

Spironolactone blocks Epstein–Barr virus production by inhibiting EBV SM protein function

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Clinically available drugs active against Epstein–Barr virus (EBV) and other human herpesviruses are limited to those targeting viral DNA replication. To identify compounds directed against other steps in the viral life cycle, we searched for drugs active against the EBV SM protein, which is essential for infectious virus production. SM has a highly gene-specific mode of action and preferentially enhances expression of several late lytic cycle EBV genes. Here we demonstrate that spironolactone, a mineralocorticoid receptor antagonist approved for clinical use, inhibits SM function and infectious EBV production. Expression of EBV viral capsid antigen is highly SM dependent, and spironolactone inhibits viral capsid antigen synthesis and capsid formation, blocking EBV virion production at a step subsequent to viral DNA replication. In addition, spironolactone inhibits expression of other SM-dependent genes necessary for infectious virion formation. We further demonstrate that molecules structurally related to spironolactone with similar antimineralocorticoid blocking activity do not inhibit EBV production. These findings pave the way for development of anti-herpesvirus drugs with new mechanisms of action directed against SM and homologous essential proteins in other herpesviruses.

Epstein–Barr virus | spironolactone | posttranscriptional regulation | herpesvirus | antiviral

Epstein–Barr virus (EBV) is a human lymphotropic herpesvirus associated with several types of lymphoid and epithelial malignancies and lymphoproliferative syndromes (for a review, see ref. 1). EBV infects the majority of humans worldwide and establishes a persistent, lifelong latent infection in memory B cells. Occasional reactivation from latency occurs, and EBV enters a lytic phase of replication, during which a tightly regulated series of lytic genes is expressed, culminating in lysis of the host cell and release of infectious viral progeny. All herpesviruses share these abilities to establish asymptomatic latent infection and reactivate intermittently.

All herpesviruses also express a regulatory protein early in lytic replication that is essential for efficient gene expression and infectious virion production. The EBV member of this family, which includes herpes simplex virus ICP27, cytomegalovirus (CMV) UL69, and Kaposi's sarcoma-associated herpesvirus (KSHV) ORF57, is known as SM (2). SM acts posttranscriptionally to enhance accumulation of many lytic EBV transcripts. SM activity is highly gene-specific and preferentially enhances expression of several late lytic transcripts, including those encoding the major viral capsid antigen (VCA) and gp350, which is required for infection of B lymphocytes (3–5). SM and its homologs in other herpesviruses may also enhance transcription and translation of specific viral genes (6).

Currently, all available antiherpesvirus drugs target viral DNA polymerases and are usually highly effective, but toxicity and development of resistance limits their use (7). To discover potential novel therapeutic compounds, we applied a cell-based screening assay to identify compounds that would specifically target SM function (8). Using this assay, spironolactone (SPR), a drug in clinical use for over 50 y, was identified as inhibiting SM function and EBV virion production. SPR is an aldosterone antagonist that binds to the mineralocorticoid receptor, but comparison

with derivatives of SPR demonstrated that mineralocorticoid receptor blocking activity was not the basis of its antiviral activity. Rather, the profile of SPR's effects on EBV gene expression paralleled that of genetically inactivating SM. SM and its counterparts in other herpesviruses have no cellular homologs and act at a stage of herpesvirus replication distinct from DNA replication (9). Thus, compounds based on SPR have the potential to yield antiviral drugs with a new mechanism of action and limited toxicity.

Results

SPR Inhibits EBV SM Function. We previously described a screening assay to identify compounds that can inhibit the function of EBV SM protein, which is essential for lytic EBV replication (3, 8). The assay is based on the finding that mRNAs dependent on EBV SM protein for efficient expression confer SM dependence on heterologous genes to which they are fused. The assay uses a reporter cell line stably expressing the SM-responsive KSHV ORF59 gene fused in frame to GFP and in which the fusion product is not efficiently expressed. These also contain a doxycycline-inducible SM gene, and when SM protein is produced, the ORF59-GFP fusion mRNA is expressed and the cells exhibit strong green fluorescence (8). We screened a library of compounds of known activity and structure (10) for their ability to inhibit SM function in this assay and identified SPR as a potential inhibitor of SM function. Treatment of the reporter cells with 10 μ M SPR led to inhibition of fluorescence accompanied by almost complete absence of ORF59-GFP RNA and protein accumulation (Fig. 1A–C). We next asked whether SPR could be affecting SM localization by performing immunofluorescence microscopy for SM in the presence or absence of SPR. As seen in Fig. 1D, SPR did not affect nuclear localization of SM. The specificity of SPR activity was tested by determining the effect of SPR on an analogous construct expressing a CMV UL44-GFP fusion construct that is not SM-dependent or -responsive. SPR

Significance

Epstein–Barr virus is a human herpesvirus associated with several types of malignancy. We show that spironolactone, a drug used to block mineralocorticoid activity, also has anti-EBV activity and that it acts by inhibiting the function of an essential EBV protein SM, which preferentially increases expression of specific EBV genes. We also show that the mineralocorticoid blocking activity is not the basis for spironolactone's antiviral activity. Because the SM protein acts at steps of the viral life cycle distinct from those targeted by currently available therapies, this study paves the way for development of novel anti-EBV drugs to address emerging problems of drug resistance and toxicity.

