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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

removal of the inoculums, the cells were treated as indicated and incubated at 37 °C. Virus titers in the culture media were determined by using monolayer of Vero cells infected with 10-fold dilutions of the media for 1 h, followed by overlay with media containing 0.5% methylcellulose and incubation at 37 °C for 3–5 days. Viral foci were detected by immunostaining with 4G2 antibody (Millipore) or counting of the plaques after crystal violet staining (Chang et al., 2013b).

2.6. Cytotoxicity assay

To determine the cell viability, a MTT assay (Sigma) was performed as described previously (Chang et al., 2009). Cells were set up and incubated with various concentrations of compounds under condition that was identical to that used for antiviral and luciferase assays, except that the cells were not infected.

2.7. Real time RT-PCR assay to detect intracellular HCV RNA

Total cellular RNA was extracted from Huh7.5 cells 4 days post infection with HCV at MOI of 0.01, using TRIzol (Invitrogen). Quantitative RT-PCR (qRT-PCR) reaction was performed on LightCycler 480II (Roche) using probe and primers as described previously (Qu et al., 2011).

2.8. Immunofluorescent staining

HCV infected Huh7.5 cells were fixed with PBS containing 1% paraformaldehyde followed by incubation with 0.1% Triton X-100 for 20 min. Cells were then blocked and incubated with a monoclonal antibody against HCV NS5A protein (kindly provided by Dr. Chen Liu, University of Florida). Bound primary antibody was visualized by Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen) (Zhu et al., 2003).

2.9. Western blot assay

For detection of HCV NS5A, cells were lysed with Laemmli buffer. Proteins in the lysates were resolved by electrophoresis in 10% Tris–glycine gel and transferred onto nitrocellulose membrane (Invitrogen). The membranes were blocked and probed with antibodies against HCV NS5A (a gift of Dr. Chen Liu, University of Florida) or β-actin (Sigma) followed by incubation with IRDye secondary antibodies and imaging with Li-COR Odyssey system (Li-COR Biotechnology).

2.10. Northern blot hybridization

Total cellular RNA was extracted from GS4.1 cells with TRIzol (Invitrogen). Six micrograms of total RNA was loaded on 1% agarose gel containing 2.2 M formaldehyde and transferred onto nylon membrane. Membrane was hybridized with riboprobe specific for plus-stranded HCV replicon RNA, as described previously (Chang et al., 2008).

2.11. HCV pseudotype particle (HCVpp) assay

Plasmid pNL4.3.Luc.R$^-$E$^-$, in which firefly luciferase gene was inserted into PNL4.3 nef gene with two frameshifts rendering Env- and Vpr-, was obtained through the NIH AIDS Research and Reference Reagent Program (Connor et al., 1995; He et al., 1995). Plasmid expressing HCV (Con-1 strain) envelope proteins E1 and E2 (pcDNA3/HCV-E1E2) was constructed largely as previously reported (Tong et al., 2011). pCMV-VSV-G plasmid expressing vesicular stomatitis virus (VSV) G envelop protein was obtained from Addgene. HCV envelope E1/E2 protein- and VSV G protein-pseudotyped lentiviral particles, designated as HCVpp and VSVpp, respectively, were packaged in 293T cells. Briefly, 2×10^6 293T cells were seeded on 100 mm plate over night. Cells were co-transfected with 20 μg of pNL4.3.Luc.R$^-$E$^-$ together with 10 μg of pcDNA3/HCV-E1E2 or pCMV-VSV-G using calcium phosphate precipitation procedure. Twenty-four hours post transfection the cells were replenished with complete DMEM. Culture media were harvested at 48 h (HCVpp) or 72 h (VSVpp) post transfection and filtered through a 0.45 μm pore sized PES filter (Millipore). To determine the effects of the "hit" compound on HCV entry, Huh7.5 cells were seeded at 2×10^4 cells/well in a 96-well plate over night. The cells were infected with the pseudotyped particles in absence or presence of test compound at 37 °C for 4 h, and the inoculum and compound were then removed and replaced with complete medium. Three days post infection, luciferase activity was determined as described above.

2.12. Data analysis

Z′ factor (Z′) was calculated using the following equation: $Z' = 1 - ((3\delta_p + 3\delta_n)/|\mu_p - \mu_n|)$, where μ_p and μ_n represent mean values of uninfected control wells and infected control wells; δ_p and δ_n are the standard deviations (Zhang et al., 1999). Coefficient of variation (CV) was calculated using the following equation: $CV(\%) = \delta/\mu * 100$. The correlation of replicate plates was assessed by pair wise Pearson correlation analysis.

3. Results

3.1. Activation of the IFN-β promoter by several species of RNA viruses is quantitatively correlated with the size of the viral inoculum

Cytoplasmic RLRs and endosomal TLR3 are the primary PRRs for several RNA viruses (Loo and Gale, 2011; Negishi et al., 2008). In order to quantitatively measure the TLR3 and RLR-mediated innate antiviral immune response, a TLR3-expressing HEK293 cell line stably expressing a firefly luciferase reporter gene under the control of a human IFN-β promoter (Li et al., 2005), designated 293TLR3/IFNβLuc, was established (Guo et al., 2012). Because the cell line has an intact intrinsic RLR pathway and a reconstituted TLR3 pathway (Guo et al., 2012; Yoneyama et al., 2005), the reporter gene should respond to infection by viruses that are able to activate one or both of the pathways. Indeed, as shown in Fig. 1, infection of the cell line with DENV, YFV, TCRV, EMCV or SenV induced luciferase expression in an inoculum size (MOI)- or hemagglutination units (HAU)-dependent manner.

