Antiviral Research 113 (2015) 49–61

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Development of a high-content screen for the identification of inhibitors directed against the early steps of the cytomegalovirus infectious cycle

Thomas J. Gardner [a,1], Tobias Cohen [a,1], Veronika Redmann [a,2], Zerlina Lau [b], Dan Felsenfeld [b], Domenico Tortorella [a,*]

[a] Icahn School of Medicine at Mount Sinai, Department of Microbiology, New York, NY 10029, USA
[b] Icahn School of Medicine at Mount Sinai, Integrated Screening Core, Experimental Therapeutics Institute, One Gustave L. Levy Place, New York, NY 10029, USA

ARTICLE INFO

Article history:
Received 16 September 2014
Revised 16 October 2014
Accepted 20 October 2014
Available online 31 October 2014

Keywords:
Human cytomegalovirus
High throughput
Therapeutics
Small-molecule screen
Antivirals
Cardiac glycoside

ABSTRACT

Human cytomegalovirus (CMV) is a latent and persistent virus whose proliferation increases morbidity and mortality of immune-compromised individuals. The current anti-CMV therapeutics targeting the viral DNA polymerase or the major immediate-early (MIE) gene locus are somewhat effective at limiting CMV-associated disease. However, due to low bioavailability, severe toxicity, and the development of drug resistant CMV strains following prolonged treatment, current anti-CMV therapeutics are insufficient. To help address this shortfall, we established a high-content assay to identify inhibitors targeting CMV entry and the early steps of infection. The infection of primary human fibroblasts with a variant of the CMV laboratory strain AD169 expressing a chimeric IE2-yellow fluorescence protein (YFP) ($AD169_{IE2\text{-}YFP}$) provided the basis for the high-content assay. The localization of IE2-YFP to the nucleus shortly following an $AD169_{IE2\text{-}YFP}$ infection induced a robust fluorescent signal that was quantified using confocal microscopy. The assay was optimized to achieve outstanding assay fitness and high *Z'* scores. We then screened a bioactive chemical library consisting of 2080 compounds and identified hit compounds based on the decrease of fluorescence signal from IE2-YFP nuclear expression. The hit compounds likely target various cellular processes involved in the early steps of infection including capsid transport, chromatin remodeling, and viral gene expression. Extensive secondary assays confirmed the ability of a hit compound, convallatoxin, to inhibit infection of both laboratory and clinical CMV strains and limit virus proliferation. Collectively, the data demonstrate that we have established a robust high-content screen to identify compounds that limit the early steps of the CMV life cycle, and that novel inhibitors of early infection events may serve as viable CMV therapeutics.

1. Introduction

Human Cytomegalovirus (CMV) is the prototypic member of the *Betaherpesvirinae* subfamily. The coevolution of CMV with humans over the past millions of years has led to the development of complex and elegant methods of immune evasion that have allowed the virus to infect a majority of the human population. Indeed, CMV causes latent and persistent infections in 60–90% of individuals (Mocarski and Pass, 2007).

While most CMV carriers are asymptomatic, virus proliferation significantly increases morbidity and/or mortality in immunocompromised individuals. This includes newborns, organ transplant recipients, AIDS patients, and the elderly. CMV is clearly a major medical problem affecting a significant number of individuals. To date, very few drugs have been developed for either prophylactic, pre-emptive, or direct treatment of CMV disease (ganciclovir, foscarnet, cidofovir, fomivirsen, and high-dose acyclovir) (Mercorelli et al., 2011). Although these drugs have some efficacy against CMV infection (Steininger, 2007), toxicity, drug-drug interactions, and drug resistance are common limitations (Chou et al., 2008). With the exception of fomivirsen, all approved anti-CMV therapeutic compounds target the viral DNA polymerase (Razonable, 2011) leading to high toxicity due to off–target effects on the host DNA polymerase. Therefore, more potent and diverse anti-CMV therapies are clearly warranted.

