

## **Development of a fluorescence-based assay to screen antiviral drugs against Kaposi's sarcoma-associated herpesvirus**

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### **Abstract:**

Tumors associated with Kaposi's sarcoma–associated herpesvirus infection include Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease. Virtually all of the tumor cells in these cancers are latently infected and dependent on the virus for survival. Latent viral proteins maintain the viral genome and are required for tumorigenesis. Current prevention and treatment strategies are limited because they fail to specifically target the latent form of the virus, which can persist for the lifetime of the host. Thus, targeting latent viral proteins may prove to be an important therapeutic modality for existing tumors as well as in tumor prevention by reducing latent virus load. Here, we describe a novel fluorescence-based screening assay to monitor the maintenance of the Kaposi's sarcoma–associated herpesvirus genome in B lymphocyte cell lines and to identify compounds that induce its loss, resulting in tumor cell death.

### **Article:**

#### ***Introduction***

Kaposi's sarcoma–associated herpesvirus (KSHV), or human herpesvirus-8, is a member of the gammaherpesvirus family, distinguished by the ability of its members to transform host cells. KSHV has been linked to multiple types of cancer, including all forms of Kaposi's sarcoma (1), primary effusion lymphoma (2), and the plasmablastic variant of multicentric Castleman's disease (3). These cancers are more prevalent in immunodeficient populations, such as transplant patients and HIV-infected individuals (3–6). In fact, Kaposi's sarcoma is the most frequent AIDS-associated cancer in the U.S. and worldwide (7).

Following primary infection, KSHV establishes latent infection in the host cell, with only a small population of cells undergoing spontaneous lytic reactivation (8, 9). During latency, a limited number of viral proteins are expressed, including the latency-associated nuclear antigen (LANA), vFLIP, vCyclin, kaposin, and K15 (10, 11). Each viral latent protein plays an important role in viral pathogenesis and KSHV-associated tumorigenesis. LANA plays a pivotal role in the maintenance and segregation of the viral genome during latency (12), and thus, is also essential for cell survival (13). Maintenance of the viral genome is absolutely dependent on the LANA protein, which tethers the latent viral episome to the host cell chromosome, ensuring that the viral genome is replicated with the host genome and is not diluted out of the expanding population of latently infected cells (14, 15). If the viral episome is lost, LANA and the other latent viral oncogenes are no longer expressed, and the tumor cell dies.

Currently, there is neither a cure nor a therapeutic vaccine for KSHV infection. Highly active antiretroviral therapy has reduced the incidence of KSHV-associated tumors in the HIV-positive population, yet Kaposi's sarcoma remains the most prevalent AIDS-associated neoplasm, even in individuals on long-term therapy (16). Ganciclovir, which specifically inhibits lytic viral replication, has reduced the incidence of KSHV-related tumors in transplant recipients (reviewed in ref. 17). However, treatment for preexisting KSHV-associated

malignancies relies on IFN- $\alpha$  administration and systemic chemotherapeutic regimens, developed for non-virus-associated cancers that target DNA replication of all dividing cells (reviewed in ref. 18). Although clinical trials are assessing new treatment options, a cure remains elusive largely due to the lack of compounds that specifically target latent proteins, which allow the virus to persist throughout the host's lifetime. Recently, Curreli et al. reported that high concentrations of glycyrrhetic acid, originally isolated from licorice (*Glycyrrhiza glabra*), could down-regulate the expression of LANA *in vitro* (19).

Nature continues to be a valuable source for new antimicrobial and anticancer pharmaceuticals (20, 21). From 1984 to 1995, >65% of new drugs in these medical fields were derived directly from natural sources or were synthesized, but modeled after a natural product lead compound (22, 23). Two anticancer chemotherapeutics derived from natural products, taxol (paclitaxel; ref. 24) and Food and Drug Administration-approved analogues of camptothecin (irinotecan and topotecan; ref. 25), together represent at least one third of the worldwide market for antineoplastic agents (26). Indeed, natural products represent a rich reservoir of chemically diverse compounds with biological relevance in many disease states (27).

This report describes the development of a fluorescence-based assay to screen for samples that inhibit latent KSHV persistence. The assay identifies samples that interfere with viral genome maintenance during latency irrespective of the specific biochemical mechanism, thus multiple targets are screened for simultaneously. In order to accomplish this, a KSHV-green fluorescent protein (GFP) recombinant virus (28) was introduced into a KSHV-negative B lymphocyte cell line (BJAB) to create a KSHV-BJAB cell line. KSHV-BJAB cells were chosen for two reasons. First, fluorescence, and hence, viral genome maintenance, could be monitored over time in live KSHV-BJAB cell cultures. Samples that interfere with viral genome maintenance could be identified by measuring an accelerated reduction in fluorescence with respect to a vehicle control because the recombinant viral genome is expelled from the dividing cell population. Second, because the BJAB cell line does not require KSHV infection for its survival but can support long-term latent viral persistence (29), using KSHV-BJAB cells uniquely allowed the distinction between samples that caused loss of the latent viral episome and those that were generally toxic to the host cell. This was essential, because naturally infected primary effusion lymphoma cell lines, such as BCBL-1 (30), require viral infection to survive, and therefore do not allow a distinction between specific antiviral and broadly cytotoxic compounds because compounds that induce loss of the virus also lead to cell death. As broadly cytotoxic compounds are often associated with multiple side effects resulting from nonselective toxicity, the utility of a live cell screen should improve the identification of compounds that may exhibit selective antiviral activity *in vivo*. In this report, samples that proved efficacious in the KSHV-BJAB cell line were also tested in the naturally infected BCBL-1 cell line to confirm that loss of the virus corresponded with primary effusion lymphoma cell death.

In addition to the application described here, this assay can be adapted for use with other viruses, such as EBV, that exist episomally in the host cell. It can be used to screen various collections (ranging from pure compounds to crude extracts) for samples that interfere with viral persistence. In this report, we used this assay to screen a small library of plant extracts and identified two that selectively induced loss of KSHV virus from infected cells.

## Materials and Methods

### Cells and Media

The KSHV-negative BJAB lymphoid cell line and the KSHV-positive BCBL-1 cell line were obtained from American Type Culture Collection. BJAB cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/mL of penicillin, and 100  $\mu$ g/mL of streptomycin. BCBL-1 cells were maintained in the same medium additionally supplemented with 1 mmol/L of NaHCO<sub>3</sub> (Life Technologies) and 0.05 mmol/L of 2-mercaptoethanol (Sigma). The BJAB-derived cell line, KSHV-BJAB, was created by nucleofection of KSHV-negative BJAB cells with the KSHV bacterial artificial chromosome containing a hygromycin antibiotic resistance marker and the GFP expression cassette (28). Cells containing the KSHV genome were selected for a minimum of 2 weeks in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/mL of penicillin,

100 µg/mL of streptomycin, and 0.2 mg/mL of hygromycin B. Unless otherwise noted, growth media and supplements were obtained from Cellgro.

### Plant Collection and Processing

Plant samples were collected in Manus Island, Papua New Guinea, in 2003 under a UIC-UPNG Memorandum of Agreement 2003–2008 and approval of the PNG BioNET/Department of Environment and Conservation dated May 21, 2003. A set of voucher herbarium specimens has been deposited at both the Herbarium of the University of Papua New Guinea, Port Moresby and the John G. Searle Herbarium, Field Museum, Chicago, IL. Taxonomic identifications were done by one of the authors (P. Piskaut) and confirmed by staff of the Lae Herbarium, Papua New Guinea and by one of the authors (D.D. Soejarto). The collection and processing strategies for these understudied plant specimens from tropical rainforests has been recently reviewed (31).