3.2. DENV activation of the IFN-β promoter is quantitatively correlated with its replication level and is dose-dependently inhibited by known antiviral compound

Having demonstrated that the IFN-β promoter reporter signals were proportional to the sizes of the viral inoculums (Fig. 1), we next determined the relationship between the levels of viral replication and reporter gene expression, using DENV as an example. 293TLR3/IFNβLuc cells were infected with DENV at an MOI of 0.1. Luciferase activity and virus titer were determined at 24, 36, 48 and 60 h post infection. As shown in Figs. 2A and B, while UV inactivated-DENV did not induce detectable IFN-β promoter activation, the levels of luciferase expression in live DENV infected cells were quantitatively correlated with virus yields. These results indicated that the virus induction of IFN-β gene expression not only relies on active viral replication, but also reports the levels of viral replication with sufficient dynamic range and therefore,

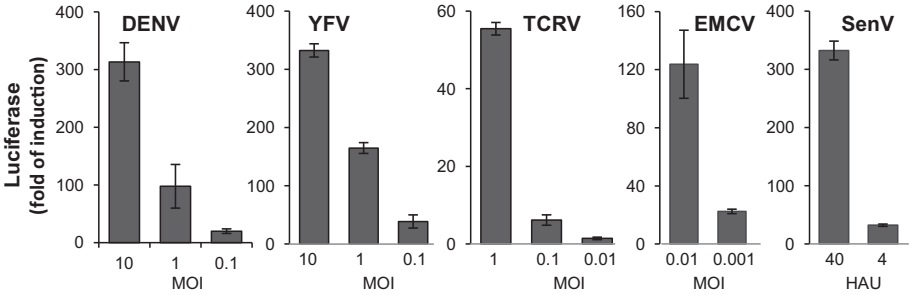

Fig. 1. Activation of IFN-β promoter in 293TLR3/IFNβLuc cells by a panel of RNA viruses. 293TLR3/IFNβLuc cells grown in 96-well plates were mock infected, or infected with indicated amount of DENV, YFV, TCRV for 48 h, or EMCV, SenV for 24 h. At least three independent experiments were performed for each virus. IFN-β promoter activities, from one representative experiment, were expressed as fold of induction of luciferase activity compared to mock infected controls (mean ± standard deviation, $n \geqslant 4$).

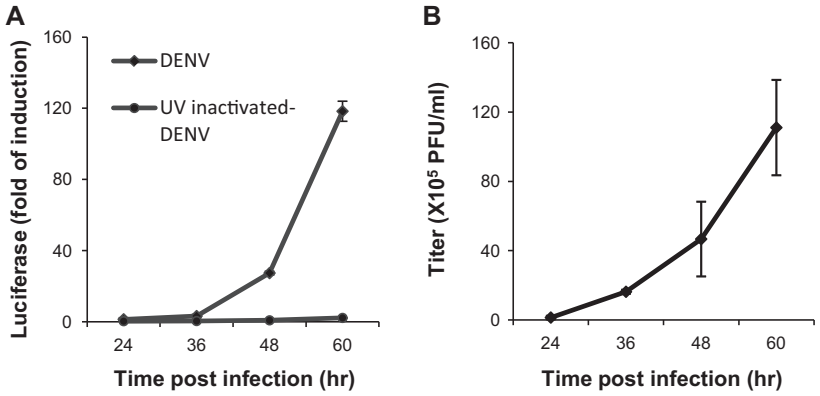

Fig. 2. DENV induced IFN-β promoter activation is quantitatively correlated with virus yield. 293TLR3/IFNβLuc cells grown in 96-well plates were infected with DENV or UV inactivated-DENV at MOI of 0.1 for 1 h. Cells were harvested at the indicated time points post-infection.(A) luciferase activity, from one of the two independent experiments, was expressed as fold of induction compared to mock infected control (mean ± standard deviation, $n = 8$). (B) Virus titer in DENV infected cell culture media was determined by plaque assay and expressed as PFU/ml (mean ± standard deviation, $n = 3$).

could potentially be used as a convenient antiviral assay to measure antiviral activity of compounds.

To confirm this notion, 293TLR3/IFNβLuc cells were infected with DENV and treated with a serial dilution of imino sugar compound IHVR17028, which inhibits virion particle assembly and secretion of DENV through inhibition of glycosylation and maturation of viral envelop protein (E protein) (Chang et al., 2013a,b). As a control, Huh7.5 cells infected with DENV was similarly treated, and DENV E protein was determined by In-cell western assay at 48 h post infection. As shown in Figs. 3A and B, IHVR17028 inhibited both luciferase expression in 293TLR3/IFNβLuc cells and viral E protein in Huh7.5 cells in a dose dependent manner, which resulted in EC_{50} values of 3.2 and 4.1 μM, respectively. These results have thus validated that the IFN-β reporter assay could be used as a convenient antiviral assay for DENV and potentially other viruses that are able to activate IFN response in this cell line.

