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Bendamustine Reactivates Latent Epstein-Barr Virus

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Keywords

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The potential of bendamustine, a purine-like alkylating agent, for treating blood cancers associated with the Epstein-Barr virus led us to examine interactions between drug and virus. Bendamustine and other alkylating agents such as chlorambucil and melphalan disrupt latency. Bendamustine and rituximab, an anti-CD20 monoclonal antibody, can combine to increase lytic replication. We discuss how this ability to reactivate latent virus informs clinical strategies.

The human Epstein-Barr virus (EBV) is associated with hematologic malignancies. This herpesvirus evades immune surveillance over the host's lifetime by entering a quiescent phase in B cells. Compared to lytic replication, during which ~100 gene products amplify virus, latency restricts transcription to ~1–10 genes [1]. EBV adopts this expression program to immortalize B cells *in vitro* and inhabit tumors of B cell malignancies like a proportion of Burkitt, Hodgkin, post-transplantation, diffuse large B cell, primary central nervous system, primary effusion, and plasmablastic lymphoma *in vivo* [1,2].

Bendamustine is a chemotherapy agent increasingly used to treat blood cancers. Recently rediscovered, this small molecule contains a mechlorethamine nitrogen mustard that generates DNA damage distinct from other alkylating drugs. Bendamustine combined with other drugs [3,4] effectively treats indolent non-Hodgkin lymphoma. Preliminary trials also show success against relapsed or refractory diffuse large B cell lymphoma [5,6]. Similarly aggressive cancers are associated with EBV infection [1,2], so the potential of bendamustine treatment for EBV-associated malignancies led us to examine interactions between drug and virus.

Bendamustine and other alkylating chemotherapy drugs induce EBV reactivation in a cell culture model of non-Hodgkin lymphoma. MutuI cells [7] were grown at 37°C with 5% CO₂ in RPMI-1640 containing 2 mM HEPES and 10% fetal bovine serum. We treated cells at a density of 0.5×10^6 /mL with bendamustine (Sigma-Aldrich or Millipore). Reactivation was

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measured by staining for the immediate-early lytic transactivator BZLF1 using the paraformaldehyde-methanol method [8] with a BZ1 antibody (Santa Cruz Biotechnology) and goat anti-mouse IgG-FITC (Santa Cruz Biotechnology). Flow cytometry was performed on a FACSCaliburDxP8 (B.D. Biosciences). Bendamustine evokes both a dose- and time-dependent response in that 2- or 3- day treatment with 10–100 μ M bendamustine induces ~10–30% reactivation (Figure 1A). Lytic induction also modestly occurs in EBV-infected Akata and Daudi cells, but not detectably in KemI, KemIII, and GM12878 cells (data not shown). Deep sequencing of the EBV transcriptome (Phan and Miranda, submitted) reveals that bendamustine increases RNA levels genome-wide by ~100-fold (Figure 1B). *BHLF1* shares a promoter with the lytic origin of replication and its expression correlates with reactivation [9]; *BcLF1*, a true late gene, requires viral DNA replication to encode a capsid protein [10]. Bendamustine increases *BHLF1* and *BcLF1* expression as well as the entire lytic program. Curious if other alkylating agents have similar effects, we treated cells with either 10 μ M chlorambucil (Sigma-Aldrich) or 10 μ M melphalan (Sigma-Aldrich) for 3 days. Both drugs also reactivate EBV (Figure 1C). We can therefore add bendamustine and other alkylators to the list of DNA-damaging agents, such as cisplatin, 5-fluorouracil, gemcitabine, and doxorubicin [11,12], capable of disrupting EBV latency.

Because bendamustine plus rituximab, an anti-CD20 monoclonal antibody, effectively treats indolent non-Hodgkin lymphoma [3,4], we investigated this combination's interaction with latent EBV. 100 μ g/mL rituximab and 10 μ M bendamustine independently induce EBV reactivation after 2 days, but combination generates even more (Figure 1D). Rituximab similarly enhances reactivation induced by dexamethasone [13]. CD20 engagement combined with other latency disruptors may be a general means of enhancing induction.

The ability of bendamustine to reactivate latent EBV informs future clinical strategies. Taken pessimistically, our work warns that drug treatment may exacerbate risk of adverse effects caused by viremia in an already weakened immune system. Impairment of lymphocyte recovery by bendamustine [14] particularly warrants heightened precautions. Personalised medicine that tailors chemotherapy regimens to EBV status of the tumor may suggest the preferred treatment. Taken optimistically, this study contends that bendamustine may be useful in lytic induction therapy, an alternative to standard cytotoxic treatment that attempts to leverage the presence of virus to target cancer cells. Latent EBV coerced into reactivation can specifically mark tumor cells for killing by cytopathic effects, epitope recognition by cytotoxic T lymphocytes, or small molecule drugs that target lytic proteins [15]. Our transcriptome profiling reveals that bendamustine induces the lytic EBV expression program, including true late genes, which demonstrates that prerequisite proteins must have been translated. These early proteins comprise the targets for multiple cure strategies. Drugs like bendamustine and other alkylating agents, which are already efficacious antitumor agents, could form the foundation of effective lytic induction therapy combinations. In whatever way this knowledge is applied, future clinical work should recognize that bendamustine reactivates latent EBV.