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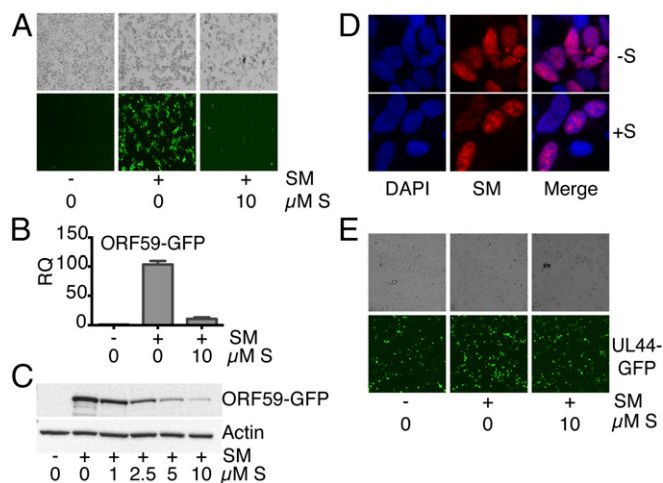


Fig. 1. Identification of SPR as a drug that inhibits EBV SM function. (A) HEK293 cells were stably transfected with a KSHV ORF59-GFP reporter plasmid and transduced with a lentivirus expressing inducible EBV SM protein. Fluorescence microscopy was performed on reporter cells in the presence or absence of SPR. Cells expressed GFP protein only when SM was expressed (+SM) but were GFP-negative when treated with 10 μ M SPR (S). (B) SPR inhibits SM-dependent ORF59-GFP RNA accumulation. The expression of ORF59-GFP fusion RNA was measured by qRT-PCR 24 h after 10 μ M SPR treatment. (C) SPR inhibits ORF59-GFP protein accumulation. Immunoblotting with anti-GFP or actin antibody was performed on lysates from cells treated as shown. (D) The reporter cell line was induced to express SM and treated with either vehicle (-S) or treated with SPR (+S). Cells were fixed 30 h after induction and stained with either DAPI (blue) or anti-SM antibodies (red). (E) 293 cells were transfected with CMV UL44-GFP and empty vector (-) or SM (+) and treated with either vehicle or 10 μ M SPR as shown.

did not affect UL44-GFP expression, indicating that its effect is SM-specific (Fig. 1E).

Antiviral Activity of SPR Is Not Due to Mineralocorticoid Receptor Blocking Activity. SPR acts to block aldosterone activity by binding to the mineralocorticoid receptor and acting as a receptor antagonist (11). To ask whether SM inhibition by SPR was related to its mineralocorticoid blocking activity, we compared its effect on SM with that of eplerenone (EPR), a chemically similar congener also used clinically as an aldosterone antagonist (12). As shown in Fig. 2, SPR displayed a dose-dependent SM inhibitory effect in the SM functional assay, whereas EPR had no effect. These results therefore indicate that mineralocorticoid receptor blockade is not sufficient for SM inhibition and that it is not likely to be related to the effect of SPR on SM activity.

SPR Exhibits Potent Structure-Dependent Antiviral Activity Against EBV. To determine whether SPR inhibition of SM activity translated into antiviral activity, we measured its effect on infectious EBV production. An epithelial gastric carcinoma cell line (AGS) infected with a GFP-expressing EBV (13) was used to test the effect of SPR and SPR-related compounds on EBV production. AGS cells were induced to permit lytic EBV replication and treated with either vehicle (DMSO) or SPR. Release of infectious EBV was then measured by incubating the supernatant from the induced AGS cells with uninfected 293 cells. EBV-infected cells express GFP, allowing them to be accurately counted by flow cytometry as previously described (5, 14). SPR led to a dose-related reduction in infectious EBV titer in the supernatant (Fig. 3A). The IC_{50} of SPR (2.1 μ M) was similar to that of acyclovir (3.4 μ M) in this assay (Fig. S1). The antiviral effect of EPR and two metabolites of SPR that also have mineralocorticoid antagonist activity (11), canrenone (CAN) and 7 α -thiomethyl SPR (TMS), was compared with that of SPR. Interestingly, CAN displayed antiviral activity, albeit less than that of SPR, whereas TMS and

EPR did not (Fig. 3B). Comparison of their structures reveals that these molecules differ only at the modifications to the number 7 carbon of the parent molecule (Fig. 3C). The residues at this position therefore appear to be critical for anti-SM- and EBV-directed antiviral activity but not for mineralocorticoid activity. All four compounds were also tested in the SM reporter assay, and SPR was highly active, with CAN displaying slight activity and EPR and TMS having no detectable activity (Fig. S2). To confirm the aldosterone blocking activity of the four compounds, a cell-based reporter assay for aldosterone function was performed and verified that all four compounds, although varying in potency, had maximal activity at concentrations of 10 μ M and above (Fig. S3).