Briefly, a pilot sample (~20 g, dry weight) was extracted with a 9:1 methanol/water solution. The resultant extract was defatted with hexanes, and the residual materials were partitioned in a 4:1:5 chloroform/methanol/water solution. Importantly, the organic-soluble fraction was washed with 1% NaCl to remove tannins (32), which are known to interfere with some biological assays. The detannified organic fraction of each sample was tested for biological activity.

### Fluorescence Assay

One hundred thousand cells from each B lymphocyte cell line were suspended in RPMI 1640 (Cellgro) supplemented with 2% fetal bovine serum (Cellgro), 100 units/mL of penicillin and 100 µg/mL of streptomycin (Cellgro) and were placed in subsequent wells of a 24-well plate. Hygromycin selection was withdrawn from KSHV-BJAB cells to prevent competition with screened samples. In the case of plant samples, the detannified organic fraction or an equal volume of DMSO was added to the growth medium at a final protein concentration of 2 µg/mL. The pure compound and positive control, glycyrrhizic acid (Sigma) dissolved in 5% ethanol at a pH of 7.2, was diluted to 2 mmol/L (1,680 µg/mL), 3 mmol/L (2,520 µg/mL), 4 mmol/L (3,360 µg/mL), and 6 mmol/L (5,040 µg/mL) in RPMI 1640 (Cellgro) supplemented with 2% fetal bovine serum (Cellgro), 100 units/mL of penicillin and 100 µg/mL of streptomycin (Cellgro). Half of the culture medium was siphoned from each well and replenished with fresh medium and identical concentrations of glycyrrhizic acid or plant sample (2 µg/mL) twice each week without passaging the cells. Mean fluorescence of the live cultures, incubated with test or control samples was measured using a Fluostar fluorimeter every 2 to 3 days. Optimal variables for detection were an excitation wavelength of 485 nm, an emission wavelength of 510 nm, and orbital well scanning.

### Real-time Quantitative PCR

Total DNA (including cellular genomic and viral DNA) was isolated from cells after 5 weeks of incubation with test samples or vehicle controls using the Promega Wizard Genomic DNA kit. Real-time quantitative PCR (RT-QPCR) was done with primers specific for the cellular *U6* gene and the viral *vGPCR* gene as previously described (33). Using this method to analyze BCBL-1 cells serially diluted in a suspension of uninfected BJAB cells, we were able to detect as few as 1 in 10,000 infected cells with 95% efficiency (Supplementary Fig. S1).<sup>6</sup> In the BJAB and BCBL-1 cells, the cytotoxicity of the plant samples was assessed using the equation  $1.9^{-\Delta CT(U6)}$  to normalize the data to the DMSO control. Results were expressed as the percentage of viability. In KSHV-BJAB cells, selective inhibition of the virus was assessed by first normalizing the cellular and viral data to the DMSO control, then determining the ratio, or selectivity index ( $SI = 1.9^{[\Delta CT(U6)-\Delta CT(vGPCR)]}$ ), of viral toxicity versus cellular toxicity.

### Immunofluorescence

After 1 week, ~100,000 BCBL-1 cells cultured with plant extract or a DMSO vehicle control were spotted on slides. Cells were fixed and permeabilized in precooled acetone at -20°C for 15 min. Slides were washed in PBS. Normal goat serum (10% in PBS) was used to block nonspecific antibody binding. The slides were incubated for 1 h at room temperature with anti-KSHV ORF-73 (LANA) rat monoclonal antibody (1:100, Advanced Biotechnologies). Slides were washed twice in PBS, then incubated for 30 min at room temperature

with anti-rat TRITC-conjugated IgG (1:100, Sigma). Slides were washed twice in PBS and once in distilled water and allowed to dry. Vectashield was applied to preserve fluorescence.

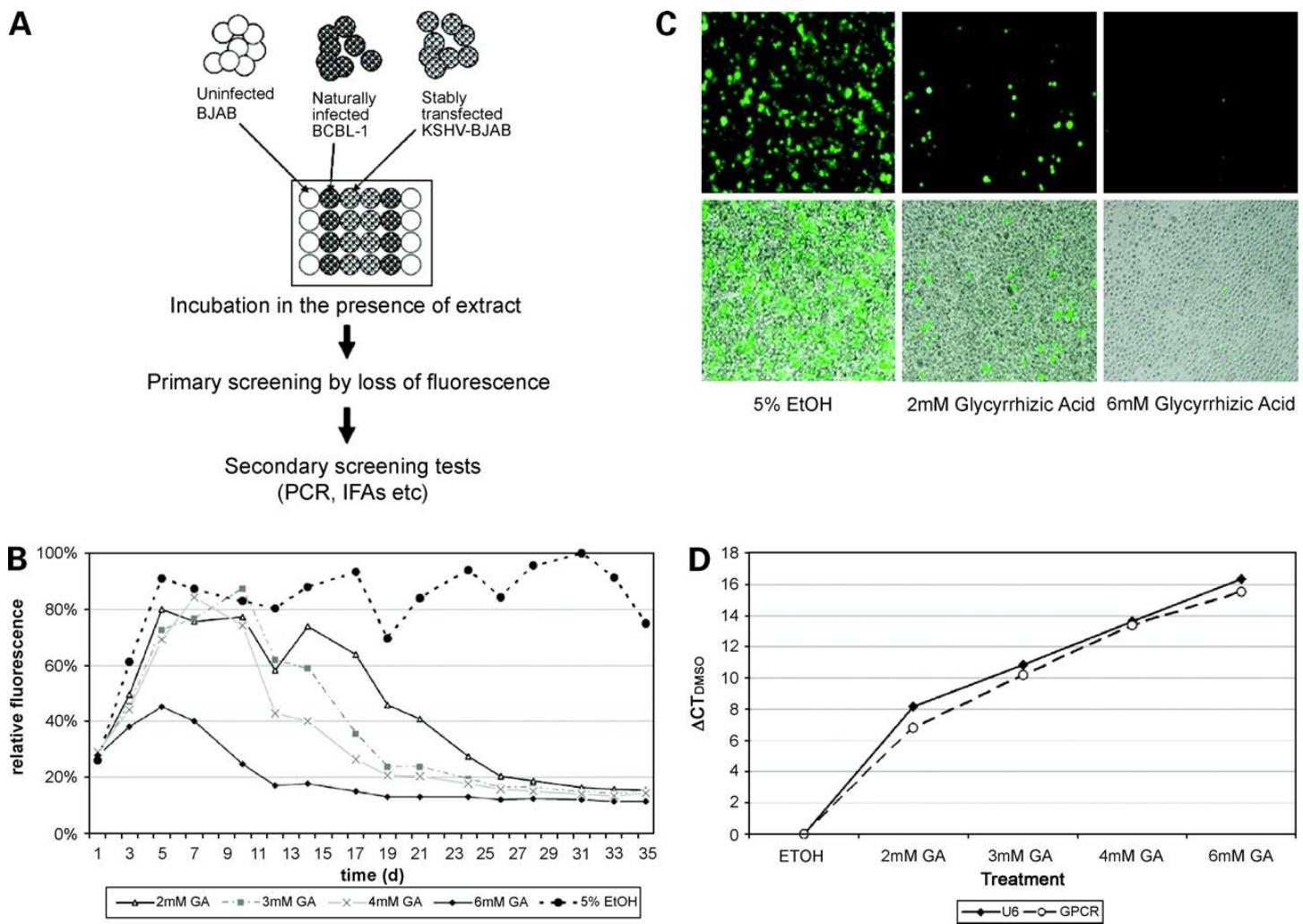
## Results

### Development of the Screening Assay

The KSHV-BJAB cell line was established by introducing the complete KSHV genome in the context of a bacterial artificial chromosome (KSHV-BAC; ref. 28) into uninfected B lymphocytes (BJAB). The KSHV-BAC contains both a mammalian hygromycin antibiotic resistance marker and the GFP expression cassette. Transfection of BAC viral DNA was previously shown to result in fully replication-competent virus and circumvented any receptor or post-entry blocks that may limit the efficiency of natural infection of B cells with KSHV (28, 29, 34). KSHV-BJAB cells were selected in 0.2 mg/mL of hygromycin B in order to achieve a stable KSHV-positive cell line.

