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A rapid DNA hybridization assay for the evaluation of antiviral compounds against Epstein-Barr virus

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Abstract

There is a need for additional therapies for Epstein-Barr virus (EBV) infections, but the routine screening of large numbers of potential inhibitors has been difficult due to the laborious nature of traditional assays. A new rapid assay was developed to evaluate compounds for antiviral activity against this virus that is both rapid and robust. Test compounds are added to cultures of Akata cells in 96-well plates that have been induced to undergo a lytic infection. Viral DNA produced during the infection is transferred to a membrane and quantified using a non-radioactive DNA hybridization assay. This assay was validated using a set of compounds with known activity against EBV and results compared favorably to an established Real-Time PCR assay. Subsequent experience with this assay has confirmed that it offers improved efficiency and robustness compared to other assays used routinely to evaluate candidate compounds for antiviral activity against EBV.

Keywords

Epstein-Barr virus; antiviral; assay; Real-Time PCR; hybridization

1. Introduction

Many techniques have been used to evaluate the antiviral activity of compounds against the herpesviruses. Since Epstein-Barr virus (EBV) replicates only in lymphocytes, plaque reduction assays were not possible and other assays have been developed. These included incorporation of radioactivity in purified viral DNA (Lin et al., 1984), dot blot assays with radioactive DNA probes (Bacon & Boyd, 1995), ELISA based assays (Bacon & Boyd, 1995,Williams et al., 2003), flow cytometry assays using intact cells (Long et al., 2003), slot blot assays with purified DNA and non radioactive DNA probes (Meerbach et al., 2000), and Real-Time PCR assays (Friedrichs et al., 2004). A more robust and efficient non-radioactive assay was sought for use in the routine evaluation of antiviral activity against EBV.

Lymphocytes latently infected with this virus are often fastidious and require regular maintenance including feeding during lengthy assays. The induction of lytic infection is also inefficient in many cell lines such that it is difficult to induce a robust and synchronous infection. A number of cell lines were tested and the efficient induction of lytic replication in Akata cells appeared to offer significant advantages (Daibata et al., 1990). These cells eliminated many of the problems encountered with other cell lines and proved to be useful in the development of a new assay. A non-radioactive hybridization based assay was developed

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that yields results similar to more labor intensive and expensive assays and a three day incubation was sufficient to measure significant accumulation of nascent viral DNA. The assay was conducted in a 96-well format and is suitable for routine testing of large numbers of samples for antiviral activity against EBV. It was optimized in terms of assay format, cell number, induction conditions, time of harvest, drug concentrations and hybridization conditions. The resulting assay was validated by different operators on different days and performed well with regard to such properties as specificity, accuracy, precision and robustness. This assay was used to assess the antiviral activity of selected compounds and results were compared to those generated with a more standard Real-Time PCR assay.

2. Materials and Methods

2.1 Cells and viruses

Akata cells were kindly provided by John Sixbey (Louisiana State University, Baton Rouge, LA). Cells were maintained in RPMI 1640, (Mediatech, Inc, Herndon, Va.) supplemented with 10% fetal bovine serum (Hyclone, Logan, Utah), L-glutamine, penicillin and gentamicin at 37°C in a humidified 5% CO_2 atmosphere. Cells were passaged 2–3 days prior to performing the assay, enumerated on a hemacytometer and diluted to a final concentration of 4×10^5 cells/ml. Latently infected cells were induced to undergo a lytic infection by adding a $F(ab')_2$ fragment of goat anti-human IgG antibody (MP Biomedicals, Aurora OH).

2.2 DNA hybridization assay

Growth media containing 50 μg/ml goat anti-human IgG antibody was added to all wells except the cell control wells of a round bottom 96-well plate. Experimental compounds were serially diluted in duplicate at 1:5 with concentrations ranging from 100 μg/ml to 0.032 μg/ml. Latently infected Akata cells were diluted to a final concentration of 4×10^5 cells/ml and 100 μl was added to each well. The plates were incubated for 72 hours after which antiviral activity was assessed using the blot hybridization assay. After incubation 100 μl of denaturation buffer (1.2 M NaOH, 4.5 M NaCl) was added to each well and 50 μl was added to the wells of a Biodot apparatus (Bio-Rad, Hercules, CA) containing an Immobilon nylon membrane (Millipore, Bedford, A). A 50 μl aliquot of denatured DNA from the cells that were induced to undergo a lytic infection was aspirated through the membrane and then 50 μl of denaturation buffer was added. The membrane was allowed to dry before beginning the hybridization process. The membrane was then incubated in DIG Easy Hyb (Roche Diagnostics, Indianapolis, IN.) at 56° C for 30 minutes. A specific digoxigenin (DIG)-labeled probe was prepared according to the manufacturer's protocol (Roche Diagnostics) using PCR primers, EBNA probe forward 5′ CCC AGG AGT CCC AGT AGT CA 3′ and EBNA probe reverse 5′ CAG TTC CTC GCC TTA GGT TG 3′. The resulting probe corresponds to coordinates 96802–97234 in EBV genome (AJ507799) and was hybridized to the blots overnight at 56°C. Blots were then washed at 56°C with 0.2X SSC with 0.1% SDS and 0.1X SSC with 0.1% SDS. Detection of specifically bound DIG probe was performed with anti-DIG antibody using the manufacturer's protocol (Roche Diagnostics). An image of the film was captured and quantified with QuantityOne software (Bio-Rad). Drug concentrations sufficient to reduce the accumulation of viral DNA by 50% (EC_{50}), were interpolated from the dose response using standard methods (Prichard and Shipman, 1990).

