeded biphasic. An early phase of lytic replication peaked at 14 days post infection (p.i.) and was accompanied and followed by long-term latency (up to 120 days p.i.). Infection depended on intact virions, since UV irradiation of the inoculums abolished all DNA and mRNA-derived signals. None of the mice developed lymphomas and thus this model cannot be used to study tumorigenesis, but KSHV lytic replication was inhibited by ganciclovir demonstrating that replication depended on the viral DNA polymerase (orf9) and hereby establishing a small animal model in which to study potential anti-KSHV drug effects.

Gamma-herpesviruses that are homologous to KSHV have been isolated and replicate in other species such as mice (murine herpesvirus 68/MHV-68) or macaques (rhesus monkey rhadinovirus/RRV, retroperitoneal fibromatosis herpesvirus RFH) [68,69]. Many more have been detected in other monkeys by PCR, but thus far have not been cultured. Since MHV-68 and RRV are easily propagated and manipulated in culture, they represent useful surrogates for KSHV. MHV-68 Rta/orf50 can reactivate human KSHV from latency and the RRV polymerase exhibits similar sensitivity to nucleotide inhibitors. Since MHV-68 and RRV plaque on fibroblasts, replication defects can be measured by single round and multiple round plaque reduction assays. RRV causes B-cell hyperplasia in SIV-infected rhesus macaques, and at some frequency KS-like lesions [70, 71]. In these animals SIV is absolutely required for disease manifestation. RFHV, another macaque rhadinovirus, is found in retroperitoneal fibromatosis (RF) lesions, which are, arguably, KS-like, but thus far this virus has not been propagated in culture. The study of animal rhadinoviruses is still in its infancy and exact correspondences between the non-human rhadinoviruses and KSHV with regard to gene regulation, latency and tropism need to be established.

However, either as homologous targets or after the creation of a chimera, which depend on one more KSHV proteins for replication, MHV-68 and RRV can be employed in whole virus drug screens.

Transgenic mouse models have been developed for individual KSHV genes, such as vGPCR [72-74], K1 [75], v-cyclin [35] and LANA (Dittmer, unpublished). In these models vGPCR and K1 activated the same molecular signaling pathways, as predicted from biochemical studies. Albeit classified as KSHV lytic genes by array analysis, ectopic expression of vGPCR and K1 in transgenic mice caused a dysplastic, highly angiogenic lesion, which underscores a role for these genes in the recruitment of inflammatory cytokines and possible paracrine functions in KS. Since any one KSHV oncogene recapitulates only one aspect of the KS phenotype a combination of many transgenes in a single mouse should represent a highly relevant model.

In conclusion, KSHV expresses many potential targets for anti-viral or anti-tumor therapy, see Table **1**. Because KSHV is found exclusively in KS or PEL tumor cells, any agent that targets a viral-viral or viral-cellular protein interaction will have a high therapeutic index.

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ABBREVIATIONS:

KSHV	=	Kaposi's sarcoma-associated Herpesvirus
LANA	=	latency-associated nuclear antigen
PEL	=	pleural effusion lymphoma
MCD	=	multi-centric Castleman's disease
TR	=	terminal repeat; EBV, Epstein-Barr Virus
SV40	=	Simian Virus 40
v-FLIP	=	viral FLICE-inhibitory protein
CDK	=	cyclin-dependent kinase
CDKI	=	CDK-inhibitor protein
HVS	=	Herpesvirus Saimiri
DD	=	death domain
DED	=	death-effector domain
IRF	=	interferon-regulatory factor
ISRE	=	interferon-responsive element
ISGF	=	interferon-stimulated gene factor
v-GPCR	=	viral G protein-coupled receptor
ITAM	=	immunoreceptor tyrosine-based activation motifs
BCBL	=	body cavity-based lymphoma
TPA	=	12-O-tetradecanoylphorbol 13-acetate

Table 1. Summary of Potential KSHV Drug Targets, Expression Pattern, Mechanism for High Through-Put Screen and Detailed Structural Information for vMIP-II[76,77, 78], vBCL [79], vIL-6[80], and v-cyclin[81].

Gene	Orf	Expression	Cellular Homolog	Function	Structure	*In vitro* Target Domain/Interaction
vCyclin	72	latent	cyclin D/E	cell cycle	Yes	vCyclin:cdk6
LANA	73	latent	-	episome maitanance		DNA binding
vFLIP	71	latent	FLICE	anti-apoptotic		DED domain
vIRF-3/LANA-2	K10.5	latent	IRF	inhibition of IFN response		vIRF:p53
vIRF-1	K9	latent	IRF	inhibition of IFN response		vIRF:p53
K15	K15	latent	-	signaling		SH3
kaposin	K12	latent	-	-		-
K1	K1	lytic	-	signaling		ITAM
vIRF-2	K11.1	lytic	IRF	inhibition of IFN response		vIRF:PKR, DNA binding
vGPCR	74	lytic	GPCR	signaling		G-coupled signaling
Ox-2	K14	lytic	Ox	NCAM adhesion		NCAM
vBCL		lytic	BCL-2	anti-apoptotic	Yes	BH3:Bac
Rta/orf50	50	lytic	-	transcription		DNA binding
vIL-6	K2	lytic	IL-6	cytokine	Yes	receptor binding
vMIP-I	K4	lytic	MIP	CC-chemokine		receptor binding
vMIP-II	K6	lytic	MIP	CC-chemokine	Yes	receptor binding

SCID = severe combined immune deficiency

PCR = polymerase chain reaction

RF = retroperitoneal fibromatosis;

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