3.3. Assay optimization and quality control for HTS

The results presented above indicate that the IFN-β promoter reporter assay can quantitatively report viral infection. Hence, it could potentially be developed into a cell-based high throughput screening assay for identification of antiviral compounds or compounds that inhibit virus-induced cytokine response. To optimize the performance of the assay in a high throughput format, we initially set out to determine the key parameters that might affect the performance of the assay. A low MOI of infection was preferred because that will allow for multiple rounds of viral infection during the experimental period and thus permit the identification of antiviral compounds interfering with any step of viral replication cycle. As shown in Table 1, under the condition of DENV infection at an MOI of 0.1 and cell density at 4×10^4 cells/well, the highest signal-to-background ratio (S/B), a Z' value of 0.75 and CV(%) of 7.9 were achieved. Because infection at an MOI of 0.1 resulted in a steady increase of luciferase S/B ratios before the development of cytopathic effect (Fig. 2A and data not shown), our HTS experiments were chosen to carry out under those conditions for 60 h.

To evaluate the performance of the HTS assay, six hypothetical replicate plates seeded with 293TLR3/IFNβLuc were processed as follow: in each plate, 1/3 of the wells were left uninfected, 1/3 of the wells were infected with DENV and mock treated, the rest of the wells were infected with DENV and treated with indicated concentration of positive control compound IHVR17028 for 60 h. As shown in Table 2 and Fig. 4A, Z' factors ranging from 0.47 to 0.74 and S/B ratios ranging from 79 to 92 were obtained from these testing plates. IHVR17028 dose-dependently inhibited luciferase activity with EC_{50} values ranging from 2.3 to 4.3 μM. These results thus suggested that the assay under the selected experimental condition has a high signal-to-background ratio and low variation. Furthermore, the Pearson's coefficient was determined ranging from 0.96 to 1.0 among the replicate plates (Fig. 4B), suggesting a high reproducibility of the HTS assay.

3.4. HTS of a 26,900 compound library

Screening a library of 26,900 compounds was carried out under the established HTS experimental condition described above. Raw luciferase values were normalized as percentage of infected/mock treated controls within each assay plate. As illustrated in Fig. 5 and 185 compounds that reduced DENV activated luciferase expression

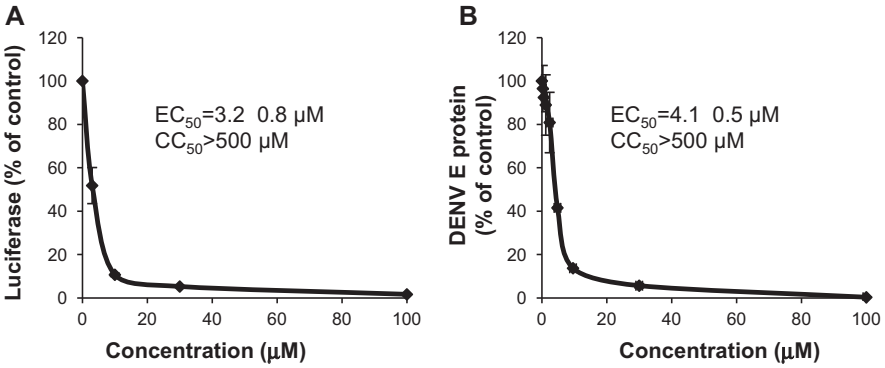

Fig. 3. DENV induced IFN-β promoter activation is dose-dependently inhibited by control antiviral compound. (A) 293TLR3/IFNβLuc cells grown in 96-well plate were infected with DENV at MOI of 0.1. Cells were either mock treated or treated with indicated concentrations of imino sugar compound IHVR17028, and harvested at 60 h post-infection to determine: luciferase activity (expressed as percentage of mock treated, mean ± standard deviation, $n = 8$). (B) Huh7.5 cells grown in 96-well plate were infected with DENV at MOI of 0.1. Cells were either mock treated or treated with indicated concentrations of IHVR17028, and harvested at 48 h post-infection to determine florescent intensity of DENV E protein by In-cell western assay (expressed as percentage of mock treated, mean ± standard deviation, $n = 3$). EC_{50} values were determined from these two assays and expressed as mean ± standard deviation. CC_{50} values were determined by MTT-based cell viability assay.

Table 1
Assay optimization in 96-well plate.

Cell Number/well	MOI of infection	S/B	CV (%)	Z′
1×10^4	0.5	59	10.7	0.66
	0.2	42	14.2	0.55
	0.1	25	19.4	0.37
2×10^4	0.5	128	7.9	0.76
	0.2	88	12.3	0.62
	0.1	60	11.1	0.66
3×10^4	0.5	144	7.8	0.76
	0.2	127	8.3	0.74
	0.1	80	8.9	0.72
4×10^4	0.5	168	13.7	0.58
	0.2	139	10.8	0.67
	0.1	108	7.9	0.75

293TLR3/IFNβLuc cells were seeded in 96-well plate at indicated density, with half of the plate infected with DENV, and another half of the plate uninfected. The firefly luciferase activities were measured 60 h post infection.
S/B, signal to background, refers to ratio of luciferase activity in infected wells to uninfected wells.
CV (%) is percentage of coefficient of variation of infected wells.
Z′, Z′ factor for statistical analysis using parameters obtained from infected and uninfected wells.

