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Selective peptide inhibitors of antiapoptotic cellular and viral Bcl-2 proteins lead to cytochrome c release during latent Kaposi's sarcoma-associated herpesvirus infection

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Abstract

Kaposi's sarcoma-associated herpesvirus (KSHV) is associated with B-cell lymphomas including primary effusion lymphoma and multicentric Castleman's disease. KSHV establishes latency within B cells by modulating or mimicking the antiapoptotic Bcl-2 family of proteins to promote cell survival. Our previous BH3 profiling analysis, a functional assay that assesses the contribution of Bcl-2 proteins towards cellular survival, identified two Bcl-2 proteins, cellular Mcl-1 and viral KsBcl-2, as potential regulators of mitochondria polarization within a latently infected B-cell line, Bcbl-1. In this study, we used two novel peptide inhibitors identified in a peptide library screen that selectively bind KsBcl-2 (KL6-7 Y4eK) or KsBcl-2 and Mcl-1 (MS1) in order to decipher the relative contribution of Mcl-1 and KsBcl-2 in maintaining mitochondrial membrane potential. We found treatment with KL6-7 Y4eK and MS1 stimulated a similar amount of cytochrome c release from mitochondria isolated from Bcbl-1 cells, indicating that inhibition of KsBcl-2 alone is sufficient for mitochondrial outer membrane permeabilization (MOMP) and thus apoptosis during a latent B cell infection. In turn, this study also identified and provides a proof-of-concept for the further development of novel KsBcl-2 inhibitors for the treatment of KSHV-associated B-cell lymphomas via the targeting of latently infected B cells.

Keywords

Kaposi's; sarcoma-associated herpesvirus; B-cell lymphoma (Bcl)-2; Latency; B cells; Peptide inhibitors; Mitochondria

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Conflict of interest

We have no conflicting financial interests.

1. Introduction

Kaposi's sarcoma associated herpesvirus (KSHV) belongs to the Herpesviridae family and is able to establish lifelong latent infections. Within infected hosts, the primary reservoir for latent KSHV infection is the B cell compartment (Ambroziak et al., 1995), although latent infection of endothelial cells, monocytes/macrophages, and dendritic cells has also been reported (Moore and Chang, 2001). Infection by KSHV is generally asymptomatic in immunocompetent individuals; however, in immunosuppressed patients, KSHV is associated with B-cell lymphoproliferative cancers, including primary effusion lymphoma and multicentric Castleman's disease (Kaplan, 2013). Thus, targeting of latently infected B cells is critical to the development of future antiviral drugs for the treatment of KSHV-associated B-cell malignancies.

To maintain latency, KSHV must regulate the homeostatic health of carrier cells with a minimal complement of expressed viral proteins. One strategy utilized by KSHV is to stimulate cell survival through increasing and/or mimicking the activity of the antiapoptotic B-cell lymphoma (Bcl)-2 family of proteins, which function to inhibit the release of cytochrome c from mitochondria and thereby block the initiation of apoptosis (Fig. 1A). KSHV has been shown to upregulate two Bcl-2 family members, Bcl-2 (Gao et al., 2011) and Mcl-1 (Graham et al., 2013), following infection in order to promote survival of latently infected B cells (Gao et al., 2011; Graham et al., 2013). KSHV also encodes a viral Bcl-2 homolog, KsBcl-2, that potently inhibits mitochondria-mediated apoptosis (Sarid et al., 1997). We previously found Mcl-1, Bcl-2, and KsBcl-2 to be expressed in a latently KSHV-infected cell line, Bcbl-1 (Cojohari et al., 2015). Despite studies showing the individual roles of cellular and viral Bcl-2 proteins in the survival of KSHV-infected cells, whether one or multiple Bcl-2 proteins play a dominant role in regulating the release of cytochrome c from mitochondria of latently infected B cells was unclear. Using BH3 profiling, a functional assay that provides information about cellular "addiction" of cancer cells on individual antiapoptotic Bcl-2 proteins (Certo et al., 2006), we identified Mcl-1 and KsBcl-2 to be responsible for the inhibition of cytochrome c release from mitochondria of Bcbl-1 cells (Cojohari et al., 2015). Interestingly, latently KSHV-infected B cell lines derived from different patients, such as BC3 cells, do not express KsBcl-2 (Widmer et al., 2002), thus highlighting the potential utility of BH3 profiling in personalized medicine as a predictive biomarker for responses to inhibitors of cellular and viral Bcl-2 proteins.