2.3 EBV Real-Time PCR

EBV DNA was purified according to the manufacturer's instructions using the Wizard SV 96 Genomic DNA Purification System (Promega, Madison, WI). The purified DNA was then subjected to Real-Time PCR to quantify viral DNA. The primers 5′-CGG AAG CCC TCT GGA CTT C-3′ and 5′-CCC TGT TTA TCC GAT GGA ATG-3′ were used with the fluorescent probe, 6FAM-TGT ACA CGC ACG AGA AAT GCG CC-TAMRA corresponding to

coordinates 155959–155981 in the EBV genome (Applied Biosystems). A plasmid containing the amplified region was diluted to produce the standards used to calculate genome equivalents and ddH_2O as a negative control. The PCR was performed in an optical 96 well plate using an ABI 7300 Real-Time PCR system. The PCR reaction contained 900nM primers, 200nM probe, 12.5 μL Taqman Universal Master Mix (Applied Biosystems, Foster City, CA), and 5 μL target DNA in a final volume of 25 μL. Amplification conditions were: 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 sec and 60°C for 1 min. Each sample was run in duplicate and the copy number was calculated using the 7300 system software.

2.4 Cytotoxicity assays

Cytotoxicity was evaluated by both MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium) and CellTiter-Glo (Promega). Assay plates for toxicity were prepared in the same manner as those for the antiviral assay, except that the cells were not induced to undergo a lytic infection and a set of control wells were added that contained only growth media. Cytotoxicity was assessed at three days, the same time that viral DNA was harvested. In the MTS assay, 20 μl of MTS was added to each well and the plate was wrapped in aluminum foil and incubated at 37°C for 4 hours. The quantity of formazan product was measured at 490 nm in a microplate reader and the CC_{50} values were calculated by standard methods (Prichard & Shipman, 1990). Cell viability was also assessed with the CellTiter-Glo Luminescent Cell Viability Assay (Promega). Briefly, assay plates were incubated at ambient temperature for 30 minutes, 50 μl of CellTiter-Glo reagent was added to each well and the plates were mixed for 2 minutes on an orbital shaker to induce cell lysis. The plates were then incubated for an additional 10 minutes at room temperature and the luminescence was quantified on a luminometer. Standard methods were used to calculate drug concentrations that inhibited the proliferation of uninduced Akata cells by 50% (CC_{50}).

2.5 Immunoblots

Proteins from cells induced to undergo a lytic infection in 96-well plates from hybridization assays described above, were solubilized in Laemli buffer, separated using 10% Tris HCl gels, and transferred to PVDF membranes. Viral proteins were specifically detected with monoclonal antibodies to EA-D and VCA (Chemicon, Temecula, CA). Bound antibodies were detected with alkaline phosphatase conjugated goat anti-mouse antisera (Southern Biotech, Birmingham, AL) and visualized with CDP* (Roche, Indianapolis IN) and Biomax film (Kodak, Rochester, NY).

2.6 Antiviral Drugs

Cidofovir (CDV) was a gift of Mick Hitchcock (Gilead Sciences, Foster City, CA). Acyclovir (ACV), ganciclovir (GCV), and thymidine (THD) were obtained from a commercial source (Sigma-Aldrich, St. Louis, MO). Maribavir (MBV), cyclopropavir (CPV) and (N)-methanocarbathymidine (MCT) were described previously (Gershburg & Pagano, 2002,Kushner et al., 2003,Prichard et al., 2006), and were obtained through the NIAID, NIH, Bethesda, MD.