SPR Inhibits Virus Production at a Stage Subsequent to EBV DNA Replication but Before Virus Release. To investigate the mechanism of SPR's antiviral action, we measured the effect of SPR on EBV DNA replication and early (E) and immediate-early (IE) gene expression, which occur before DNA replication (1). EBV-infected AGS cells were induced to replicate and were treated with SPR or vehicle. DNA replication was measured by qPCR of DNA harvested from induced cells, and EBV protein expression was assessed by Western blotting of cell lysates (Fig. 4A and C). Despite SPR having the expected inhibitory effect on infectious EBV production, SPR did not affect the increase in intracellular EBV copy number that occurs with lytic replication (Fig. 4A and B). SPR also had no effect on IE or E gene expression, including SM expression (Fig. 4C). Many E genes are required for EBV lytic DNA replication, whereas EBV late gene expression is highly dependent on EBV DNA replication (1). Preservation of IE and E gene expression and DNA replication in the presence of SPR therefore indicated that SPR was inhibiting the expression of one or more late genes required for infectious EBV production. To determine whether defective, noninfectious EBV particles were being released after SPR treatment, we measured the effect of SPR on release of extracellular EBV DNA by qPCR. As shown in Fig. 4D, SPR, but not EPR, prevented release of EBV DNA into the supernatant. To determine whether SPR had similar effects in EBV-infected B lymphocytes, we measured E and IE lytic protein expression in EBV-positive P3HR1-ZHT lymphoma cells treated with SPR (Fig. S4). SPR did not affect E or IE gene expression in lymphoma cells. However, as in epithelial cells, SPR inhibited release of extracellular EBV DNA into the supernatant (Fig. S4A). These data therefore indicate that because EBV DNA is replicated normally in the presence of SPR, subsequent stages of viral packaging, assembly, or egress may be inhibited by SPR.

SPR Prevents EBV Capsid Formation. Because SPR prevented infectious virus production despite having no effect on DNA replication, we examined the effect of SPR on virion assembly and

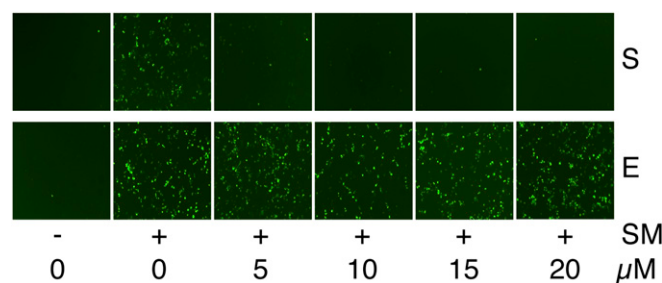


Fig. 2. Inhibitory effect of SPR on SM activation of gene expression is independent of mineralocorticoid receptor antagonist activity. The ORF59-GFP reporter cell line was either mock-treated (-) or treated with dox (+) to induce SM expression, which is required for GFP expression. The induced cells were also treated with either DMSO vehicle (0) or various concentrations of SPR (S) or the mineralocorticoid receptor antagonist EPR (E), and cells were examined for GFP expression by fluorescence microscopy 24 h after treatment. Drug concentrations are shown below the panel.

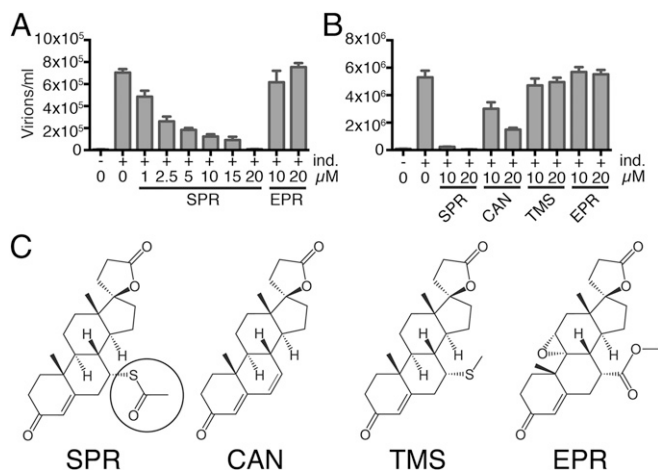


Fig. 3. Antiviral activity of SPR in epithelial cells. (A) EBV lytic replication was induced with TPA (+ ind.) in an GFP-EBV-infected gastric carcinoma cell line (AGS), which was then treated with SPR or EPR at various concentrations as shown. Virion production was measured by passage to 293 cells and quantitated by flow cytometry. EBV titer expressed as the number of GFP-transducing units per mL is shown on the y axis. (B) Antiviral activities of CAN, TMS, and EPR (E) were compared with SPR as in A. Cells were either untreated (-) or treated (+) to induce lytic replication (ind.). (C) Structures of SPR, CAN, TMS, and EPR are shown. The thioacetyl group at C-7 of SPR is circled.