by greater than 75% at 10 μM concentration and/or that demonstrated more potent inhibitory effect than control compound were nominated as primary "hits". All the primary "hit" compounds were subsequently tested in a serial dilution ranging from 10 to 1 μM to evaluate their effect on DENV-induced luciferase activity as well as cytotoxicity in 293TLR3/IFNβLuc reporter cells. As illustrated in Fig. 5, a total of 46 compounds were confirmed to selectively inhibit DENV induced luciferase expression in a dose-dependent manner. These 46 confirmed "hits" were next evaluated by testing their antiviral activities against DENV with an In-cell western assay (Qu et al., 2011; Yu et al., 2012) in Huh7.5 cells. This experiment categorized the confirmed "hits" into two groups. A group of 14 compounds selectively inhibit the production of DENV E protein by greater than 50% at 10 μM and thus are antivirals. The remaining 32 compounds that do not apparently affect DENV E protein production in Huh7.5 cells, but dose-dependently reduce the virus activated IFN-β promoter activities in the reporter cells, suggesting they are most likely inhibitors of virus-elicited innate immune response pathways.

While the characterization of the innate immune pathway inhibitors will be reported elsewhere, the antiviral compounds were further tested for their effects on IFN-β promoter activities induced by representative positive stranded RNA virus (EMCV) and negative stranded RNA virus (TCRV) to determine their antiviral spectrum. Among the 14 antiviral compounds, 11 of the compounds were also active in inhibition of EMCV and TCRV induced IFN-β promoter activity and are thus potentially broad-spectrum antivirals.

Table 2
Assay performance in pilot experiment.

Plate #	S/B	Z′	IHVR17028 EC_{50} (μM)
1	79	0.73	3.5
2	81	0.47	3.9
3	85	0.74	2.3
4	91	0.61	3.2
5	84	0.71	4.3
6	92	0.58	2.7

293TLR3/IFNβLuc cells were seeded in 96-well plate at density of 4×10^4. 4 columns of the wells were uninfected. 8 columns of the wells infected with DENV at MOI of 0.1, with 4 columns of the wells each column treated with 3, 10, 30 or 100 μM of IHVR17028 (positive control), and another 4 columns of the plate mock treated. The firefly luciferase activities were measured 60 h post infection.
S/B, signal to background, refers to ratio of luciferase activity in infected control wells to uninfected wells.
EC_{50} values were calculated based on wells infected and treated with doses of IHVR17028.
Z′, Z′ factor for statistical analysis using parameters obtained from infected control wells and uninfected wells.

3.5. Characterization of antiviral compound BPU

Since we are particularly interested in discovering virus-specific antivirals, one of the three antiviral compounds that only inhibited DENV, but not EMCV and TCRV, 1-(6-ethoxybenzo[d]thiazol-2-yl)-3-(3-methoxyphenyl) urea, a benzothiazolylphenyl urea compound (BPU) (Fig. 6A), was chosen to highlight the "hit" evaluation studies. The EC_{50} values of BPU against DENV determined by IFN-β reporter assay and In-cell western assay were 2.0 μM and 3.8 μM, respectively (Fig. 6B-D). Moreover, we further demonstrated that BPU not only inhibited the infection of DENV (Fig. 7A), as judged by reduction of virus yield, and another flavivirus YFV (Fig. 7B), as judged by reduction of virus E protein, it also efficiently inhibit HCV infection of Huh7.5 cells, as demonstrated by significant suppression of HCV RNA replication by a quantitative RT-PCR assay (Fig. 7C). On the contrary, consistent with the results obtained with the IFN-β reporter assays (Fig. 6B), BPU did not apparently inhibit the replication of TCRV and EMCV, as determined by virus yield

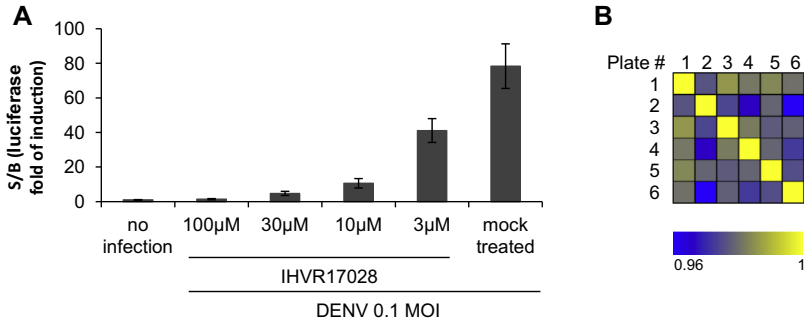

Fig. 4. Assay performance using hypothetical replicates. 293TLR3/IFNβLuc cells were seeded in 6 of 96-well plate at density of 4×10^4/well. 4 columns of the wells were uninfected ($n = 32$). 8 columns of the wells infected with DENV at MOI of 0.1, with 4 columns each column treated with 3, 10, 30 or 100 μM of IHVR17028 ($n = 8$), and another 4 columns mock treated ($n = 32$). The firefly luciferase activities were measured 60 h post infection. (A) S/B values, the fold of luciferase induction in infected wells relative to uninfected wells, were calculated from 6 plates, and expressed as mean ± standard deviation. (B) Correlation heat map showing pairwise Pearson's coefficient of data on all 6 replicate plates.