The early stage of a CMV infection includes virus attachment and entry followed promptly by expression of the immediate early gene products. The Major Immediate Early transcript (MIE) is generated shortly following infection from spliced products creating

* Corresponding author at: Department of Microbiology, Mount Sinai School of Medicine, One Gustave L. Levy Place, Box 1124, New York, NY 10029, USA.
[1] Authors contributed equally.
[2] Washington University, School of Medicine, Department of Pathology and Immunology, St. Louis, MO 63130, USA.

http://dx.doi.org/10.1016/j.antiviral.2014.10.011
0166-3542/

the IE1 and IE2 gene products. IE2 is a transactivator for early viral proteins (Cherrington and Mocarski, 1989; Meier and Stinski, 1997) that can complex with host factors of the cellular transcription machinery. IE1 stimulates a variety of viral and cellular promoters through its ability to interact with multiple transcriptional regulators, disrupt repressive nuclear architecture, and work synergistically with IE2 to activate viral promoters (Meier and Stinski, 1997). These are essential viral gene products whose expression is a critical first step for transcription of the CMV genome. Therefore, assessment of IE gene expression can serve as a rapid proxy for the measurement of CMV infection. We and others have utilized CMV virus strains encoding fluorescent chimeric proteins to measure IE expression in infected cells (Gardner et al., 2013; Straschewski et al., 2010).

In this study, we employed a CMV strain that expresses a yellow fluorescent protein (YFP), fused to the IE2 transcript ($AD169_{IE2\text{-}YFP}$), to develop a sensitive and specific assay to identify inhibitors of virus infection. The assay, which is cell-based and multiparametric, represents a high-content approach to identify antiviral compounds (Gasparri, 2009). Based on fluorescent protein expression in CMV-infected fibroblasts, we identified and validated a panel of small molecule compounds that block diverse, early events of CMV infection *in vitro*. Secondary assays confirmed the inhibition of viral gene expression when cells were treated with the hit compounds. Additionally we show that our lead compound, the cardiac glycoside convallatoxin, was capable of limiting CMV proliferation and production of infectious virus. Together, we present a rapid, high-content platform for identifying agents that target the early events of a CMV infection.

2. Materials and methods

2.1. Cell lines, antibodies, viruses and chemicals

Human lung fibroblasts (MRC5) (ATCC, Manassas, VA) were cultured in complete Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U of penicillin–streptomycin/ml and 100 U of HEPES/ml at 37 °C in a humidified incubator with 5% CO_2. CMV $AD169_{IE2\text{-}YFP}$ and $TB40/E_{FLAG\text{-}YFP}$ were propagated as previously described (Gardner et al., 2013). Infectious virus yield was assayed on human fibroblasts by median tissue culture infective dose ($TCID_{50}$). The $TCID_{50}$ results were used to estimate infectious particles/ml to infect cells at the desired multiplicity of infection (MOI). Cycloheximide was purchased from MP Biomedicals, Solon, Ohio (Cat. No. 100183).

2.2. High-content screen

MRC5 cells were seeded and treated the following day for one hour with controls and samples. Virus was added to the cells and adsorbed for 1 h at 37 °C, spin-infected at 1200 rpm for one hour at room temperature and returned to 37 °C incubator. At 16 h post infection (hpi), cells were fixed with 4% Paraformaldehyde (PFA) for 30 min at 4 °C. Cells were stained with 25 μg/mL Hoechst in phosphate buffered saline (PBS) with 1% bovine serum albumin (BSA) and 0.1% saponin for one hour at RT. Cells were then washed twice with PBS and images were collected using Channel 1 (excitation at 405 nm; emission detection at 442–447 nm) and Channel 2 (excitation at 488 nm; emission detection at 502–548 nm) to detect DAPI and YFP respectively on a Molecular Devices ImageXpress Ultra (IXU) plate-scanning confocal microscope. Positive cells were identified based on the following criteria: primary stain (nuclei) between 6 and 21 μm and at least 4500 gray levels above local background and secondary stain (IE2-YFP) was only present in the nucleus, between 6 and 21 μm and at least 1000 gray levels above background. MetaXpress software (Molecular Devices, Sunnyvale, CA) was used to calculate the fluorescent intensity (W2 stained average intensity/well). These values were utilized to determine Robust *Z*-scores (Zhang et al., 1999). The median fluorescent intensity value was calculated for each plate (excluding controls), and used to determine the % fluorescent intensity of each well.