Mechanistically, antiapoptotic Bcl-2 proteins block the release of cytochrome c by binding and sequestering activator BH3-only proteins (aBH3), such as Bid and Bim (Fig. 1A) (Thomas et al., 2010). aBH3 activate proapoptotic effectors Bax and Bak, which form channels on the mitochondrial outer membrane allowing for the release of cytochrome c and cell death by apoptosis. Repression of aBH3 proteins by antiapoptotic Bcl-2 proteins can be relieved by competitive inhibition with sensitizer BH3-only proteins (sBH3), such as Bad, Bik, Noxa, Hrk, Puma, and Bmf. Although cellular and viral Bcl-2 proteins share several binding partners, differences within their BH3 binding groove also allow for distinct protein-protein interactions (Flanagan and Letai, 2008; Foight and Keating, 2015; Kvensakul and Hinds, 2013). Sequence identity within the binding groove is >60% between Bcl-2, Bcl-xL, and Bcl-w (Foight and Keating, 2015). In contrast, Bcl-A1 and Mcl-1 only

show 30% and 39% with the Bcl-2/xL/w subgroup. The viral Bcl-2 homologs KsBcl-2 and BHRF1 [the Epstein-Barr virus (EBV) homologue] share low sequence identity with each other and with human homologs. Based on these differences, we screened computational designed BH3 peptide libraries using bacterial surface display to identify BH3 binding peptides that selectively bound to KsBcl-2 (Foight and Keating, 2015). Peptide KL6-7 Y4eK was found to specifically bind to KsBcl-2 (K_d : 2.9 nM), while showing no affinity towards BHRF1 or to the other Bcl-2 proteins, and very weak binding to Mcl-1 (K_d : ~7800 nM) (Table 1). Foight et al. (2014) also developed a peptide, MS1, that binds to both KsBcl-2 and Mcl-1 but not to other Bcl-2 family members (Foight et al., 2014), which is in accord with KsBcl-2 being most closely related to Mcl-1 (Flanagan and Letai, 2008; Foight and Keating, 2015).

The inability of KL6-7 Y4eK and MS1 peptide inhibitors to freely cross the cytoplasmic membrane preclude the use of any cell killing assays to directly examine their cytotoxic effect. Consequently, we tested the ability of KL6-7 Y4eK and MS1 to stimulate the release of cytochrome c from mitochondria isolated from Bcbl-1 cells using a modified BH3 profiling assay. BH3 profiling is a functional assay based on the principle that BH3 proteins bind to antiapoptotic Bcl-2 proteins with different selectivity and affinity (Certo et al., 2006). The strength and the selectivity of protein–protein interactions among Bcl-2 proteins directly correlates with the ability of BH3 proteins to bind and antagonize Bcl-2 proteins from blocking cytochrome c release (Certo et al., 2006; Del Gaizo Moore and Letai, 2013; Flanagan and Letai, 2008; Kvensakul and Hinds, 2013; Ni Chonghaile and Letai, 2008). To perform our modified BH3 profiling assay, mitochondria were isolated from Bcbl-1 cells and then exposed to a select panel of BH3 domain peptides derived from BH3-only proteins, KL6-7 Y4eK, or MS1. Procedural details are similar to those described by Ryan et al. (2010). Briefly, Bcbl-1 and Akata (a latently EBV-infected B-cell line) cells were lysed in mitochondria isolation buffer [250 mM sucrose, 10 mM Tris–HCl (pH 7.4), 0.1 mM EGTA] and passed once through a 27-gauge needle or a Dounce homogenizer. After samples were centrifuged at $600 \times g$ for 10 min, the resulting supernatant was centrifuged at $10,000 \times g$ for 10 min to obtain mitochondria. Mitochondria were then resuspended in experimental buffer [125 mM KCl, 10 mM Tris–MOPS (pH 7.4), 5 mM glutamate, 2.5 mM malate, 1 mM KPO₄, and 10 μ M EGTA–Tris, (pH 7.4)] to a concentration of 0.3–0.5 mg/ml protein and exposed to BH3 domain peptides of Bid, Noxa B, or Bnip3 at 100 μ M or to KL6-7 Y4eK or MS1 at 10 μ M for 40 min at room temperature. Following treatment with BH3 peptides, mitochondria were separated and cytochrome c concentration measured in the pellet and supernatant fractions by ELISA.