egress. Electron microscopy was performed on EBV-infected AGS cells after induction of EBV replication and SPR treatment to examine the potential effect of SPR on virion formation. As shown in Fig. 5, SPR completely abolished EBV capsid formation. Whereas intranuclear EBV capsids at various stages of formation were readily detectable in EBV-infected AGS cells (32% of induced cells), no capsids were found in SPR-treated cells (0%). Viral DNA replication was again unaffected by drug treatment (Fig. S5). These data therefore suggested that SPR inhibits expression of one or more proteins required for EBV capsid formation but did not rule out additional effects on other late genes.

SPR Inhibits Expression of an Essential SM-Dependent Late Lytic Capsid Gene. We have recently shown that SM preferentially enhances expression of several late lytic genes but is not required for efficient expression of IE or E genes (9). To investigate the effect of SPR on EBV late gene expression, we compared the effect of SPR on EBV protein expression from several EBV genes of different temporal classes, by immunofluorescence microscopy. As expected, SPR had no effect on expression of IE gene Z or E genes EA or SM (Fig. 6). However, SPR did strongly inhibit expression of VCA, which is highly dependent on SM for efficient expression (4, 9). Although the percentage of BMRF1 (EA-D)-, ZTA-, and SM-expressing cells was unaffected or actually increased slightly with SPR treatment, the percentage of VCA-positive cells decreased by 73% ($P < 0.0001$; Fig. 6B). To confirm these results and examine SPR effects on additional SM-dependent genes, qPCR for several EBV lytic genes was performed using RNA from SPR-treated AGS cells. As shown in Fig. S6A, the effect of SPR on late lytic genes was gene-specific, inhibiting expression of SM-dependent mRNAs, such as BDLF1, BLLF1 (gp350), and BcLF1 (VCA), but having little effect on BFRF3 or BALF4, which are not SM-dependent (9). The effect of SPR on these genes in EBV-infected lymphoma cells was similar to that observed in AGS epithelial cells (Fig. S6B). We also compared the effect of SPR on SM-responsive and SM-unresponsive transcripts in cotransfection assays (Fig. S7) and demonstrated that SPR inhibits only SM-responsive gene expression.

Concordance Between the Effects of SPR Treatment and SM Deletion on the EBV Transcriptome. We have comprehensively characterized the specificity of SM effects on the EBV transcriptome by comparing the transcriptome of SMKO EBV-infected cells with that of cells infected with wt EBV and SM-rescued SMKO EBV (9). The effect of SM is highly gene-dependent and primarily enhances expression of several essential late genes, including VCA and gp350 (4, 5, 9). It was therefore possible to conduct a similar global comparison between the effects of SPR on WT EBV-infected cells and the effect of genetically knocking out SM function. We treated AGS (epithelial cells) and P3HR1-ZHT (lymphoma cells) with SPR or vehicle and harvested RNA from the infected cells 48 h after induction of lytic EBV replication. The gene expression patterns in the presence and absence of SPR were then determined by high-throughput sequencing and transcriptomic analysis in which read counts for each EBV gene were determined. The results were then computed as binary ratios between gene expression in the absence or presence of SPR and gene expression in the presence or absence of SM. As shown in Fig. 7, many of the genes highly inhibited by SPR in both cell types were also highly SM-dependent. To examine the overall correlation between the effects of SM deletion and SPR, an unsupervised clustering analysis of the differential expression data was performed. Although the effect of SPR was not identical in each cell line, there was a high degree of concordance between the effect of SPR and the effect of inactivating SM (Fig. S8), with a Pearson's correlation coefficient of 0.68 and 0.63 between the effect of SM knockout and SPR treatment in P3HR1

