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Research paper

A standardized approach to the evaluation of antivirals against DNA viruses: Orthopox-, adeno-, and herpesviruses

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ABSTRACT

The search for new compounds with a broad spectrum of antiviral activity is important and requires the evaluation of many compounds against several distinct viruses. Researchers attempting to develop new antiviral therapies for DNA virus infections currently use a variety of cell lines, assay conditions and measurement methods to determine in vitro drug efficacy, making it difficult to compare results from within the same laboratory as well as between laboratories. In this paper we describe a common assay platform designed to facilitate the parallel evaluation of antiviral activity against herpes simplex virus type 1, herpes simplex virus type 2, varicella-zoster virus, cytomegalovirus, vaccinia virus, cowpox virus, and adenovirus. The automated assays utilize monolayers of primary human foreskin fibroblast cells in 384-well plates as a common cell substrate and cytopathic effects and cytotoxicity are quantified with CellTiter-Glo. Data presented demonstrate that each of the assays is highly robust and yields data that are comparable to those from other traditional assays, such as plaque reduction assays. The assays proved to be both accurate and robust and afford an in depth assessment of antiviral activity against the diverse class of viruses with very small quantities of test compounds. In an accompanying paper, we present a standardized approach to evaluating antivirals against lymphotropic herpesviruses and polyomaviruses and together these studies revealed new activities for reference compounds. This approach has the potential to accelerate the development of broad spectrum therapies for the DNA viruses.

1. Introduction

Effective antiviral therapies are available for a few DNA viruses, but no approved therapies exist for most infections with these agents. The search for new compounds with a broad spectrum of antiviral activity is important and requires the evaluation of many compounds against several viruses. A fundamental challenge to the discovery and development of new therapies for the DNA viruses is simply the assessment of antiviral activity against this set of diverse viruses, many of which are fastidious and require specialized cells and long incubation times. The analytical approach used in our laboratory includes the use of rapid primary screening assays designed to assess the specific inhibition of biologically relevant viral functions, such as the production of cytopathic effects (CPE), or the accumulation of progeny DNA in the course of low multiplicity infections. This eliminates the requirement for engineered virus strains and facilitates the evaluation of different virus strains including clinical and drug resistant isolates (Prichard, 2009; Prichard et al., 2009). Many methods have been reported for the evaluation of antiviral activity against these viruses and range from traditional plaque reduction assays, to CPE-based assays with viability indicators such as neutral red, MTT, alamar blue, and CellTiter-Glo, as well as assays utilizing reporter viruses, quantitative polymerase chain reaction (qPCR), and yield reduction methods (Chou et al., 2005; Dal Pozzo et al., 2008; Prichard et al., 1990, 2013; Tardif et al., 2014; Toth et al., 2018; Zhukovskaya et al., 2015).

Herein, we describe the assessment of a set of common reference compounds across three virus families using a common approach that evaluates the antiviral activity of compounds in monolayers of primary human foreskin fibroblast (HFF) cells. For each virus, this automated 384-well assay platform determines antiviral activity and cytotoxicity in the same assay plate to ensure identical compound exposure and measures both CPE and cell viability with CellTiter-Glo to ensure that the data are directly comparable. The efficiency and precision that this system affords ensures that the results are highly reproducible and significantly reduces the hands-on time for the analysts to reduce the potential for repetitive strain injury. This body of work documented the effectiveness of this approach across three families of DNA viruses and provided an efficient means to compare antiviral efficacy directly in the same cell type. Antiviral activity for a set of reference compounds is reported here against herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), varicella-zoster virus (VZV), cytomegalovirus (CMV), vaccinia virus (VACV), cowpox virus (CPXV), and adenovirus (AdV) and facilitated a broad assessment of activity against this set of viruses revealing novel activities.

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2. Materials and methods

Reference compounds. A set of 16 reference compounds was selected on the basis of their reported antiviral activity including: cidofovir (CDV), foscarnet (PFA), ganciclovir (GCV), acyclovir (ACV), penciclovir (PCV), fluoroiodoarabinosyl adenine (FIAU), idoxuridine (IDU), bromodichlororibobenzimidazole (BDCRB), tecovirimat (ST-246), azidothymidine (AZT), filociclovir (FCV, cyclopropavir), 4-thioidoxuridine (4-thio-IDU), N -methanocarbathymidine (N-MCT), L -Bromovinyl-Hydroxymethyl-Dioxolan Uracil (L-BHDU), adefovir (PMEA) and brincidofovir (CMX001). This set includes representative compounds that are active against a single virus or family (such as ST-246 or BDCRB), as well as those that are active against all the viruses tested (as with CDV). CDV, ACV and GCV were purchased from the University of Alabama Hospital Pharmacy, and PFA, PCV, FIAU, IDU, AZT, and PMEA were purchased from Sigma-Aldrich (St. Louis, MO). Other compounds were obtained from the following sources: BDCRB (Dr. John Drach), FCV (Dr. Terry Bowlin), L-BHDU (Dr. David Chu), N-MCT (Drs. Robert Glazer and Victor Marquez), 4-thio-IDU (Dr. Jack Secrist), ST-246 (Dr. Dennis Hruby), and CMX001 (HDP-CDV from Dr. Karl Hostetler).