3. Results

3.1 Antiviral Assays

The DNA hybridization assay was validated using a set of control drugs and was conducted on different days and with trained operators. These experiments confirmed that the assay was robust and yielded EC_{50} values that were reproducible on different days and among different operators (data not shown). Resulting EC_{50} values for control drugs were also within a prescribed range based on results obtained within the laboratory and those published by others

(Zacny et al., 1999). The validated assay was then used to assess the antiviral activity of a set of control compounds with high, moderate and no activity to compare the hybridization assay in parallel with an established Real-Time PCR assay similar to one reported previously (Friedrichs et al., 2004). Control drugs included CDV, ACV, GCV, MBV and CPV with THD serving as a negative control. Each experiment yielded at least a 10-fold increase in the genome copy number indicating that the cells had been successfully induced to undergo a lytic infection and at least 20% of the cells had detectable VCA as determined by fluorescent microscopy.

Both assays produced good dose-response curves and yielded EC_{50} values that were similar. Dose response curves for representative drugs with very high, high, and moderate potency were generated by hybridization assay and Real-Time PCR (Fig. 1). These data confirm that both assays measure DNA accumulation and yield similar dose-response curves and EC_{50} values. Both assays were used to evaluate a set of drugs and a summary of results from three experiments conducted on different days are shown in Table 1. Average EC_{50} values for each drug were calculated in both assay systems and no statistical differences were observed as determined by a Student's t-test ($p > 0.15$). These results confirm that the hybridization assay performs as expected and yields results that are similar to those from a more standard assay.

3.2 Effect of drugs on VCA and EA-D in Akata cells

ELISA and FACS assays utilize monoclonal antibodies to measure expression of viral proteins. Expression of the viral proteins VCA and EA-D were assessed in western blots to correlate their expression with viral replication in Akata cells. In this system, essentially no differences were observed between induced and uninduced cells, for either VCA or EA-D (Fig. 2). This result is likely the result of lower sensitivity of the antibody based method on the limited number of cells. Thus, viral DNA proved to be a superior endpoint for evaluating antiviral activity in this assay format.

3.3 Evaluation of cytotoxicity

The effect of these drugs on cell viability was measured by both a standard MTS assay reported previously (Williams-Aziz et al., 2005), and the CellTiter-Glo assay. Both assays performed well and no toxicity was observed for CDV, ACV, and THD. Both assays detected some toxicity for CPV, MCT, and MBV with MBV exhibiting the greatest toxicity in this system (Table 1).

4. Discussion

DNA hybridization assays are very specific and are commonly used in the assessment of antiviral activity. Although these are used routinely for other viruses, a 96-well based assay for EBV has been difficult in large part because of the inefficient induction of lytic infection. The utilization of Akata cells in this assay allowed us to conduct the entire assay in 96-well plates and yielded results that were similar to other assays that typically required cells to be grown in larger volumes. The 72 hour incubation time was sufficient to detect antiviral activity, yet was short enough such that no media additions were required. This rapid assay is a single cycle assay that measures the induction of viral DNA synthesis, which is by far the most common mechanism of action of antiviral drugs. Other multi-cycle assays will be required to assess the activity of drugs that inhibit later steps in viral replication. It is also possible that this assay could be used to identify molecules that are capable of inducing a lytic infection by omitting the anti-human IgG antibody.

The hybridization assay yields results that are very similar to the Real-Time PCR assay, which was encouraging since both assays were designed to quantify viral DNA. Both assays are useful for assessing antiviral activity, but the hybridization assay has the advantage of greater

throughput and lower cost per assay. The Real-Time PCR assay is a rapid and important confirmatory assay for compounds that exhibit activity in the DNA hybridization assay. Viral DNA synthesis proved to be a better correlate of viral replication than either VCA or EA-D. These data, however, were generated in Akata cells with conditions that were highly optimized for DNA accumulation and do not suggest that the levels of these proteins do not increase during lytic infection. The poor correlation of the protein levels may reflect the lower sensitivity of the antibody based methods and it is possible that other assays could be optimized for expression of these late viral proteins.

Both cytotoxicity assays performed well in Akata cells. The conversion of MTS to the colored product is less efficient in lymphocytes compared to most other cell lines so longer incubation times are required. The CellTiter-Glo assays yield a better signal to noise ratio than MTS, but both assays yield similar CC_{50} values.

5. Conclusions

The studies presented here support the use of the DNA hybridization assay for the routine evaluation of compounds with activity against EBV. This assay is robust and yields efficacy values that are not statistically different from values obtained with a Real-Time PCR assay, as determined by Student's t test. This assay can facilitate the evaluation of large numbers of compounds against EBV and may accelerate the development of new drugs for the treatment of EBV infections.