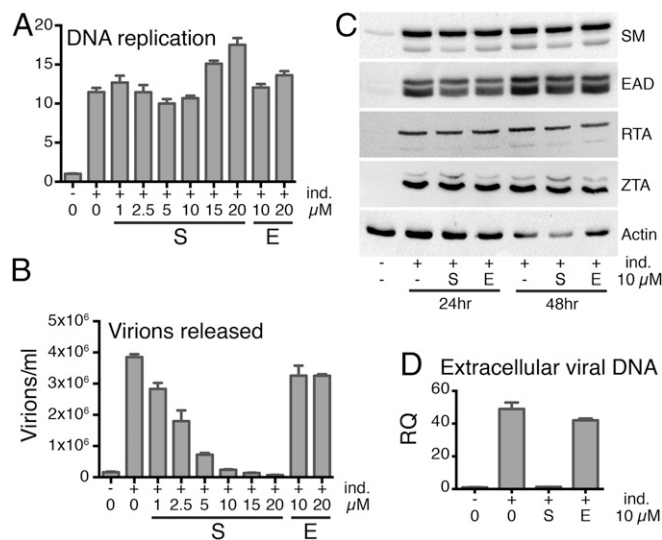


Fig. 4. SPR inhibits virus production at a lytic replicative stage after DNA replication. (A) Antiviral effect of SPR is not due to inhibition of viral DNA replication. EBV-positive AGS gastric carcinoma cells were transduced with a lentivirus expressing EBV transactivator ZTA protein under control of a doxycycline-inducible promoter to generate a cell line in which EBV replicates robustly when treated with doxycycline (AGSiZ). The AGSiZ cell line was either mock treated (-) or treated (+) with doxycycline to induce lytic DNA replication (ind.) and also treated with SPR or EPR at concentrations shown. DNA copy number was measured by qPCR of cell pellets 70 h postinduction. RQ, relative quantity of intracellular viral DNA. (B) SPR but not EPR specifically inhibits infectious virus production. Serial dilutions of virus-containing supernatant were used to infect 293 cells, and the number of GFP-positive cells representing infectious viral particles was measured by flow cytometry. (C) SPR does not affect expression of IE or E EBV proteins. Protein cell lysates from cells treated with SPR (S) or EPR (E) were immunoblotted using anti-SM, EA-D, RTA, ZTA, or actin antibodies at 24 and 48 h after induction of replication. (D) SPR but not EPR inhibits production of extracellular EBV. Extracellular EBV DNA was measured by DNA qPCR in cell supernatants harvested 5 d after SPR (S) or EPR (E) treatment. RQ, relative quantity of DNA.

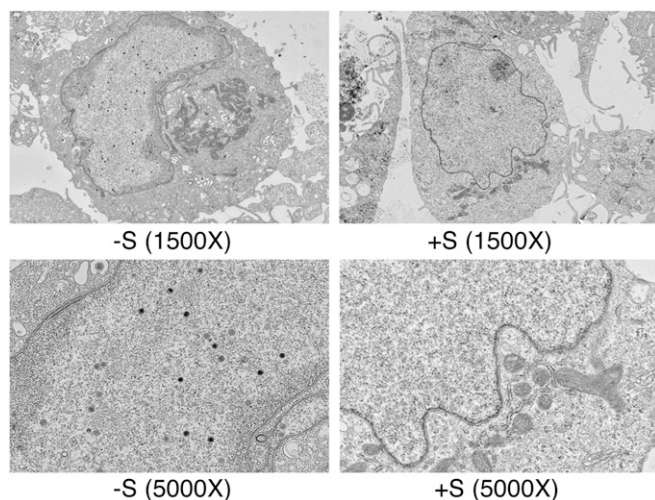


Fig. 5. SPR inhibits EBV capsid formation. AGSiZ cells were induced to permit EBV replication with doxycycline and treated with 10 μ M SPR (+S) or vehicle alone (-S). The cells were harvested, fixed at 4 d postinduction, and analyzed by electron microscopy. Viral particles were seen in vehicle-treated cells (-S) as shown in magnifications of 1,500 $\times$ and 5,000 $\times$. No viral particles were detectable in the SPR-treated samples (+S).

and AGS cells, respectively. These data therefore indicate that a major aspect of the antiviral effect of SPR is mediated by its inhibitory effect on SM and its target-specific mode of action.

Discussion

In this study, we describe the ability of SPR to inhibit EBV SM protein function, decreasing accumulation of SM target RNAs and thereby preventing production of infectious EBV particles. This property of SPR is not based on its mineralocorticoid receptor blocking activity, as congeners with antialdosterone activity did not possess anti-SM or antiviral activity. The activity of SPR was structure-dependent, with specific substitutions at the 7

position of SPR altering anti-SM and anti-EBV activity. Consistent with its anti-SM activity, the effect of SPR on EBV gene expression was highly concordant with the effect of mutationally deleting SM from the EBV genome. The most highly SM-dependent and SPR-sensitive genes are late lytic genes, and most encode either tegument proteins or glycoproteins (4, 5, 9). In addition, two genes that are essential for encapsidation before tegument incorporation and virion envelopment, the major VCA and the minor capsid gene (BDLF1) product, are also highly SM-dependent (9). Inhibition of VCA expression thus represents the most proximate block in EBV production due to SPR, and SPR completely prevented capsid formation despite adequate EBV DNA replication.

The mechanism(s) of action of SM protein remains incompletely characterized. SM binds EBV RNA and affects RNA stability (15–19). Although it preferentially enhances accumulation of some EBV mRNAs, SM action likely depends on inherent characteristics of inefficiently expressed RNAs, such as stability or nuclear exportability (8, 9). Further, the possibility that SM exerts transcriptional effects on one or more of its target genes has not been excluded. ORF57, the KSHV homolog of SM, may also act transcriptionally as well as posttranscriptionally (6, 20). SPR may directly interact with SM or cellular partners of SM to block function. Even if SPR does not directly affect the ability of SM to interact with target RNAs, it may still act to interfere with formation of RNA-binding protein complexes recruited by SM. SPR could also act indirectly by inhibiting expression of cellular proteins required for SM function at the transcriptional level. SPR has been shown to exert inhibitory effects on several transcription factors in mononuclear cells independently of mineralocorticoid receptor antagonism (21). Establishing the exact mechanism(s) by which SPR inhibits SM function will therefore require further investigation of SM's mechanism of action.