KSHV-BJAB cells harboring the KSHV-BAC express GFP, thus providing a means to screen for samples that interfere with latent viral genome maintenance. Inhibition of viral genome maintenance results in loss of fluorescence as the viral episome is lost from the dividing cell population. Because KSHV episome loss occurs at cell division (35), multiple cell division cycles must take place before a significant loss of fluorescence can be observed. The design, as a multicycle assay, also increased the sensitivity and allowed us to prioritize potential lead samples based on their effectiveness.

To screen for samples with activity against latent KSHV infection, hygromycin selection was removed from the KSHV-BJAB cells to prevent competition with the screened samples. Cells were incubated with test and control samples for 5 weeks. Culture medium and samples were replenished twice each week without passaging the cells. Primary screening of samples was achieved by measuring the mean fluorescence of the GFP-positive KSHV-BJAB cells every 2 to 3 days (Fig. 1A). Mean fluorescence was plotted over time to identify samples that accelerated loss of fluorescence in the KSHV-BJAB cell line with respect to a vehicle control. The uninfected BJAB cell line served as an additional control against broad-spectrum cytotoxic effects of the tested samples. A naturally infected KSHV-positive primary effusion lymphoma cell line, BCBL-1 (30), was also included to confirm the antiviral effects of lead samples by real-time QPCR and indirect immunofluorescence assays for LANA (Fig. 1A).



**Figure 1.**

Validation of the assay. **A**, cells from three B lymphocyte cell lines (BJAB, KSHV-BJAB, and BCBL-1) were incubated in RPMI containing test or control samples. Samples were initially screened for loss of fluorescence in the KSHV-BJAB cells, suggestive of loss of virus. To verify loss of virus from the cells, additional assays were done including real-time PCR viral load assays and immunofluorescence assays for LANA. **B**, incubation of KSHV-BJAB cells with a known inhibitor of KSHV latency, glycyrrhizic acid, leads to a dose-dependent decrease in mean fluorescence as compared with an ethanol control. **C**, digital images of the KSHV-BJAB cells incubated with glycyrrhizic acid (2 and 6 mmol/L) or an ethanol control. *Top*, fluorescence images; *bottom*, merged fluorescent and bright-field images. **D**, real-time QPCR using primer sets to amplify viral (vGPCR) and cellular (U6) regions of DNA isolated from BCBL-1 cells incubated with glycyrrhizic acid (GA) or an ethanol (*EtOH*) control. Dose-dependent increases in the cycle threshold for viral (○) and cellular (□) template as compared with the ethanol control were evident.

### Validation of the Screening Assay

To validate the assay, a known inhibitor of KSHV latency, glycyrrhizic acid was tested. At 3 mmol/L, glycyrrhizic acid has been shown to down-regulate the expression of LANA from KSHV (19). KSHV-BJAB cells were incubated with 2 to 6 mmol/L of glycyrrhizic acid or an ethanol control (final ethanol concentration, 0.6%). Culture medium was replaced twice each week with fresh medium plus glycyrrhizic acid without passaging the cells. In the ethanol control or 2 to 4 mmol/L glycyrrhizic acid-treated KSHV-BJAB cultures, fluorescence increased from days 1 to 7 as KSHV-BJAB cells proliferated. After day 7, fluorescence leveled off in the ethanol control cultures, whereas cells incubated with glycyrrhizic acid showed a dose-dependent decrease in mean fluorescence (Fig. 1B). At 6 mmol/L, glycyrrhizic acid inhibited the initial proliferative burst of KSHV-BJAB cells, most likely due to broad cytotoxicity. By day 18, the majority of KSHV-BJAB cells

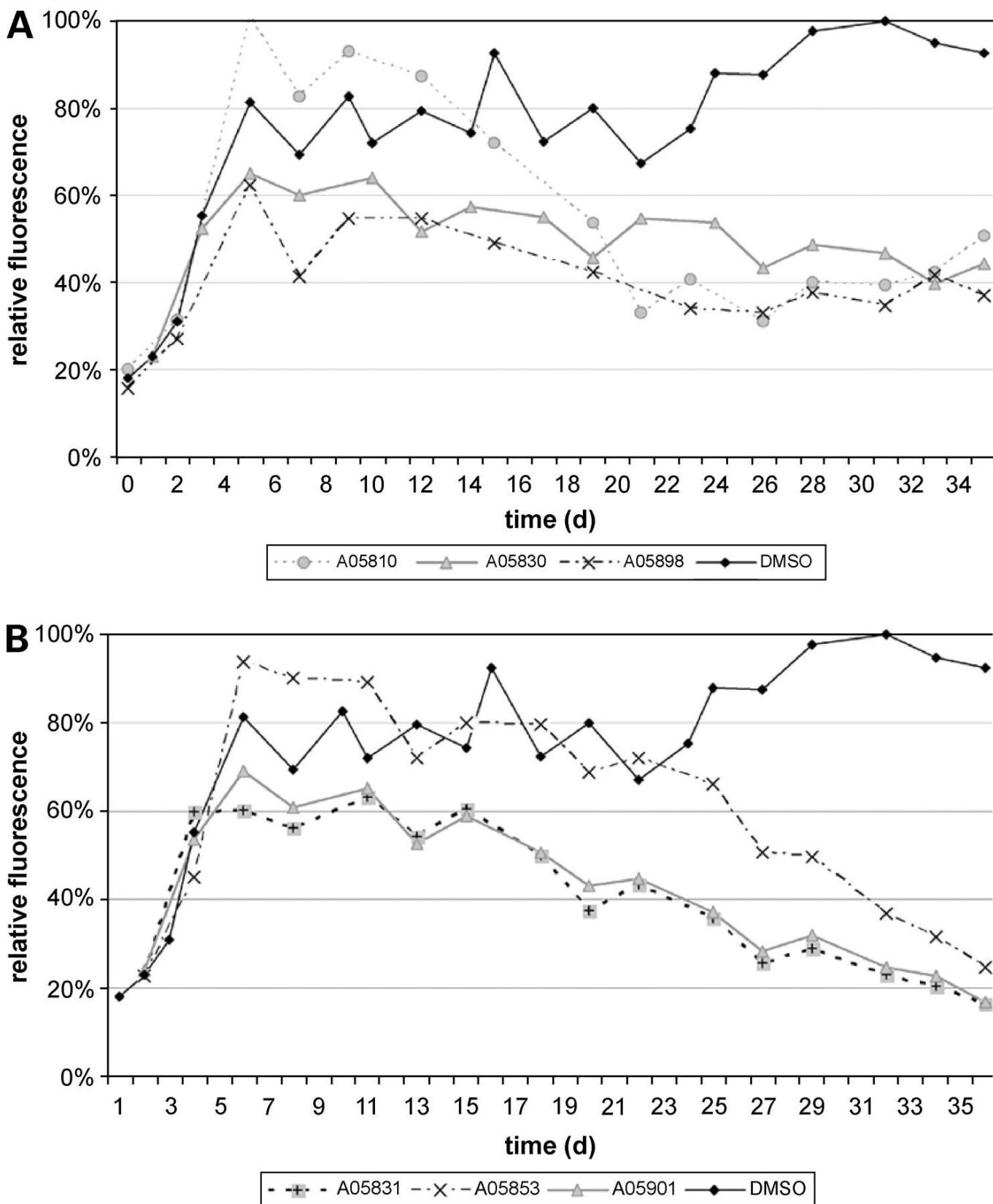
incubated with 2 mmol/L of glycyrrhizic acid no longer exhibited green fluorescence, but remained viable (Fig. 1C, *middle*), in contrast to the gross cytotoxicity observed at 6 mmol/L of glycyrrhizic acid (Fig. 1C, *right*). On day 18, uninfected BJAB cells also remained viable at 2 mmol/L of glycyrrhizic acid, but lost viability at 6 mmol/L of glycyrrhizic acid (data not shown).