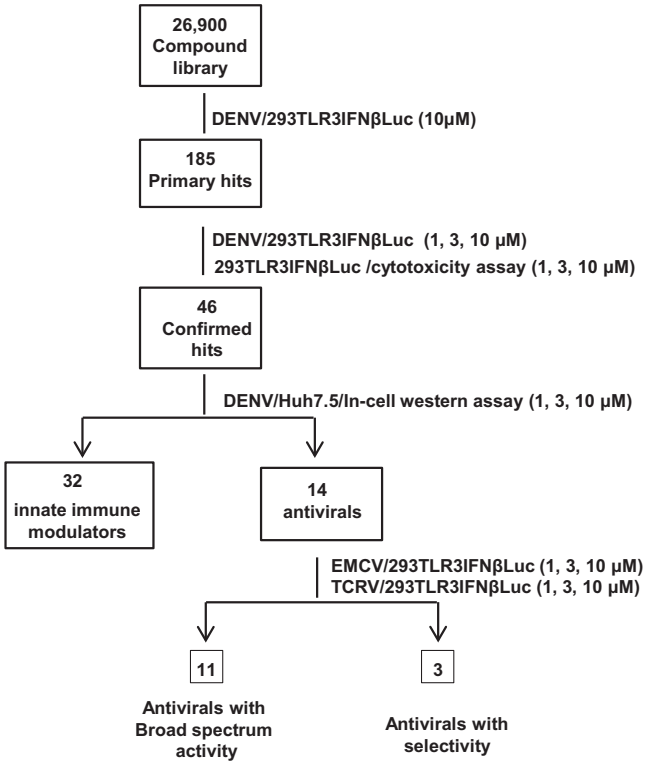

Fig. 5. Strategy of HTS of 26,900 small molecule compound library. Stepwise screening and characterization strategies are shown to indicate virus/cell line/assay and test concentration. Numbers in box represent number of hits identified in each screening step.

reduction assays (data not shown). Hence, BPU appears to be a specific inhibitor of viruses in the family of *Flaviviridae*.

3.6. BPU inhibits HCV entry into host cells

In order to elucidate the antiviral mechanism, we took advantage of available tools in HCV research in our laboratory to map the viral replication step targeted by BPU. As shown in Fig. 8, while BPU potently inhibited HCV replication when the treatment was started immediately after infection at a low MOI (0.01) (Fig. 8A and C), the compound did not apparently affect HCV replication when the treatment was initiated after the virus had spread to infect all the cells in cultures (4 days post infection) (Fig. 8B and D). However, as expected, IFN-α efficiently inhibited HCV replication under both conditions. Furthermore, BPU did not inhibit the replication of HCV subgenomic replicon in Huh7 cells (Fig. 8E). These results thus implied that BPU most likely inhibited an early or late event of HCV replication, such as entry and uncoating or virion particle assembly and secretion, but not viral protein biosynthesis/processing and RNA replication. To distinguish these two possibilities, we further demonstrated that BPU selectively inhibited the infection of HCV envelope proteins E1 and E2-pseudotyped lentiviral particles (HCVpp), but not VSV G protein-pseudotyped lentiviral particles (VSVpp). Hence, our results indicated that BPU most likely inhibited HCV entry into host cells.

4. Discussion

Although IFN-β reporter assay has been widely used for studying virus induction of innate immune responses, we report herein that this simple assay can also be used for screening and identification of compounds with antiviral or innate immune response-modulating activities in a high throughput manner. Our results demonstrated that this high-throughput screening platform has the following unique features:

First, the reporter assay is sensitive and robust. It allows the screening to be performed with a low MOI (0.1) of infection (Fig. 4 and Table 2), which supports multiple rounds of viral infection and thus allows for the identification of compounds that inhibit any step of viral replication cycle. On the contrary, many other commonly used cell-based antiviral assays, such as those based on virus induced cytopathic effect (CPE), frequently require high MOI of infection, which may preclude the identification of compounds targeting late events of viral replication cycle. In addition, although in the current study the screen was performed in 96-well plate, the assay can be easily miniaturized into 384-well format and adapted to using of liquid handling.

Second, in addition to identify compounds with antiviral activity, the IFN-β reporter assay can also be used for simultaneously identification of compounds that inhibit virus-elicited proinflammatory cytokine response. This is because the activation of IFN-β gene expression relies on coordinative activation of IRF3/IRF7, NFκB and MAPK pathways, and the later two pathways are also required to induce other inflammatory cytokines, therefore, monitoring the IFN-β gene activation by viral infections should cover the most, if not all, important innate immune signaling pathways. Although it was not a focus of the current study, we have demonstrated that the virus activated IFN-β promoter activity can be dose-dependently inhibited by multiple known inhibitors of these pathways ((Guo et al., 2012) and data not shown). In fact, our pilot