As expected, the aBH3 peptide corresponding to Bid, which directly binds and activates proapoptotic proteins Bax and Bak, induced cytochrome c release from mitochondria of cells latently infected with either KSHV (Bcbl-1) or EBV (Akata) (Fig. 1B, C). It should be pointed out that basal% cytochrome c release from mitochondria not treated with peptides was subtracted from% cytochrome c release from mitochondria treated with BH3 peptides, KL6-7 Y4eK, or MS1. The negative peptide control Bnip3, a human BH3-only protein that does not bind to antiapoptotic Bcl-2 proteins nor activate Bax or Bak, did not perturb mitochondrial polarization. Consistent with our previous findings that Mcl-1 contributes to

cell survival (Cojohari et al., 2015), Noxa B peptide treatment at 100 M stimulated weak cytochrome c release from mitochondria of Bcbl-1 cells. KL6-7 Y4eK at 10 M induced cytochrome c release similar to Bid peptide at 100 M in Bcbl-1 cells (Fig. 1B) while inducing minimal release from Akata cells (Fig. 1C), which are dependent on Bcl-xL for survival (Cojohari et al., 2015). Similarly, MS1 also stimulated cytochrome c release while not inducing release from Akata mitochondria. However, the amount of cytochrome c released in response to MS1 was similar to that released in response to KL6-7 Y4eK, indicating that KsBcl-2 is likely the major regulator of mitochondrial membrane potential while Mcl-1 plays a supporting role. In accord, we previously found that high concentrations of only one out of three Mcl-1 selective inhibitors was required to induce apoptosis of Bcbl-1 cells when compared to other Mcl-1-dependent cell types (Cojohari et al., 2015).

2. Conclusion

Our previous studies demonstrated that, unlike other herpesviruses including human cytomegalovirus (HCMV) and EBV, KSHV expresses its viral homologue of Bcl-2, KsBcl-2, to regulate cytochrome c release during a latent infection (Cojohari et al., 2015). However, because BH3 profiling analysis also indicated the involvement of Mcl-1 in maintaining mitochondrial polarization, the relative contribution of each antiapoptotic Bcl-2 protein was unclear. Using KL6-7 Y4eK, a novel peptide that selectively targets KsBcl-2 (Foight and Keating, 2015), our data indicate that KsBcl-2 is the major regulator of mitochondrial membrane potential during latent infection of B cells. Thus, the further development of viral Bcl-2-specific inhibitors to increase efficacy and allow for translocation across the plasma membrane may hold promise for the treatment of herpesvirus-related cancers, while minimizing the side effects traditionally associated with conventional Bcl-2 targeted chemotherapies. Recently, a protein inhibitor of BHRF1 was shown to have efficacy in suppression of tumor growth in a mouse xenograft model of EBV-associated lymphoproliferative disease (Procko et al., 2014). Overall, our study demonstrates the targeting of KsBcl-2 as a potentially viable anti-viral strategy to specifically kill latently KSHV-infected B cells.

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Fig. 1.

KsBcl-2 selective peptide inhibitors induce cytochrome c release from mitochondria of latently KSHV-infected B cells. (A) A model depicting the control of mitochondrial depolarization by the cellular Bcl-2 proteins and herpesvirus Bcl-2 homologues. (B) Bcbl-1 and (C) akata mitochondria were treated with 100 μ M BH3-only peptides or 10 μ M KL6-7 Y4eK (KL6-7) and MS1. Shown is the mean of 3 independent experiments; error bars show standard deviation.

Table 1

Dissociation constant values (K_d) for peptide inhibitors of Mcl-1 and herpesvirus Bcl-2 homologs.

Peptide	Sequence	K_d (nM)		
		Mcl-1	KsBcl-2	BHRF1
Bid	EDIIRNIARHLAQVGDSMDR	<10 ^a	112 ^a	99 ^a
Noxa B	PADLKDECAQLRRIGDKVNL	28 ^a	–	–
Bnip3	VVEGEKEVEALKKSADWVSD	–	–	–
KL6-7 Y4eK	RPQIWIHQGLQRLGDGLNAYKAR	7800 ^b	2.9 ^b	–
MS1	RPEIWMQTQGLRRLGDEINAYYAR	1.9 ^c	2.9 ^c	–

Sources for K_d values:

^a Flanagan and Letai, 2008;

^b Foight and Keating, 2015;

^c Foight et al., 2014.