The antiviral effect of glycyrrhizic acid was verified by viral load assays. Total cellular and viral genomic DNA was isolated from BCBL-1 cells or KSHV-BJAB cells incubated with glycyrrhizic acid or an ethanol control for 7 days. As expected, the cycle threshold for both the cellular (U6) and viral (vGPCR) primer sets increased in a dose-dependent manner, indicating a simultaneous reduction of both viral and cellular DNA (Fig. 1D). This was expected as glycyrrhizic acid interfered with viral latency, leading to loss of viral DNA and, because the KSHV genome is required for BCBL-1 cell survival, a loss of cellular DNA as well. Glycyrrhizic acid selectively inhibited the virus in KSHV-BJAB cells, which do not depend on the virus for survival, with reductions in viral load on average 17 times greater than reductions in cellular DNA (data not shown). These results served to validate our assay. However, cumulative cytotoxic effects became evident in all glycyrrhizic acid cultures, including the uninfected BJAB cultures, after 18 days of treatment (data not shown). Therefore, despite its initial selective antiviral effect, glycyrrhizic acid is unlikely to be a good drug candidate given its cumulative broad cytotoxicity. Moreover, the relatively high concentration required for a positive response *in vitro* could be difficult to achieve *in vivo*.

### **Identification of Antiviral Plant Extracts**

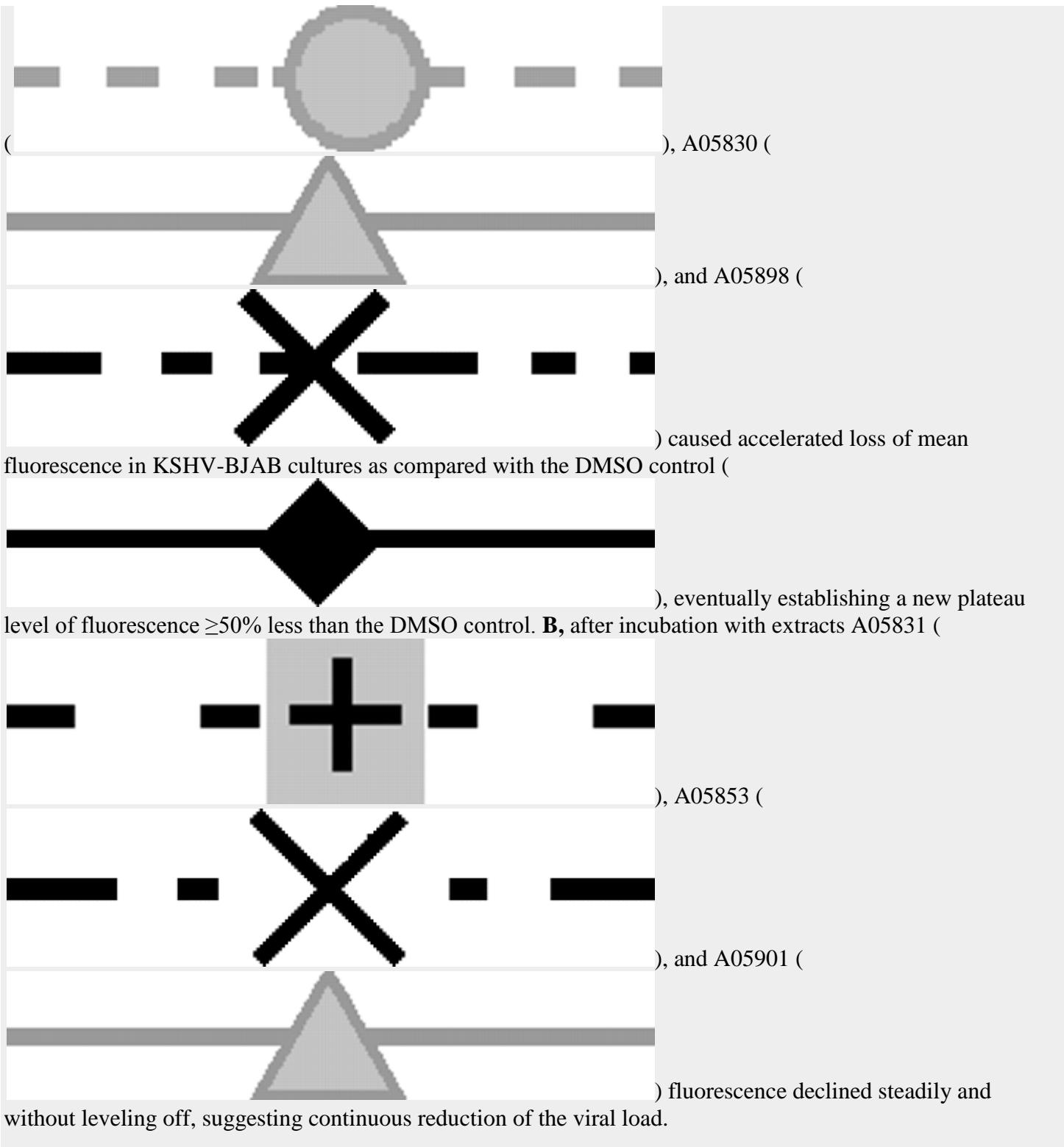
Having validated the assay, a screening set of 81 plant extracts was tested for anti-KSHV activity. All extracts were dissolved in DMSO and were tested at a final concentration of 2 µg/mL in RPMI. An equal volume of DMSO served as a vehicle control. Culture medium was replaced twice each week with fresh medium plus plant extract, without passaging the cells. Mean fluorescence measurements from KSHV-BJAB cultures were taken every 2 to 3 days. Fluorescence from KSHV-BJAB cells treated with DMSO plateaued within 1 week as the cells achieved equilibrium. Sixty-two extracts exhibited insignificant changes in the fluorescence of KSHV-BJAB cultures with respect to the DMSO control. Eight extracts marginally reduced fluorescence, whereas six extracts consistently decreased fluorescence by at least 50% of the DMSO control without apparent cytotoxicity (Supplementary Table S1).<sup>6</sup> Four extracts exhibited acute and one delayed (cumulative) cytotoxic effects irrespective of the cell's infection status.

**Extracts that Decreased Fluorescence.** Figure 2 displays the mean fluorescence graphs of six extracts (A05810, A05830, A05831, A05853, A05898, and A05901) that consistently decreased fluorescence by ≥50% of the DMSO control in four separate trials. Two distinct trends were observed within this group of six extracts. Fluorescence of KSHV-BJAB cells incubated with extracts A05810, A05830, and A05898 (Fig. 2A) peaked within 7 days, then decreased steadily until achieving a new plateau level of fluorescence at least 50% less than the DMSO control, but still above background. Fluorescence from KSHV-BJAB cells incubated with extracts A05831, A05853, and A05901 (Fig. 2B) peaked within 7 days, then steadily declined throughout the experiment.