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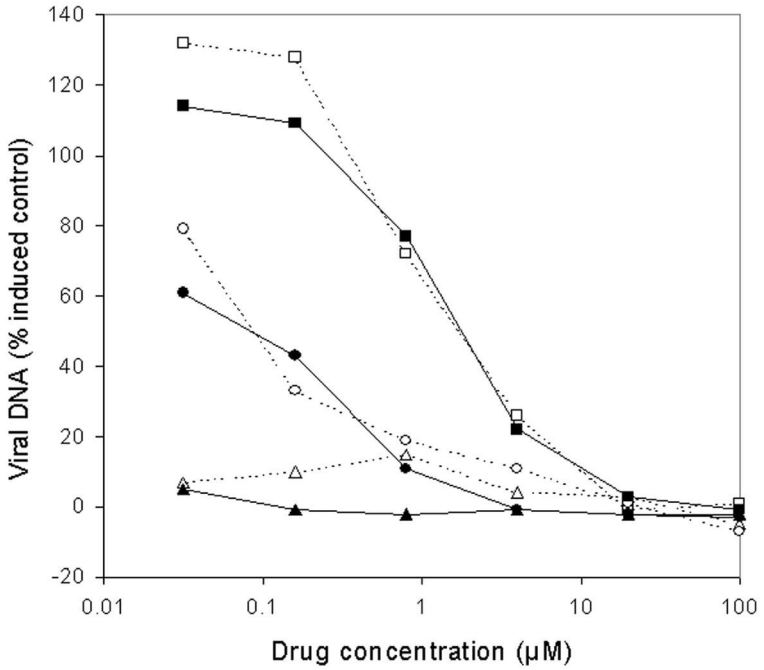

Figure 1.

Dose response curves for selected compounds as determined by hybridization assay and RealTime PCR. Antiviral activity of three selected compounds with different potencies was determined by the hybridization assay (open symbols) and by RealTime PCR (filled symbols). Dose response curves are shown for CDV (squares), CPV (circles) and (N)-MCT (triangles).

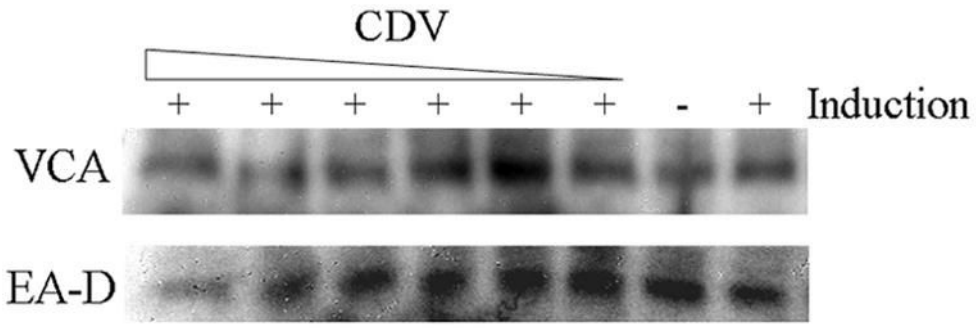

Figure 2.

Effect of CDV on EA-D and VCA. Proteins from cells induced to undergo a lytic infection and treated with CDV were separated on SDS polyacrylamide gels, transferred to PVDF membranes and VCA and EA-D were detected with monoclonal antibodies. Proteins in the first six lanes were from isolated from wells treated with 100, 20, 4, 0.8, 0.16, 0.04 μM CDV, respectively. Proteins in lanes 7 and 8 were isolated from uninduced and induced untreated Akata cells, respectively.

Table 1

Antiviral activity and cytotoxicity of selected compounds against EBV

Compound	Hybridization Assay		Real-Time PCR assay		Cytotoxicity	
	EC_{50}[a]	SI[b]	EC_{50}[a]	SI[b]	MTS[c]	CellTiter Glo[d]
CDV	2.5 ± 0.5	>40	2.6 ± 1.8	>38	>100 ± 0	>100 ± 0
ACV	5.9 ± 3.2	>17	2.0 ± 1.4	>50	>100 ± 0	>100 ± 0
MBV	0.08 ± 0.05	50	0.28 ± 0.4	14	4.0 ± 1.6	16 ± 6.4
MCT	<0.032 ± 0	>212	<0.032 ± 0	>212	6.8 ± 4.5	27 ± 20
CPV	0.22 ± 0.17	209	0.08 ± 0.04	575	46 ± 32	82 ± 5
THD	>100 ± 0	1	>100 ± 0	1	>100 ± 0	>100 ± 0

[a] Concentration required to inhibit viral DNA synthesis by 50%, in units of μM with standard deviation values shown. Results are the average of least three separate experiments conducted on different days.

[b] Selective index calculated using cytotoxicity values obtained in the MTS assay.

[c] Concentration required to inhibit proliferation of Akata cells using MTS shown in units of μM with standard deviation values.

[d] Concentration required to inhibit proliferation of Akata cells using CellTiter-Glo in units of μM with standard deviation values shown.