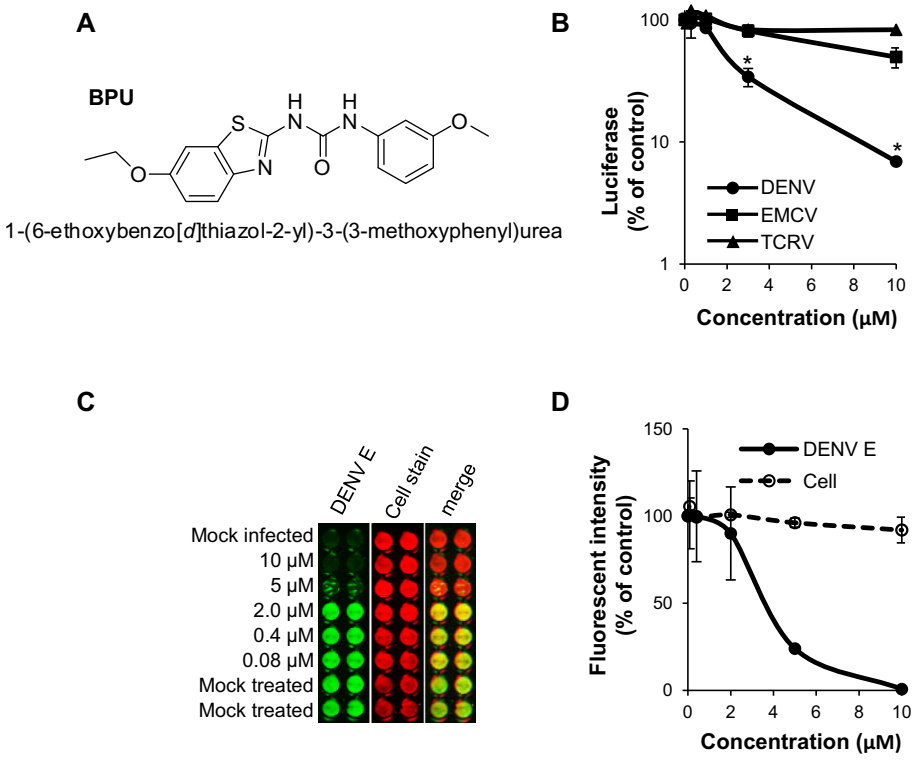

Fig. 6. Identification of representative antiviral compound. (A) Structure of a representative antiviral hit BPU. (B) Antiviral activity of BPU, determined by IFN promoter reporter assay. 293TLR3/IFNβLuc cells, infected with DENV (MOI of 0.1), TCRV (MOI of 0.1), or EMCV (MOI of 0.01), were either mock treated, or treated with the indicated concentrations of BPU. Cells were harvested at 60 h (DENV), 48 h (TCRV), or 24 h (EMCV) to determine the luciferase activities. The representative result of three independent experiments was expressed as percentage of mock treated controls (mean ± standard deviation, $n = 8$). P values were calculated using 2-tailed student's t-test. * indicates $P < 0.001$, compared to mock treated control. (C) Representative image from four independent experiments to determine anti-DENV activity of BPU by In-cell western assay. Huh7.5 cells grown in 96-well plate were either mock infected or infected with DENV at MOI of 0.1, and mock treated or treated with indicated concentration of BPU for 48 h. DENV envelop (E) protein expression was revealed by In-cell immunostaining (green). Cell viabilities were determined by DRAQ 5 and Sapphire 700 staining (red). (D) Quantification of DENV E and cell stain in In-cell western assay using Li-COR Odyssey. Fluorescent intensity data were expressed as percentage of mock treated controls.

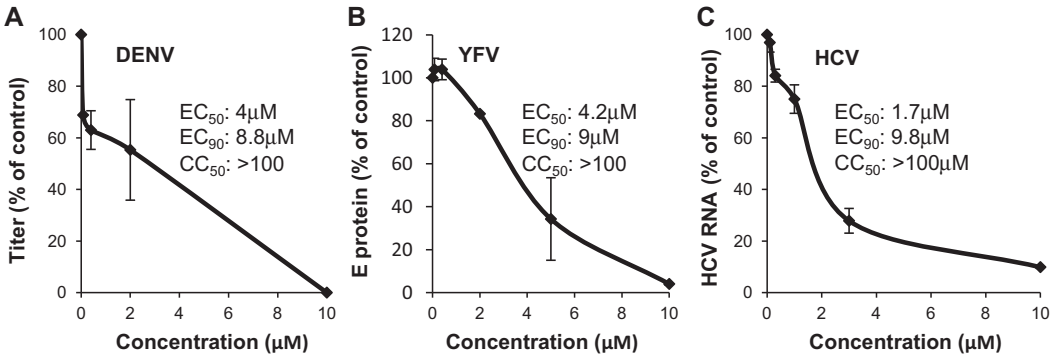

Fig. 7. Antiviral spectrum and activity of BPU. Huh7.5 cells were infected with DENV and YFV at MOI of 0.1, or HCV at MOI of 0.01. Immediately after 1 h incubation with the virus, the infected cells were either mock treated or treated with indicated doses of BPU. (A) For DENV, culture media were collected at 48 post infection, and measured for virus titers using plaque assay. (B) For YFV, cells were fixed 48 h post infection, and measured for YFV E protein using In-cell western assay followed by quantification of fluorescent intensity. (C) For HCV, total RNA was extracted from cells 4 days post infection to determine HCV RNA by qRT-PCR assay. Values were expressed as percentage of mock treated controls (mean ± standard deviation, $n \geqslant 3$). Dose-dependent inhibition curves were generated to calculate EC_{50} and EC_{90} values. CC_{50} values were determined by MTT-based cell viability assay.

screening has indeed identified multiple compounds with such properties (Fig. 5). The compounds that inhibit viral induction of cytokine response could be candidates for the development of drugs that alleviate viral pathogenesis related to "cytokine storm", such as encephalomeningitis in West Nile virus infection (Wang et al., 2004), hemorrhagic fevers in the infection of DENV, Ebola virus and many other viruses (Paessler and Walker, 2013; Pang et al., 2007; Wauquier et al., 2010) as well as severe pneumonia in influenza A virus or SARS-coronavirus infection (Cheng et al., 2011; Oslund and Baumgarth, 2011; Theron et al., 2005). Alternatively, these compounds could also serve as chemical genetic probes for studying virus–host interaction. Unlike siRNA-mediated knockdown and other genome-wide gene discovery technologies, this chemical genetic approach is likely to identify host functions that are potentially "drugable" (de Chassey et al., 2012; Panda and Cherry, 2012).