**Figure 2.**

Initial screening for antiviral activity is based on loss of fluorescence. Mean fluorescence of live B lymphocyte cultures incubated with potential antiviral compounds is measured every 2 to 3 d for 5 wks. **A**, extracts A05810



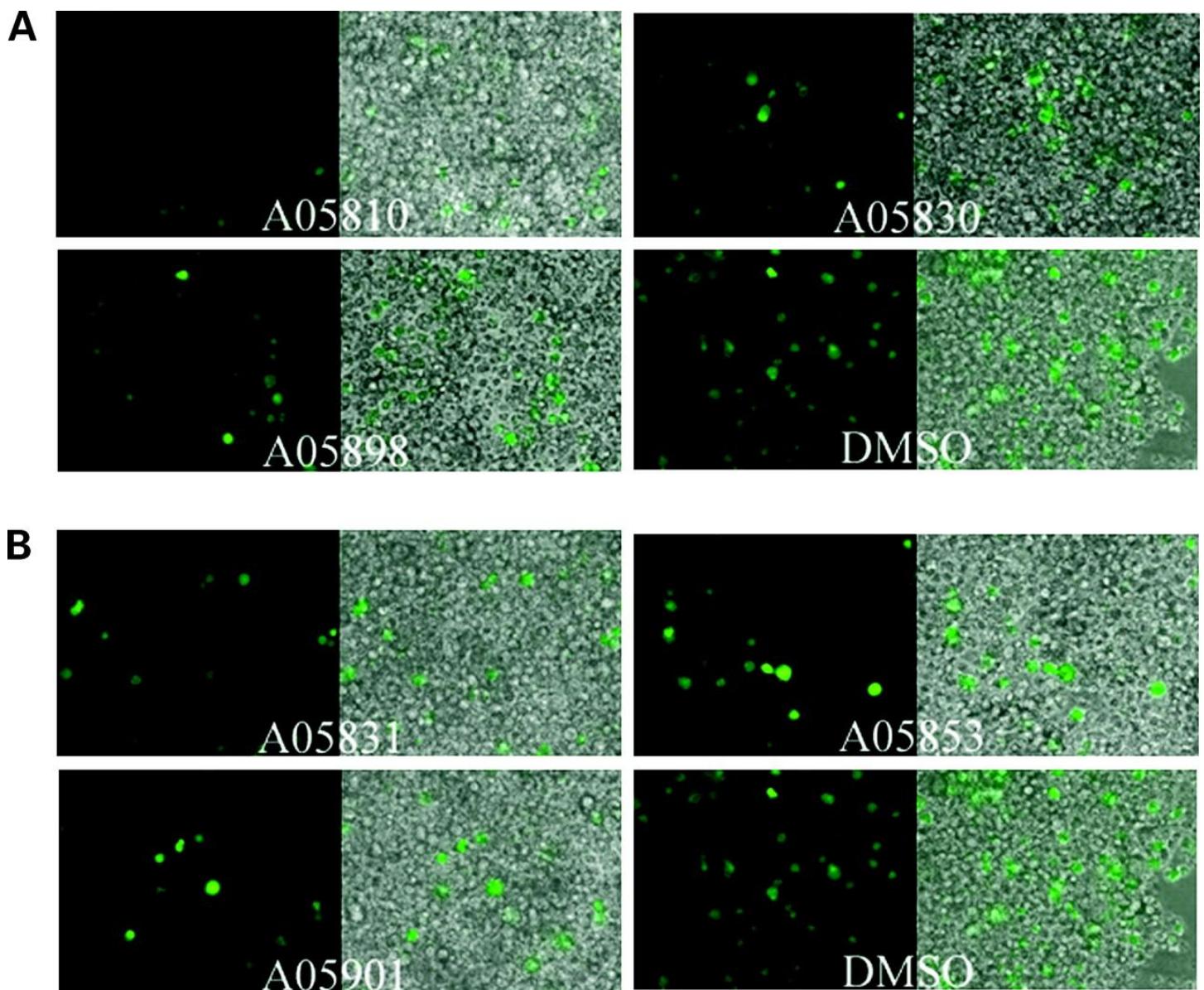
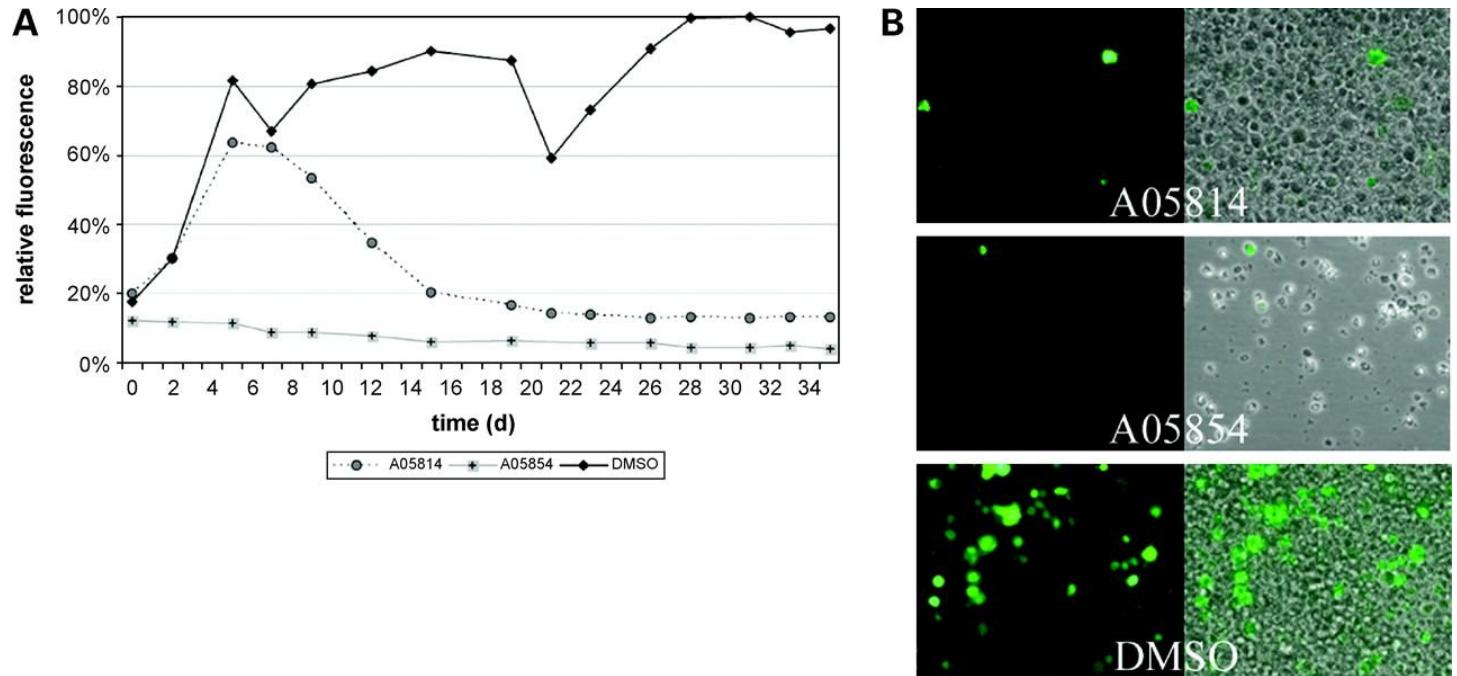


Figure 3 displays digital images of KSHV-BJAB cells after 20 days of incubation with the six aforementioned extracts and the DMSO control. Roughly equivalent numbers of cells were present in each field. However, the number of GFP-positive cells was reduced  $\geq 50\%$  by each of the extracts as compared with the DMSO control. No visually apparent cytotoxic effects of these extracts were evident at day 20. Furthermore, these extracts were assessed at day 14 in uninfected BJAB cells (Supplementary Fig. S2A)<sup>6</sup> and in three unrelated cancer cell lines (Supplementary Fig. S2B)<sup>6</sup> and were found to have limited cytotoxicity in these assays. These six extracts that repeatedly decreased the fluorescence of KSHV-BJAB cells may interfere with episomal maintenance and were chosen for further analyses.