Optimal cell plating density, positive control concentration and MOI were determined by pilot assays testing the following ranges for each parameter: 500–3000 cells/well, cycloheximide concentrations of 0.5–50 μg/μL and MOI 0.5–15. The *Z′* factor as described in (Zhang et al., 1999) was used to evaluate assay fitness using the equation $Z' = 1 - [(3\sigma_{c+} + 3\sigma_{c-})/(|\mu_{c+} - \mu_{c-}|)]$. The ideal conditions were determined to be 3000 cells/well at an MOI of 0.5, with a positive control (cycloheximide) concentration of 9.8 μM (samples measured in quadruplicate).

The bioactive chemical library at the Mount Sinai Integrated Screening Core was purchased from Microsource Discovery (Gaylordsville, CT) and consisted of 2080 total compounds. Aurora 384 black well plates (Brooks Automation, Chelmsford, MA) were seeded with 3000 cells/well and maintained at 37 °C, 5% CO_2 overnight. The following day the respective chemical compound source plate (final 5.6 μM) and controls (cycloheximide, final 9.8 μM) were transferred by pin tool (V & P Scientific) into previously-seeded plates (in duplicate) and incubated for one hour at 37 °C. Cells were then mock-infected or infected with $AD169_{IE2\text{-}YFP}$ at an MOI of 0.5 for one hour at 37 °C and then spin-infected at 1200 rpm for one hour at RT. Plates were then returned to the incubator and fixed and stained using Hoechst reagent (25 μg/mL) at 16 h post infection. Each library source plate was screened in duplicate. Images of four fields/well were acquired by the IXU plate-scanning confocal microscope. Image segmentation and analysis was carried out using MetaXpress.

2.3. Determining half maximal effective concentration (EC50) of hit compounds

MRC5 cells (10,000 in 100 μL) were plated in each well of a 96-well plate (Greiner, Monroe, NC). The following day, media was replaced with DMEM with varying concentrations (0–5 μM) of the compounds (final 100μL) in sextuplicate (1 h @ 37 °C) and then cells were infected with $AD169_{IE2\text{-}YFP}$ (MOI:3). Following a one hour inoculation cells were washed with media and then fresh media without drug was added back to the wells. At 18 hpi, the plates were analyzed with an Acumen ^{e}X3 cytometer for the number of IE2-YFP positive cells/well based on IE2-YFP fluorescent intensity/well (Gardner et al., 2013). Fluorescent emission above 2 standard deviations of background was registered as positive signal. Any fluorescent signal larger than 5 μM, smaller than 300 μM, and separated from any other emission by at least 0.5 μM in both *x* and *y* axes was classified as an "event". Using DMSO treated cells (0.1%) infected with $AD169_{IE2\text{-}YFP}$ as 100% infection, the percent infection of cells pre-treated with increasing amounts of compound was determined. For $TB40/E_{FLAG\text{-}YFP}$ infections, ARPE-19 cells were plated (10,000 in 100 μL) as above. The following day, media was replaced with DMEM with varying concentrations (0–10 nM) of convallatoxin (final 100 μL) in sextuplicate and then cells were infected with $TB40/E_{FLAG\text{-}YFP}$. At 120 hpi, plates were analyzed with an Acumen ^{e}X3 cytometer as above. The EC50 values were calculated using Prism 5's nonlinear fit log (inhibitor) vs. response (three parameters) analysis of the average % infection values.

2.4. Determining half maximal toxicity concentration (CC50)

MRC5 or ARPE-19 cells (10,000 in 100 μL) were plated in each well of a 96-well plate. The following day, media was replaced with DMEM with varying concentrations (0–10 μM) of the compounds