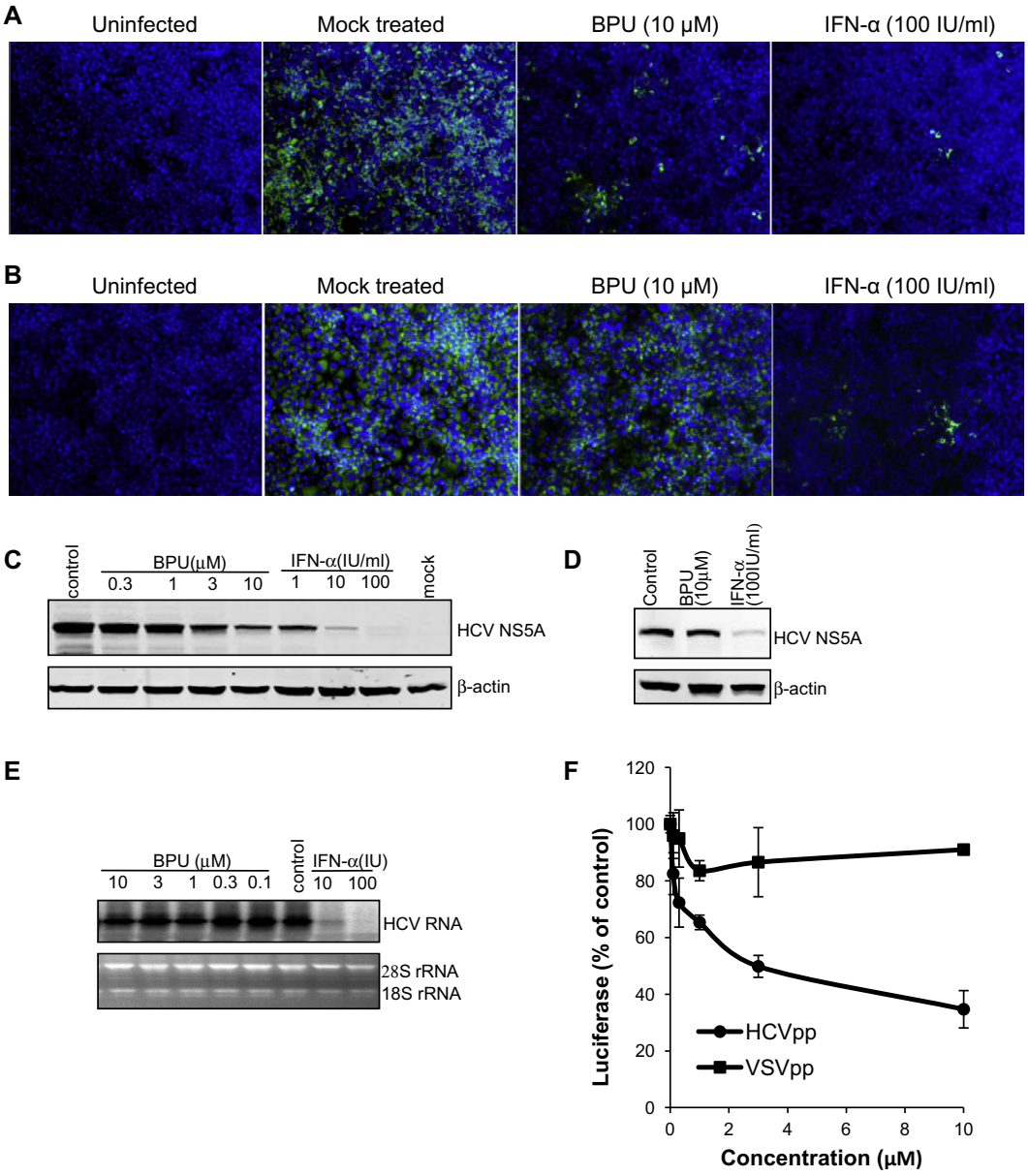

Fig. 8. BPU inhibits HCV entry. Huh7.5 cells were infected with HCV at MOI of 0.01. Cells were treated for 4 days, either immediately after 1 h of incubation with the virus (A and C), or starting on day 5 post infection, when HCV replication was fully established with 100% of the cells being infected (B and D). Cells were either mock treated, or treated with indicated concentration of BPU or IFN-β. HCV NS5A protein was detected with an indirect immunofluorescence assay. Cell nuclei were stained with DAPI (A and B). Alternatively, levels of intracellular HCV NS5A were determined by a Western blot assay using β-actin as loading control (C and D). (E) To determine the effect of BPU on HCV replicon replication, GS4.1 cells were mock treated or treated with indicated concentrations of BPU for 3 days. IFN-α treatment served as a positive control. Total RNAs were analyzed by Northern blot hybridization to detect HCV RNA. rRNAs served as loading controls. (F) To determine the effect on virus entry, Huh7.5 cells grown in 96-well plate were infected with HCVpp, and simultaneously treated with the indicated concentrations of BPU during the 4 h incubation with the pseudovirus. The compound and the pseudovirus were then removed and cells were cultured with fresh medium for additional 72 h. Luciferase activities were determined and expressed as percentage of mock treated control (mean ± standard deviation, $n = 4$). VSVpp was used as control.