**Cytotoxic Extracts.** In contrast to the extracts that showed a consistent decrease in fluorescence without visually apparent cytotoxicity, four extracts, as represented by extract A05854 in Fig. 4, were acutely and uniformly cytotoxic to all three B lymphocyte cell lines tested regardless of their infection status. Additionally, one extract (A05814) was found to have cumulative nonspecific cytotoxic effects. As before, fluorescence from DMSO-treated KSHV-BJAB cells increased sharply as cells proliferated, whereas the fluorescence of KSHV-BJAB cells incubated with the acutely cytotoxic extract (A05854) failed to increase at all and quickly achieved baseline levels (Fig. 4A). Extract A05814 initially permitted cell proliferation concomitant with escalating fluorescence measurements (Fig. 4A). However, by day 14, fluorescence had plummeted to near-background

levels. Whereas the cytotoxicity of extract A05854 was unmistakable (Fig. 4B) at day 20, with only few apoptotic cells present, many more cells were present in the A05814 culture (Fig. 4B), with apoptotic changes just becoming visually apparent at this time point. Key to the distinction and subsequent exclusion of these cytotoxic extracts from further analyses was their nonselective toxicity to uninfected BJAB and infected BCBL-1 cells alike (data not shown). Thus, the inclusion of uninfected BJAB cultures in this assay distinguishes generally cytotoxic samples from those that are selectively active against KSHV-infected cells and allows us to separate specific antiviral activity in our samples from acute and delayed cytotoxic activity.



**Figure 4.**

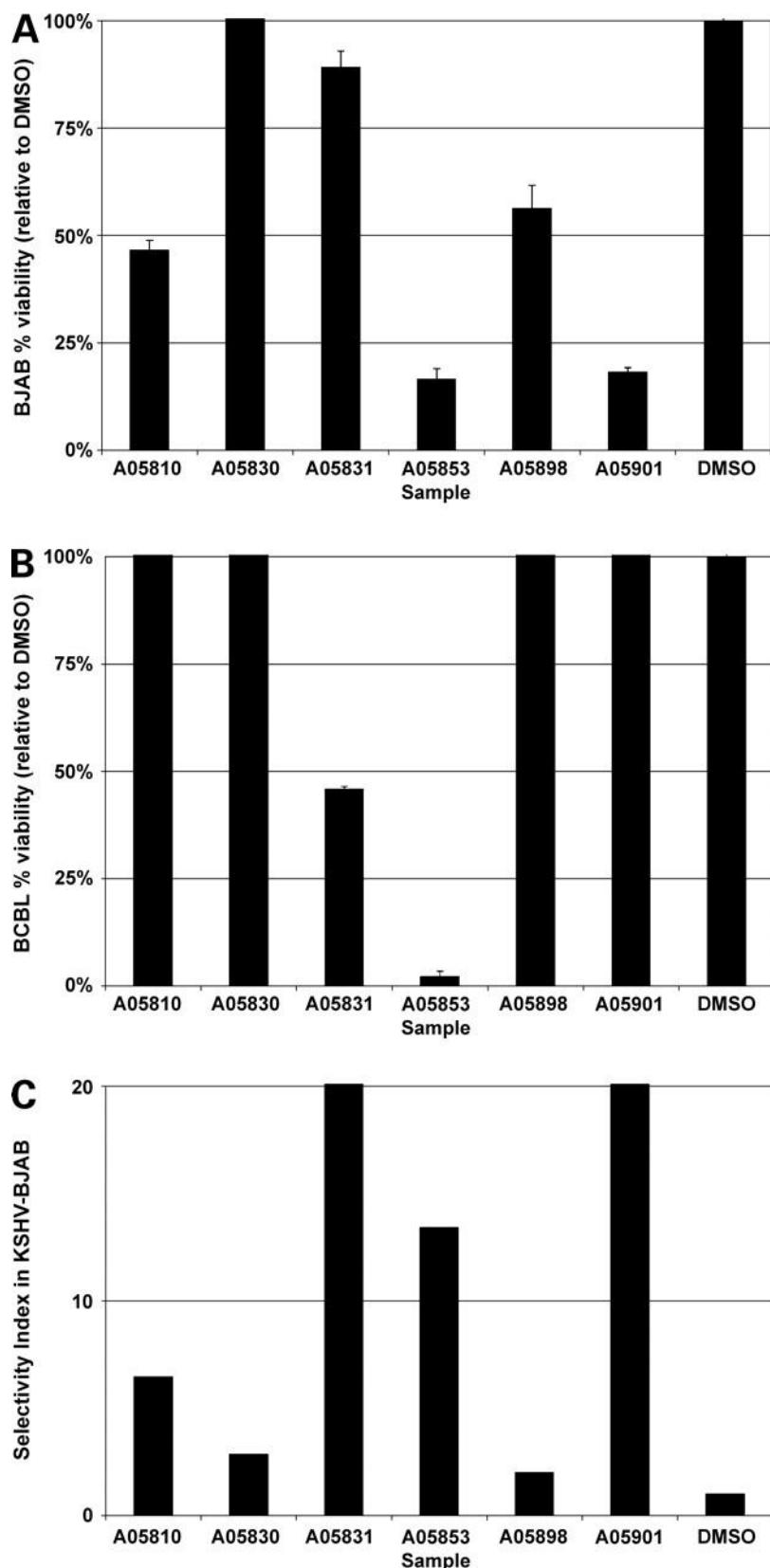
Five extracts exhibited cytotoxicity to all three B lymphocyte cell lines. **A**, mean fluorescence graph representative of acute and delayed cytotoxic samples, A05854 ( $\circ$ ) and A05814 ( $\blacksquare$ ), respectively. **B**, digital images of KSHV-BJAB cells incubated with acutely cytotoxic sample A05854 or delayed cytotoxic sample A05814. *Left*, fluorescence images; *right*, merged fluorescent and bright-field images.

### Confirmation of Antiviral Activity by Real-time QPCR

The six extracts (A05810, A05830, A05831, A05853, A05898, and A05901; Figs. 2 and 3) that decreased fluorescence with respect to the DMSO control in the initial fluorescence-based screen were selected for further study. After incubation with test or control samples for 5 weeks, viral genomic and host chromosomal DNA was isolated from BJAB, KSHV-BJAB, and BCBL-1 cells. Each culture began with an identical number of cells and was not split during the course of the experiment. The isolated DNA was resuspended in the same volume of buffer, and an equal volume of the isolated DNA was used as a template for real-time QPCRs with viral (vGPCR) and cellular (U6) primer sets. Thus, any increases in cycle threshold for the cellular primer set were likely due to the cytotoxic effects of the extract, whereas changes in the viral cycle threshold were due to changes in the cell-associated viral load. After normalizing the raw data to the DMSO control, either toxicity in BJAB and BCBL-1 cultures or selectivity in KSHV-BJAB cultures were ascertained.

As expected, the non-template control yielded no signal with either primer set after 40 cycles. Likewise the cycle threshold (CT) for the viral primer set in uninfected BJAB cells was 40, indicating the absence of viral DNA, whereas the cellular primers (directed against the U6 gene) gave a consistent signal (mean  $CT = 22 \pm 1$ , across the six extracts) similar to the DMSO control ( $CT = 21 \pm 1$ ). Although BJAB cultures remained at least 80% viable at day 14, as measured by trypan blue exclusion (Supplementary Fig. S2A),<sup>6</sup> cytotoxic effects became apparent by QPCR at 5 weeks (Fig. 5A). BJAB cells treated with extracts A05830 and A05831 remained viable (109% and 89% viable, respectively, as compared with the DMSO control). Extracts A05810

and A05898 reduced viability by ~50%, whereas extracts A05853 and A05901 reduced viability by >80% compared with the DMSO control.



**Figure 5.**

Real-time QPCR allows potential hits to be stratified based on selectivity and specificity. Real-time QPCR was done using viral (vGPCR) and cellular (U6) primer sets. For uninfected BJAB (**A**) and naturally infected BCBL-1 cells (**B**), cell viability of extract-treated cells was normalized to the DMSO control using the equation:

$1.9^{-\Delta CT(U6)}$ . C, cellular and viral DNA in extract-treated KSHV-BJAB cells were first normalized to DMSO-treated KSHV-BJAB cells. Then, the selectivity index was calculated as the ratio of changes in viral DNA to changes in cellular DNA.

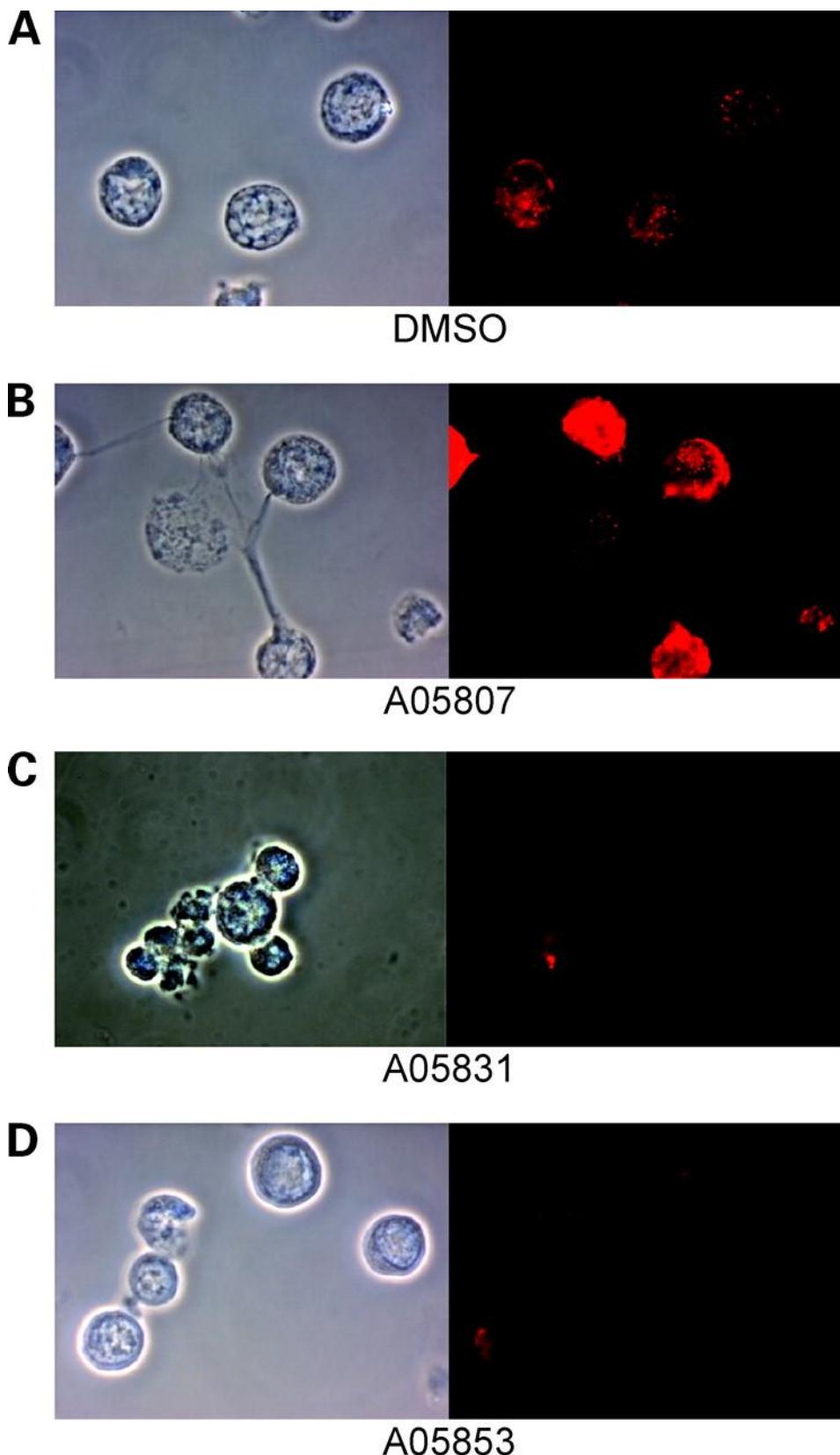
Next, naturally infected BCBL-1 B lymphoma cells were evaluated. Each cell contains approximately 70 copies of the KSHV episome (36) and its maintenance is vital for BCBL-1 survival. If all viral genomes are lost, the cell dies. Given the interdependence of viral genome maintenance and cell survival in BCBL-1 cells, we analyzed only the cytotoxicity of the extracts in BCBL-1 cells compared with the DMSO control (Fig. 5B). Two of the six extracts—A05831 and A05853—were toxic to BCBL-1 cells, reducing viability to 46% and 2%, respectively. These extracts exhibited expectable increases in both viral and cellular cycle thresholds compared with DMSO (Supplementary Table S1).<sup>6</sup>

Finally, the selectivity of each extract was assessed in KSHV-BJAB cells (Fig. 5C), representing the key innovation of this report because nonspecific cytotoxic effects can be uncoupled from changes in viral load as KSHV-BJAB cells do not require the virus for survival. Here, the KSHV-specific primers (directed against the *vGPCR* gene) detected drastic changes in KSHV viral DNA resulting in CT values ranging from  $28 \pm 1$  to  $39 \pm 1$  in extract-treated cells and  $25 \pm 1$  in DMSO-treated cells, whereas the cellular DNA (*U6* gene) remained largely unchanged (mean CT =  $22 \pm 1$  across six extracts; CT =  $20 \pm 1$  in DMSO-treated cells). Changes in cellular and viral genomic DNA were first individually normalized to DMSO-treated cells. The selectivity index of each extract was then determined by the ratio of viral DNA reduction to host DNA reduction, i.e., cytotoxic effects. Three extracts—A05831, A05853, and A05901—were highly selective as indicated by selectivity indexes >10, indicating that extract-mediated reductions in viral DNA were 232, 13, and 152 times greater, respectively, than reductions in KSHV-BJAB cellular DNA.

Taken together, the information gleaned from each QPCR experiment was used to prioritize samples according to the selectivity of their antiviral activity and the specificity of their cytotoxic effects. Specifically, extracts that exhibit (a) high selectivity (selectivity index >10 in KSHV-BJAB cells), (b) large reductions in BCBL-1 viability (>50%), and (c) minimal cytotoxic effects in BJAB cells would receive higher priority in subsequent analyses. Of the three highly selective extracts we identified, only extracts A05831 and A05853 were toxic to BCBL-1 cells, resulting in 54% and 98% reductions in BCBL-1 viability, respectively. Whereas extract A05831 was mildly toxic to uninfected BJAB cells (11% reduction in cell viability), A05853 was significantly toxic (84% reduction in cell viability) and may require further refinement to achieve priority status. Nonetheless, the fluorescence-based screening assay and the QPCR data suggest that extracts A05831 and A05853 selectively interfere with viral genome maintenance, and were chosen for further study as described below. However, it should be noted that of all the extracts tested, extract A05831 was most selective for viral genome loss with minimal cytotoxicity in BJAB cells.