Substitutions at C-7 of SPR appear to be critical for antiviral activity. The fact that CAN and SPR retain activity but TMS does not suggests that the molecules may be acting as an electrophilic trap. The thioacetate at C-7 in SPR may serve as an acyl donor, or it may be eliminated to the γ,δ -unsaturated form, producing CAN *in vivo*. Thus, the target nucleophile would react to produce an acylated variant or undergo a Michael-addition to

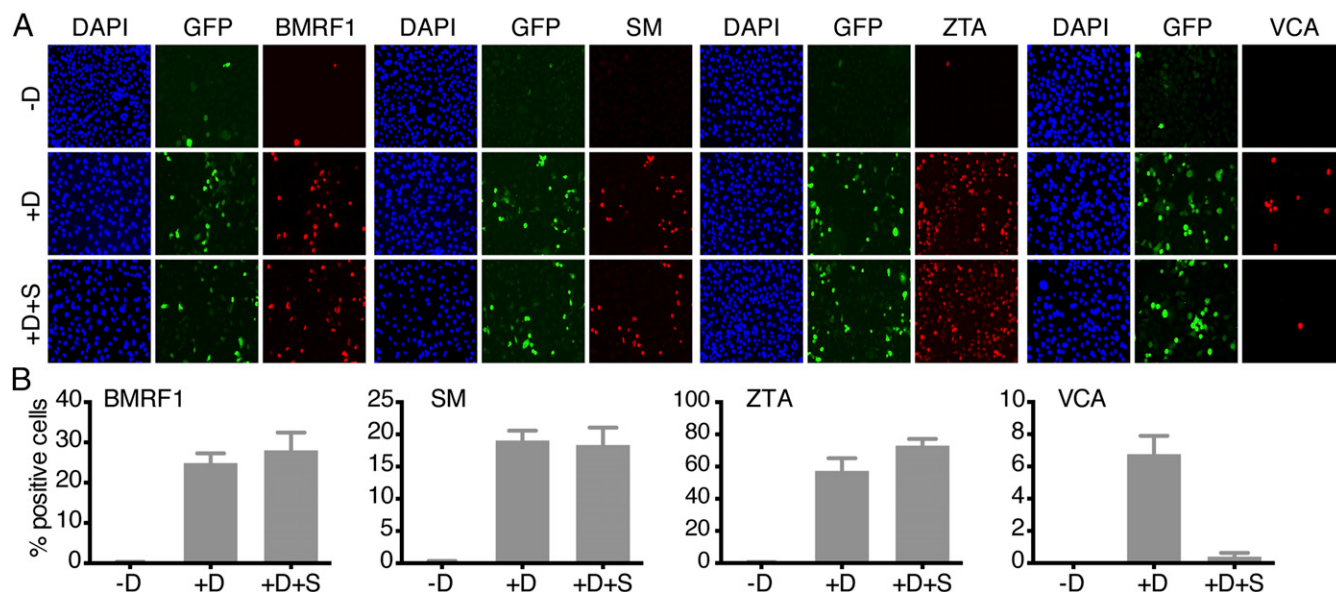


Fig. 6. SPR inhibits EBV capsid protein expression. AGSiZ cells were either mock-treated (-D), treated with doxycycline (+D) to induce replication, or induced and also treated with SPR (+D+S) for 48 h. The cells were fixed and stained with BMRF1, SM, ZTA, and VCA antibodies and analyzed by immunofluorescence microscopy. DAPI-stained nuclei (blue), GFP-positive EBV-infected cells (green), and specific antigen expression (red) are shown. (A) Representative images for expression of each protein are shown. (B) Percentage of cells positive for each protein is shown.