We are aware of the possibility that certain compounds identified with antiviral activity might indirectly inhibit viral replication by either enhancing virus-elicited IFN response or attenuate the viral suppression of IFN response. However, because Huh7.5 cells used for our antiviral assay in the secondary screen are deficient in both TLR3 and RIG-I pathways, it is unlikely that the antiviral compounds we identified in this study inhibit viral replication via such a mechanism.

Third, it is well known that many species of viruses, such as influenza A virus and Rift Valley fever virus, have evolved ability to inhibit the PRR-mediated cytokine response, which is essential for the establishment of viral infection *in vivo* and critical for viral pathogenesis (Habjan et al., 2008; Mibayashi et al., 2007; Rajsbaum et al., 2012; Siu et al., 2009). Thus the IFN-β promoter reporter assay might also be used to screen for compounds that can restore the innate immune response induced by these viruses. For example, a novel small molecule inhibitor of influenza A viruses has been discovered in screening of compounds that restore IFN response to influenza virus infection in a MDCK cell-based IFN-β promoter reporter assay (Ortigoza et al., 2012).

Forth, the assay is resource efficient. Because activation of the IFN response is a common property of many different viruses, the assay can serve as a broadly applicable HTS platform for any virus that can infect the cells. Along this line, the robustness of

the reporter assay also makes it feasible for parallel screening with multiple viruses. In fact, such a multiplex viral screening should reveal virus-specific and broad-spectrum antiviral compounds as well as compounds disrupting virus-specific or common innate immune response pathways. The information obtained from the multiplex viral screening will help prioritize the active compounds for further development as therapeutics and/or molecular probes. For instance, the compounds that hit the common "choke" points of host functions required for replication of many different viruses can potentially serve as candidates for development of broad-spectrum antiviral drugs, or experimental probes to reveal the commonly required host functions for viral replication or induction of host antiviral response (Ma-Lauer et al., 2012).

Finally, characterization of representative antiviral "hits" from our pilot HTS clearly demonstrated the feasibility of the reporter assay for discovery of antiviral compounds. BPU is an antiviral compound that inhibits the infection of viruses from at least two genera of the *flaviviridae* family, but not EMCV and TCRV, the representative positive and negative-stranded RNA viruses, respectively. Mechanistic analysis further demonstrated that the compound inhibited the entry of HCV, and possibly other flaviviruses, by targeting common entry factor(s) shared by these viruses. Chemically, BPU has an *N*-benzothiazolyl *N*′-phenyl urea core structure with two alkoxy substitutions (Fig. 6A). Several analogs with this core structure have been found to have antiviral activity against HIV (D'Cruz et al., 2002; Paget et al., 1969), inhibit Type III secretion systems in pathogenic bacteria (PCT Int. Appl. 2009, WO2009145829A1), as well as inhibit the ubiquitin ligase (PCT Int. Appl. 2005, WO 2005037845A1). However, the biological activity of compounds with the unique alkoxy substitutions in BPU has not been reported. Although BPU is not a highly potent antiviral compound, its structure is tractable for chemical modification and structure–activity relationship studies for future development into potential antiviral therapeutic.

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References

Blight, K.J., McKeating, J.A., Rice, C.M., 2002. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. J. Virol. 76 (24), 13001–13014.

Chang, J., Block, T.M., Guo, J.T., 2013a. Antiviral therapies targeting host ER alpha-glucosidases: current status and future directions. Antiviral Res. 99 (3), 251–260.

Chang, J., Guo, J.T., Jiang, D., Guo, H., Taylor, J.M., Block, T.M., 2008. Liver-specific microRNA miR-122 enhances the replication of hepatitis C virus in nonhepatic cells. J. Virol. 82 (16), 8215–8223.

Chang, J., Schul, W., Butters, T.D., Yip, A., Liu, B., Goh, A., Lakshminarayana, S.B., Alonzi, D., Reinkensmeier, G., Pan, X., Qu, X., Weidner, J.M., Wang, L., Yu, W., Borune, N., Kinch, M.A., Rayahin, J.E., Moriarty, R., Xu, X., Shi, P.Y., Guo, J.T., Block, T.M., 2011. Combination of alpha-glucosidase inhibitor and ribavirin for the treatment of dengue virus infection in vitro and in vivo. Antiviral Res. 89 (1), 26–34.

Chang, J., Wang, L., Ma, D., Qu, X., Guo, H., Xu, X., Mason, P.M., Bourne, N., Moriarty, R., Gu, B., Guo, J.T., Block, T.M., 2009. Novel imino sugar derivatives demonstrate potent antiviral activity against flaviviruses. Antimicrob. Agents Chemother. 53 (4), 1501–1508.

Chang, J., Warren, T.K., Zhao, X., Gill, T., Guo, F., Wang, L., Comunale, M.A., Du, Y., Alonzi, D.S., Yu, W., Ye, H., Liu, F., Guo, J.T., Mehta, A., Cuconati, A., Butters, T.D., Bavari, S., Xu, X., Block, T.M., 2013b. Small molecule inhibitors of ER alpha-glucosidases are active against multiple hemorrhagic fever viruses. Antiviral Res. 98 (3), 432–440.

Cheng, X.W., