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An interferon-beta promoter reporter assay for high throughput identification of compounds against multiple RNA viruses

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ABSTRACT

Virus infection of host cells is sensed by innate pattern recognition receptors (PRRs) and induces production of type I interferons (IFNs) and other inflammatory cytokines. These cytokines orchestrate the elimination of the viruses but are occasionally detrimental to the hosts. The outcomes and pathogenesis of viral infection are largely determined by the specific interaction between the viruses and their host cells. Therefore, compounds that either inhibit viral infection or modulate virus-induced cytokine response should be considered as candidates for managing virus infection. The aim of the study was to identify compounds in both categories, using a single cell-based assay. Our screening platform is a HEK293 cell-based reporter assay where the expression of a firefly luciferase is under the control of a human IFN-β promoter. We have demonstrated that infection of the reporter cell line with a panel of RNA viruses activated the reporter gene expression that correlates quantitatively with the levels of virus replication and progeny virus production, and could be inhibited in a dose-dependent manner by known antiviral compound or inhibitors of PRR signal transduction pathways. Using Dengue virus as an example, a pilot screening of a small molecule library consisting of 26,900 compounds proved the concept that the IFN-β promoter reporter assay can serve as a convenient high throughput screening platform for simultaneous discovery of antiviral and innate immune response modulating compounds. A representative antiviral compound from the pilot screening, 1-(6-ethoxybenzo[d]thiazol-2-yl)-3-(3-methoxyphenyl) urea, was demonstrated to specifically inhibit several viruses belonging to the family of *flaviviridae*.

1. Introduction

Viral infectious diseases remain to be a major public health challenge worldwide. While significant progress has been made during the last two decades in the development and clinical application of antiviral drugs against several medically important viruses, including human immunodeficiency virus (HIV), hepatitis B virus (HBV) and hepatitis C virus (HCV) (Fung et al., 2011; Jilek et al., 2012; Yang et al., 2011), antivirals against many RNA viruses that cause hemorrhagic fevers and respiratory tract diseases are either limited in efficacy or not yet available (Chang et al., 2013a). To fulfill this unmet medical need, we intended to develop convenient assays for high-throughput discovery of compounds that either inhibit the replication of the viruses or alleviate their pathogenesis, which are usually resulted from uncontrolled cytokine response to viral infection and sometime life-threaten.

To achieve this goal, we have designed and implemented a cell-based high throughput assay that can be applied to many types of viruses and is suitable for simultaneous identification of compounds that either inhibit viral replication or modulate virus-induced cytokine response. The principle of the assay is based on the innate immune recognition of virus infection by host innate pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), NOD-like receptors, C-type lectins and many others (Takeuchi and Akira, 2010). Activation of

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PRRs by molecular patterns of viruses, such as viral nucleic acids, oligomers of envelope proteins and nucleocapsids, induces cellular responses leading to production of type I interferons (IFN), proinflammatory cytokines and chemokines (Takeuchi and Akira, 2008, 2010). Because activation of IFN response is a common feature of many different viruses, we established a HEK293 cell-based reporter system where expression of a firefly luciferase gene is under the control of human IFN-β promoter to quantitatively monitor the virus activated host innate immune response. Specifically, the stable cell line used in this study, 293TLR3/IFNβLuc, has intrinsic RLR pathway and a reconstituted TLR3 pathway, which are primary PRRs for many RNA viruses (Guo et al., 2011; Loo and Gale, 2011; Negishi et al., 2008), and therefore should be responsive to the infection of these viruses.

Indeed, we have demonstrated that infection of 293TLR3/IFNβLuc cell line with RNA viruses from five different families efficiently activated IFN-β promoter driven luciferase production. Interestingly, because the levels of reporter gene expression upon the virus infection were quantitatively correlated with the levels of virus replication and progeny virion production, we have thus speculated and subsequently demonstrated that the level of luciferase expression in virally infected cells can serve as a simple and quantitative readout of viral replication activity in a high throughput manner. As we anticipated, a pilot screening of 26,900 compounds convincingly demonstrated that the IFN-β promoter reporter assay can be used as a broadly applicable platform for discovery of compounds with antiviral activity against many different viruses as well as compounds that inhibit virus-induced cytokine response. A representative antiviral compound from the pilot screening, 1-(6-ethoxybenzo[d]thiazol-2-yl)-3-(3-methoxyphenyl) urea, was characterized in detail and demonstrated to specifically inhibit several viruses belonging to the family of *flaviviridae*.

2. Materials and methods

2.1. Cell culture, viruses and reagents

Huh7.5 cells were maintained in Dulbecco's modified minimal essential medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (Blight et al., 2002). TLR3-expressing HEK293 (293TLR3HA, Invivogen)-derived stable reporter cell line that expresses firefly luciferase under the control of a human IFN-β promoter (293TLR3/IFNβLuc) was established and cultured as described previously (Guo et al., 2012). Dengue virus (DENV, serotype 2, New Guinea C), yellow fever virus (YFV, strain 17D), encephalomyocarditis virus (EMCV), Tacaribe virus (TCRV, strain TRVL-11,573) and HCV (genotype 2a, JC1 strain) were described previously (Chang et al., 2011, 2009, 2013b; Jiang et al., 2010; Qu et al., 2011; Zhou et al., 2011). Sendai virus (SenV, strain 52) was purchased from ATCC. HCV subgenomic replicon-containing Huh7 cell line (GS4.1) was described previously (Guo et al., 2001).

IHVR17028, an imino sugar compound with known anti-DENV activity (Chang et al., 2013b; Du et al., 2013), was synthesized in house with >95% purity. Compound benzothiazolylphenyl urea (BPU) was purchased from ChemDiv. IFN-α2b was from PBL, Inc.

2.2. Luciferase reporter assay

293TLR3/IFNβLuc cells were seeded in black wall/clear bottom 96-well plate (Corning Inc.). The firefly luciferase activities, under different experimental conditions, were measured by adding equal volume of Steady-Glo reagent (Promega), followed by luminometry in a TopCounter (Perkin Elmer).

2.3. High throughput screen (HTS)

A library containing 26,900 small molecular compounds from ChemDiv (Dougherty et al., 2007) was screened. 293TLR3/IFNβLuc cells were seeded in 96-well plates at 4×10^4/well and cultured overnight. For each plate, column 1 wells were mock-infected and served as uninfected controls. The column 12 wells were infected with DENV at a multiplicity of infection (MOI) of 0.1, with four of the wells treated with 1% DMSO to serve as mock treated controls, which had no effect on readout compared to that without DMSO (data not shown), and the remaining four wells treated with 10 μM of IHVR17028 to serve as positive drug treatment controls. Each of the remaining 80 wells in columns 2–11 were infected with DENV at an MOI of 0.1 and treated with a library compound at 10 μM with 1% DMSO. The treatment was initiated immediately following infection. The luciferase activities were determined at 60 h post infection. Although the assay should be liquid-handling compatible, due to the use of infectious virus, the HTS was performed manually using multi-channel pipette. Typically, fifty of 96-well plates containing 4000 of library compounds were tested in a single experiment. In addition, a control plate, with half of the plate infected with DENV at MOI of 0.1 and half of the plate uninfected, was included in each experiment. Z' factor for each plate was calculated using data from infected control wells and uninfected control wells, which ranged from 0.15 to 0.7. Z' factor for each experiment was calculated based on parameters obtained from control plate, which ranged from 0.4 to 0.8.

The compounds that reduced luciferase activity by 75% of the mock treated controls, and/or demonstrated the same or superior potency in comparison with the positive control drug IHVR17028, were scored as primary "hits". The primary "hits" were subjected to further evaluation of dose-dependent effect on luciferase activity in DENV-infected 293TLR3/IFNβLuc reporter cells as well as cytotoxicity in uninfected cells by a MTT assay (Promega). The compounds that dose-dependently reduced DENV-induced luciferase expression with EC_{50} values of less than 10 μM, but reduced cell viability less than 25% at 10 μM were considered as confirmed "hits". All the confirmed "hits" were evaluated with an In-cell western assay to identify compounds that inhibit DENV infection in a human hepatoma cell line, Huh7.5. These compounds with antiviral activity against DENV were further tested for their activities on EMCV and TCRV induced IFN-β reporter expression in 293TLR3/IFNβLuc cells to determine their antiviral spectrum. The confirmed "hits" that did not inhibit the virus infection are considered as candidates of the innate immune pathway inhibitors.

2.4. In-cell western assay

Antiviral activity against DENV and YFV was evaluated with an In-cell western assay essentially as described previously (Jiang et al., 2010; Yu et al., 2012). Briefly, Huh7.5 cells in a 96-well plate were infected with DENV or YFV at an MOI of 0.1, and mock-treated (1% DMSO) or treated with a serial dilution of compound for 48 h. Cells were fixed and incubated with a mouse monoclonal antibody (4G2, Millipore) against flavivirus envelope (E) proteins, followed by incubation with anti-mouse IRDye 800CW-labeled secondary antibody together with two reagents for cell staining (DRAQ5 from Biostatus and Sapphire700 from Li-COR). The DENV or YFV E protein was visualized in Li-COR Odyssey in 800 channel as green. The cell viability was determined in 600 channel as red. The fluorescence signal intensity was quantified with Li-COR Odyssey.

2.5. Virus yield reduction assay

To determine DENV yield, 293TLR3/IFNβLuc cells or Huh7.5 cells were infected with DENV at an MOI of 0.1 for 1 h. After