### Assessing LANA Expression by Immunofluorescence

We used an indirect immunofluorescence assay against KSHV LANA as a first step toward elucidating the antiviral mechanism(s) behind extracts A05831 and A05853. BCBL-1 cells were incubated with plant extracts for 7 days, then stained with an antibody directed against LANA (anti-KSHV ORF-73) followed by fluorophore-conjugated anti-idiotypic immunoglobulins. Cells incubated with either the DMSO control (Fig. 6A ) or extract A05807 (Fig. 6B) that showed no antiviral effect in the screening assays, displayed characteristic speckled nuclear staining. In BCBL-1 cells that were incubated with extracts A05831 or A05853 (Fig. 6C and D, respectively), anti-LANA immunofluorescent staining was decreased to near-background levels, indicating that loss of viral genome correlated with loss of LANA expression.



**Figure 6.**

Indirect immunofluorescence assays for LANA investigate possible mechanisms of interference with viral genome maintenance. BCBL-1 cells incubated with plant extract or a DMSO control were spotted on slides and fixed with precooled acetone. Incubations with anti-KSHV ORF-73 (LANA) monoclonal antibody followed by TRITC-conjugated anti-rat IgG were used to detect the presence of LANA. *Left*, bright-field images; *right*, fluorescent staining of the KSHV LANA. **A**, DMSO control; **B**, extract A05807; **C**, extract A05831; and **D**, extract A05853.

## Discussion

Eradicating latently infected cells represents the ultimate goal in the therapy of KSHV-associated malignancies, in which loss of the viral episome expectedly leads to tumor cell death. Currently, treatment of Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease typically includes chemotherapeutic agents that target all replicating cells, failing to distinguish between virally infected and uninfected cells. Such regimens are associated with severe side effects, including myelotoxicity and pancytopenia, which can become life-threatening in an already immunocompromised population. Because KSHV infection remains in a latent state in the majority of infected tumor cells, drugs that target latent viral proteins may be more effective than current regimens at both preventing and treating disease and may have an added benefit of fewer side effects.

This report describes an assay designed to identify samples that induce viral episome loss, irrespective of the specific mechanism, and without generalized cytotoxicity. The design hinges on a two-step screen. The first step identifies samples that cause loss of the latent virus in a cell line (KSHV-BJAB) that does not depend on the virus for viability. The second step validates those hits in a cell line (BCBL-1) that does depend on the virus for survival.

The initial screening step employs a B lymphocyte cell line (KSHV-BJAB) carrying the KSHV-BAC and expressing GFP. KSHV-BJAB cells are incubated in medium containing test samples and are monitored for loss of fluorescence (i.e., loss of the viral episome). Seven percent of 81 screened plant extracts consistently reduced fluorescence by  $\geq 50\%$  in KSHV-BJAB cells as compared with a DMSO control. An additional 6% of the extracts were cytotoxic to all B lymphocyte cell lines tested, regardless of their KSHV infection status. Eighty-seven percent had no significant effect (data not shown).

Because nonspecific promoter silencing could diminish fluorescence in KSHV-BJAB cells, virus-specific effects were verified by real-time QPCR for the viral genome. Using primer sets for both a viral (vGPCR) and a cellular (U6) gene and DNA templates isolated from BJAB, KSHV-BJAB, or a primary effusion lymphoma cell line, BCBL-1, after incubation with the test samples, the cytotoxicity and selectivity of each extract was assessed. Six extracts, identified as potential hits ( $\geq 50\%$  reduction of fluorescence) in the first screening step, were tested. Extracts A05831 and A05853 showed selective activity against latent virus, as the viral episome was lost from a model infection (KSHV-BJAB) at least 10 times more efficiently than host chromosomal DNA (corresponding to cell death). Furthermore, both extracts resulted in at least a 50% reduction in cell viability from naturally infected tumor cells (BCBL-1), as compared with a DMSO control. Extract A05831 receives higher priority for further study because it is relatively nontoxic in uninfected BJAB cells, whereas extract A05853 may prove too nonselectively toxic in its unrefined state.

In principle, compounds that interfere with viral genome maintenance may target cellular or viral proteins required to maintain latency. Samples exerting their effects by targeting viral proteins are preferred because a specific antiviral effect may be less toxic to other highly replicating cells and, presumably, would have fewer side effects than currently available chemotherapeutics. KSHV LANA is a likely viral target for antiviral samples because it is essential for KSHV genome maintenance. In a complex with multiple cellular proteins (37–43), LANA tethers the viral genome to the cellular chromosome, ensuring that the two are replicated coincidentally and are segregated equally to each daughter cell. Samples that target LANA may accomplish their antiviral effect by one or more means, including (a) transcriptional down-regulation, (b) degradation or posttranslational modification, (c) sequestration outside the nucleus, (d) interference with binding to the host chromosome or the viral genome, or (e) similarly targeting cellular proteins that complex with LANA. Additionally, other viral latent proteins may also be involved in viral genome maintenance and may also be targeted.

To explore the mechanism(s) by which extracts A05831 and A05853 propel episomal loss, a LANA immunofluorescence assay was done on BCBL-1 cells incubated with the two lead extracts, identified by the initial fluorescence-based screening step and verified by QPCR. Both extracts resulted in near-background levels of LANA immunofluorescent staining. Thus, these plant extracts may contain one or more compounds

that down-regulate the transcription of LANA, accelerate its degradation, or cause posttranslational modifications that render it undetectable by this antibody. Therefore, extracts A05831 and A05853 are prime candidates for further experimentation. Because each extract likely contains hundreds of compounds, future studies will employ a bioactivity-directed fractionation strategy to purify and identify the antiviral constituent(s).

Loss of fluorescence mediated by culturing KSHV-BJAB cells with antiviral samples is dependent on two things: loss of the KSHV-BAC, which contains the GFP gene and degradation of GFP that is made prior to loss of the gene. GFP has a reportedly long half life, ranging from 26 to 80 h in eukaryotic cells (44–46), which contributed to the length of the initial screen, which was further extended to 5 weeks in order to assess cumulative cytotoxicity. Analysis of the data, however, showed that the most potent inhibitors already displayed a significant effect by day 20, and the second screening step allowed for the earlier detection of cumulative cytotoxic effects.

In modern medicine, many highly effective therapeutic agents such as camptothecin and taxol/paclitaxel, were first isolated from plant extracts and have revealed novel targets and mechanisms for antitumor drug action. Although certain herbal extracts were recently found to reactivate KSHV (47), the novel assay described in this report identifies plants as a rich source for antiviral compounds that may cure KSHV infection by interfering with latent viral episome maintenance. Although a relatively small sample set was tested, the results are representative of the discovery potential for samples with therapeutic promise, as an overall hit rate of 2% is consistent with other natural product screens, in which hit rates typically range from 0.5% to 5%, regardless of the biological target. Indeed, small molecule libraries derived from plant or other natural product sources may prove to be repositories for antiviral agents with varied targets and activities against a breadth of currently incurable viral infections.

### Acknowledgments

We thank A. Douglas Kinghorn, of the Ohio State University, for access to the plant samples, which were collected with the consent of the Papua New Guinea Government's Department of Environment and Conservation, the Papua New Guinea Institute of Biodiversity, and the local people of Manus Province. We thank Yuka Nakanishi for supplemental cytotoxicity assays and sample preparation.

### Footnotes

- ↩6 Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).
- **Grant support:** NIH grants CA096500, HL083469, DE018281, and CFAR (2P30AI050410), and an American Heart Association grant 0640041N (B. Damania), NIH grants CA109232 and DE018304, and a Lymphoma Society Translational Science award 6021-06 (D.P. Dittmer). B. Damania is a Leukemia & Lymphoma Society Scholar and Burroughs Wellcome Fund Investigator in Infectious Disease. Supported in part by the Virology training grant 5T32AI007419 and Cancer Cell Biology training grant 2T32CA071341 (T.K. Nun). Financial support for the collection and processing of plant materials also came from the NIH (U19-CA52956). Partial support via a Research Scholar Grant from the American Cancer Society RSG-02-024-01-CDD (N.H. Oberlies).
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