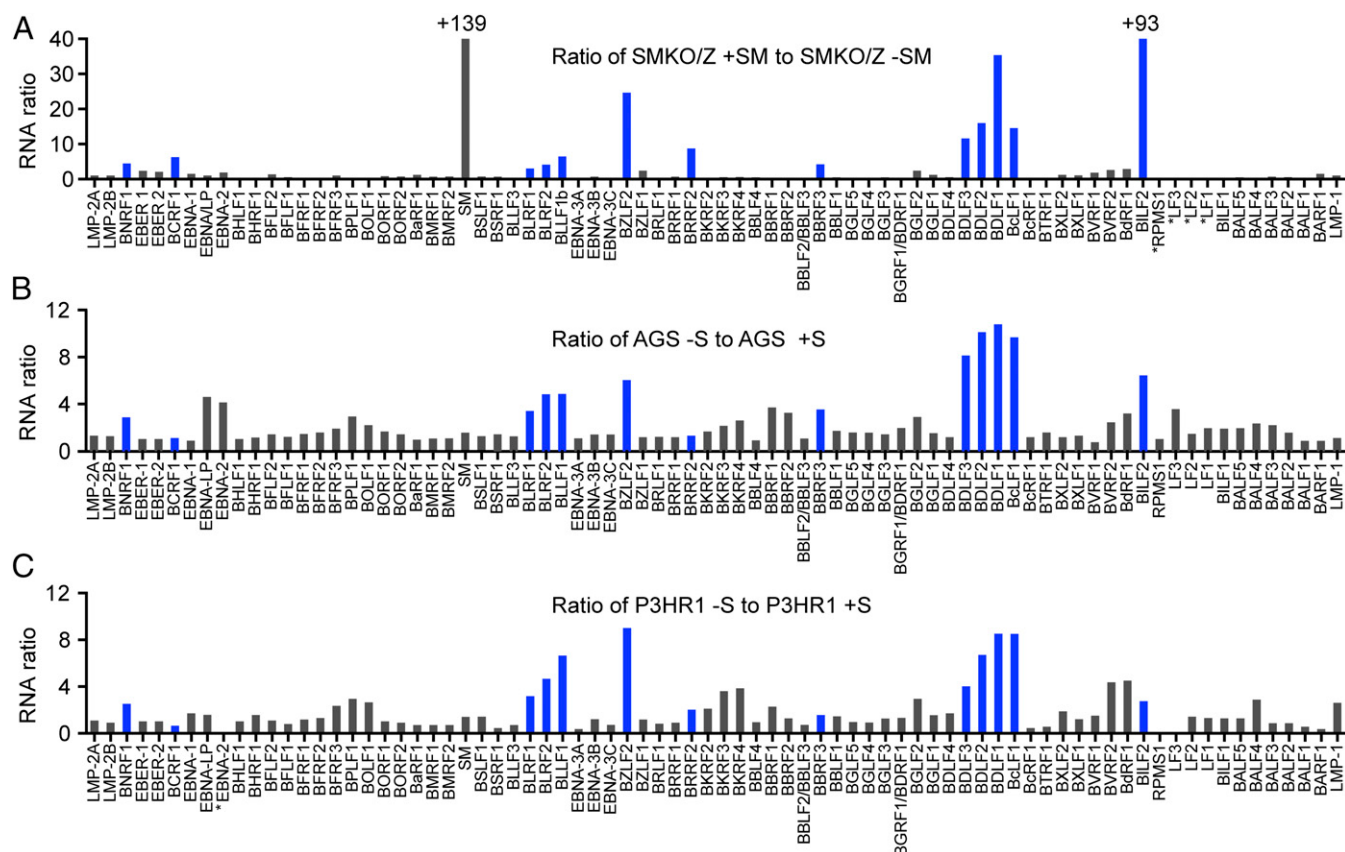


Fig. 7. Comparison of the effect of SPR treatment to SM knockout on EBV gene expression in epithelial and lymphoid cells. (A) SMKO EBV-infected 293 cells were transfected with ZTA to induce replication or transfected with both ZTA and SM to rescue SM-dependent gene expression. RNA was prepared from cells 48 h postreplication induction. High-throughput RNA sequencing was performed to determine the effects of SPR or SM knockout on the lytic EBV transcriptome. Differential expression of SM-dependent genes is shown as the ratio of RNA expression in SMKO EBV transfected with Z+SM versus SMKO Z alone. Genes whose expression is most highly dependent on SM are marked in blue. (B) Epithelial AGSIZ cells were treated with doxycycline to induce replication. The differential expression of SPR-sensitive genes during EBV replication is shown as the ratio of RNA in vehicle-treated (–S) versus that in SPR-treated cells (+S). The most SPR-sensitive genes therefore have the highest ratios of RNAs. (C) Comparison of SPR-treated versus untreated EBV-infected P3HR1-ZHT lymphoma cells was performed as in B except that cells were treated with tamoxifen to induce lytic replication. +, values greater than limit of y axis; *, genes in the region that were deleted in B95.8 and P3HR1 genomes.

produce a CAN-alkylated adduct. The thiomethylether in TMS cannot transfer an acyl group, and elimination is less likely based on the pK_a of the methanethiolate. Either rationale would explain the lack of activity with TMS.