Preparation and culture of human foreskin fibroblast (HFF) cells. The preparation of HFF cells was described by methods published previously (Prichard et al., 2013) and were derived from human foreskin tissue obtained from the University of Alabama at Birmingham tissue procurement facility with approval from the Institutional Review Board. Briefly, the tissue was stored at 4 °C in cell culture medium consisting of minimum essential media (MEM) with Earle's salts supplemented with 10% fetal bovine serum (FBS; HyClone, Inc., Logan, UT) and standard concentrations of L-glutamine, amphotericin B (Fungizone), and vancomycin. The tissue was then placed in a phosphate-buffered saline solution, minced, and rinsed to remove the red blood cells. Tissue fragments were then resuspended in a trypsin-EDTA solution and incubated at 37 °C to disperse the cells, which were then collected by centrifugation. Cell pellets were then resuspended in 4 ml culture medium, placed in a 25-cm^2 tissue culture flask, and incubated at 37 °C for 24 h. The culture medium was then replaced with fresh medium, and condition of the cells was monitored daily until a confluent cell monolayer was formed. The HFF cells were then expanded through serial passages in standard growth medium of MEM with Earle's salts supplemented with 10% FBS, L-glutamine, penicillin, and gentamicin. Each lot of cells is confirmed to be free of mycoplasma infection and routinely passaged or used for assays at or before passage 10.

Virus strains. The HSV-2 strain G (VR-734), VZV strain Ellen (VR-1367), CMV strain AD169 (VR-538) and AdV5 (VR-1516) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The Copenhagen strain of VACV and CPXV strain Brighton, were kindly provided by John W. Huggins (Department of Viral Therapeutics, Virology Division, United States Army Medical Research Institute of Infectious Disease). The E-377 stain of HSV-1 was a gift of Dr. Jack Hill, Burroughs Wellcome.

Antiviral Assays: Antiviral and cytotoxicity data were obtained in a series of three to five separate experiments for each virus to provide an accurate estimate of antiviral activity and statistical data. Each assay included positive and negative control compounds as well as infected and uninfected controls to ensure the integrity of the experiment. The concurrent assessment of cytotoxicity was performed in each assay plate using the same number of cells and equivalent levels of compound exposure so that accurate selective index (SI) values could be obtained. All liquid handling steps were performed on a BioMek 4000 and significantly increased the efficiency of the assays and reduced hands-on time of analysts.

The CPE reduction assays were performed in monolayers of HFF cells in 384-well plates maintained in assay media consisting of MEM with Earle's salts, 2% FBS and standard concentrations of L-glutamine, penicillin and gentamycin. Briefly, 5000 cells were seeded into 384-well microtiter plates and were subsequently incubated at 37 °C in a humidified 5% CO_2 incubator for 24 h to allow the formation of confluent monolayers. Dilutions of test compounds were prepared directly in the plates in a series of 5-fold dilutions in duplicate wells to yield final concentrations that range from 300 to 0.1 μM or from 10 to 0.003 μM depending of the efficacy of the compounds. This compound dilution scheme provides a large dynamic range of compound concentrations to facilitate the detection of antiviral activity of unknown compounds with either weak or potent antiviral activity.

Cell monolayers were infected at an MOI of approximately 0.005 PFU/cell with HSV-1 or -2, CMV, VACV, CPXV, or AdV5 or with approximately 200 VZV-infected HFF cells by methods similar to those we have reported previously (Kern et al., 2009; Prichard et al., 2009; Toth et al., 2018). Infected cells were then incubated further at 37 °C until 100% CPE was observed in the virus control wells. Cytopathology was determined by the addition of CellTiter-Glo reagent (Promega, Madison, WI). Concentrations of test compounds sufficient to reduce CPE by 50% (EC_{50}) were interpolated from the experimental data using standard methods in Microsoft excel. Cytotoxicity was also determined with CellTiter-Glo and concentrations of the compounds that decreased cell viability by 50% (CC_{50}) were also calculated from the data and SI values were calculated as the CC_{50}/EC_{50} as a measure of antiviral activity. Data from different experiments performed on different days were used to calculate average EC_{50} and CC_{50} values as well as standard deviation values. When values did not vary, as in the case of toxicity being > 300 μM in each of 5 determinations, there was no standard deviation and was indicated with a value of zero by convention.

3. Results

An overarching goal of this series of studies was to assess the antiviral activity of a set of reference compounds and characterize the performance of the assay platform described here. Results were compared to those reported in the literature as well as with established assays in our laboratory to gauge the accuracy of the efficacy. Specific objectives included the evaluation of a set of 15 reference compounds against each of the 7 viruses and included compounds with known antiviral activity as well as those that were reported to be ineffective against some viruses. Data from all the studies are summarized graphically and are shown in Figs. 1 and 2 to present results in a format that allows a rapid comparison of the spectrum of antiviral activity for all the compounds. Salient results are as follows: i) each of the assays could detect compounds with specific antiviral activity, ii) some compounds, such as CDV, exhibited broad spectrum antiviral activity and inhibited every virus tested, iii) some compounds, such as BDCRB or ST-246, were highly specific and inhibited a single virus or a single family of viruses, and iv) each of the assays yielded data that were accurate and reproducible between experiments performed on different assay days.

Results from individual assays are summarized in Tables 1–8 to provide a statistical analysis of the accuracy and reproducibility of the EC_{50} and CC_{50} values and compare them to values reported in the literature. The level of variation observed with the current assay was acceptable and generally superior to that yielded in our laboratory from CPE assays utilizing crystal violet or neutral red dyes (data not presented). Most of the EC_{50} values cited in the literature were derived from plaque reduction assays and oftentimes used alternate cell substrates. Thus, values obtained in the CPE assays were expected to be close to results in the literature, but not the same. Nonetheless, the automated 384-well assays yielded values that were typically very close to those values reported in the literature and indicated that the assays could estimate accurately EC_{50} and CC_{50} values to determine the relative potency of test compounds.

Evaluation of Antiviral Activity against HSV-1 and HSV-2. The set of 15 control compounds was evaluated against HSV-1 and HSV-2 in