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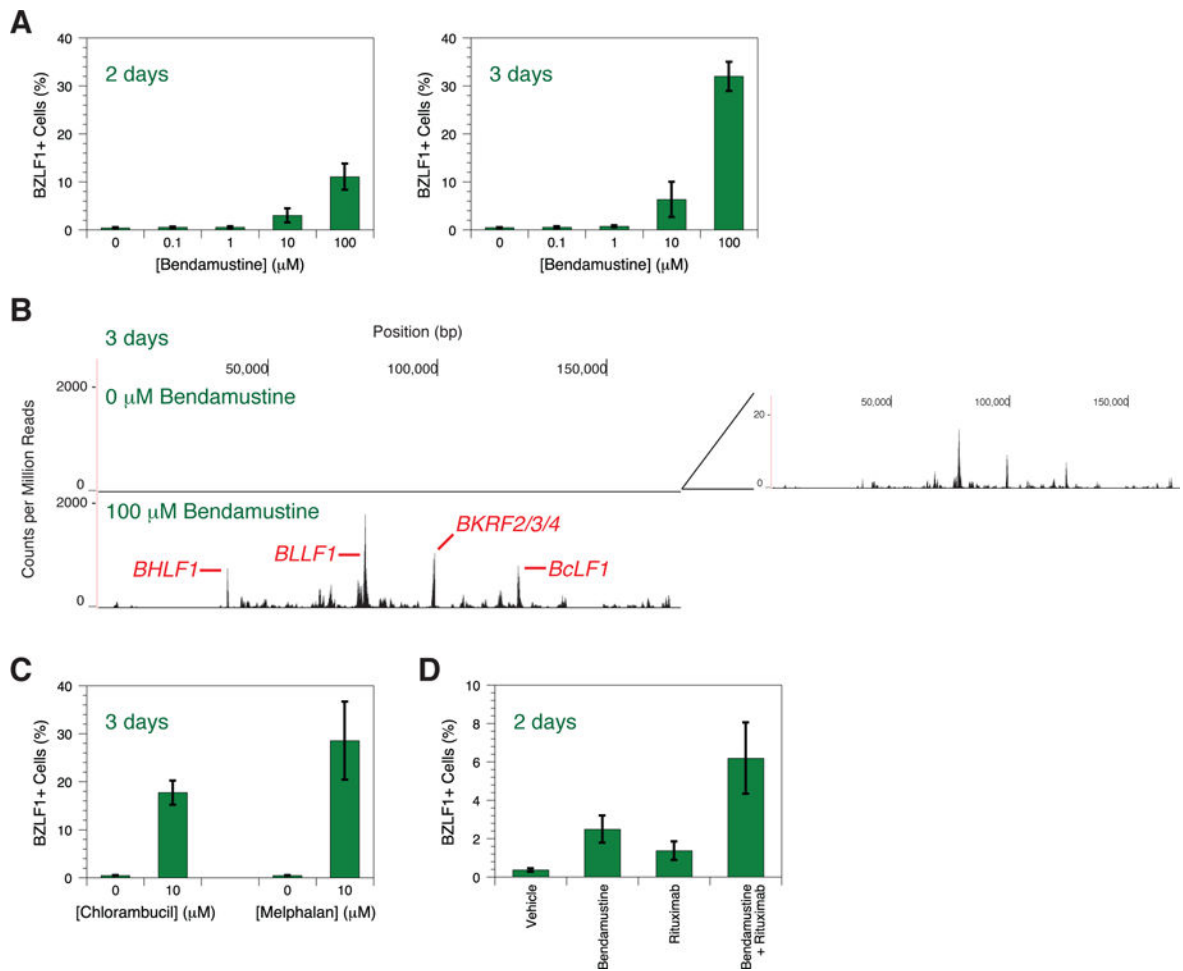
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**FIG 1.**

EBV reactivation induced by bendamustine and other alkylating agents. (A) Time- and dose-dependent response of EBV reactivation upon bendamustine treatment. BZLF1 expression in MutuI cells was measured by flow cytometry. Error bars represent the standard deviation of $n = 4$ replicates. (B) Deep sequencing of the EBV transcriptome in response to bendamustine treatment. The X axis denotes nucleotide position and the Y axis denotes the number of counts per million mapped reads. An inset of the top panel depicts low levels of transcription not visible on the scale used to compare conditions. Specific lytic genes are annotated. One representative experiment from two independent replicates is shown. (C) EBV reactivation upon chlorambucil and melphalan treatment. BZLF1 expression in MutuI cells was measured by flow cytometry. Error bars represent the standard deviation of $n = 4$ replicates. (D) Increased reactivation induced by bendamustine plus rituximab. BZLF1 expression in MutuI cells was measured by flow cytometry. Error bars represent the standard deviation of $n = 4$ replicates.