Although SPR has been used clinically for decades as a diuretic and to treat heart failure, hyperaldosteronism, and edematous states, several side effects limit its use (22, 23). These include effects on reproductive and other metabolic functions. It should be noted that the IC_{50} of SPR ($\sim 2.1 \mu M$) for blocking infectious EBV production is lower than that of acyclovir ($3.4 \mu M$) in the same assay (Fig. S1). Although the steady-state levels of SPR achieved in vivo are likely to be lower than the concentrations used in vitro in our study (24), the metabolite CAN has a much longer half-life in vivo (24) and may potentiate the activity of the parent compound. Nevertheless, because the mineralocorticoid antagonist activity of SPR does not seem to be related to its anti-EBV activity, SPR provides an ideal lead compound for developing antiherpesvirus drugs without SPR's known side effects. Resistance to currently available antiherpesvirus drugs such as acyclovir and ganciclovir is an emerging problem in the treatment of immunocompromised patients (25). Cross-resistance to other agents active against viral DNA polymerase, such as foscarnet and cidofovir, not infrequently complicates therapy (25). In addition, these agents have significant myelosuppressive or nephrotoxic toxicities. Drugs directed against other viral proteins such as SM therefore have the potential to significantly extend the

antiviral armamentarium. Use of combination therapy with agents directed against different steps in the viral life cycle also has the potential to limit the emergence of drug resistance. Each human herpesvirus expresses an SM homolog that is essential for virus production (3, 26–28). Although they generally do not cross-complement each other in rescuing viral replication (3, 26, 29), they are structurally and functionally similar. SPR and its derivatives therefore have the potential to be active against other human herpesviruses. Further, the screening assay that we have used to specifically identify SM inhibitors can be easily adapted to screen for agents active against SM homologs from other herpesviruses (8).

In summary, we have shown that SPR specifically acts to inhibit EBV SM protein function, which is required for expression of several late lytic EBV genes and infectious EBV production. The effect of SPR parallels the effect of knocking out SM function, which is critical for expression of EBV VCA and therefore results in an inability of the virus to make viral capsids. In addition to preventing capsid formation, SPR inhibits expression of other SM-dependent EBV genes important for infectivity, including gp350, thereby inhibiting EBV at several steps in virion production. SPR may be used as the template for development of antiherpesvirus drugs that act at stages of herpesvirus replication distinct from that of all other currently available drugs.

Materials and Methods

Cell Lines. HEK293 cells stably carrying an ORF59-GFP fusion gene that also inducibly expresses SM-dependent GFP were maintained as previously described (8). P3HR1-ZHT is an EBV-positive lymphoma cell line that contains the EBV BZLF1 gene fused to the hormone-binding domain of the 4-hydroxytamoxifen (4HT) receptor that allows robust induction of EBV replication with 4HT treatment (30). The gastric carcinoma cell line AGS was infected with GFP-expressing EBV Akata BX1 (31). AGS Akata BX1 cells stably express EBV transactivator protein BZLF1 under control of a doxycycline-inducible promoter (AGSIZ). See [Supporting Information](#) for details of AGSIZ cell line construction using lentivirus transduction (32).

Induction Reagents and Antiviral Compound Screening. EBV lytic replication in AGS Akata BX1, AGSIZ, and P3HR1-ZHT cells was performed by addition of 12-O-tetradecanoylphorbol-13-acetate (TPA), doxycycline, or 4HT, respectively. HEK293 reporter cells carrying ORF59-GFP and inducible SM were induced to express SM with 1 μ g/mL doxycycline. SPR (Sigma, S3378), CAN (TOCRIS Bioscience, 3281), TMS (TRC Toronto, T353500), and EPR (Sigma, E6657) were used at various concentrations from 0 to 20 μ M as indicated. Screening was performed using ORF59-GFP-SM cells, an assay developed and validated for high throughput screening of SM inhibitors as previously described (8).

Quantification of Infectious Virion Production and Viral DNA Replication. EBV lytic replication in AGS and AGSIZ cells was induced by TPA or doxycycline treatment, respectively, and also treated with either DMSO control or with SPR, CAN, TMS, or EPR for 5 d. Filtered cell supernatants were used to infect 293T cells, and the numbers of GFP-positive 293T cells representing infectious viral particles were quantitated by flow cytometry as described previously (14, 33). For P3HR1-ZHT cells, cell supernatants were harvested at

5 d after induction of replication, and EBV DNA released in the supernatant was measured by qPCR. To measure DNA replication, cell pellets were harvested at 70 h after treatment as described above, and EBV DNA copy number was measured by qPCR as described previously (5, 14).

Electron Microscopy. AGSIZ cells were treated with doxycycline to induce lytic replication and also treated with SPR. Cells were harvested at 4 d post-treatment and examined by electron microscopy. At least 150 cells were examined from three different preparations.

RNA Sequencing and Data Analysis. cDNA libraries were prepared from poly(A) RNA and were sequenced on a HiSeq2000 instrument with 50 cycle single end reads. Sequenced reads obtained from EBV-infected AGS and P3HR1 cells were aligned to the EBV Akata (GenBank accession no. KC207813.1) and P3HR1 genomes (GenBank accession no. DQ279927.1). Differential gene expression was measured using USeq's Defined Region Differential Seq application as described previously (14). Heat maps showing clustering of SM-dependent and SPR-sensitive genes and $\log_2$ transformed fold changes were clustered using the R package pheatmap (cran.r-project.org/web/packages/pheatmap/index.html). The distance method was "euclidean" and the clustering method was "complete."

Additional methods are included in [Supporting Information](#).

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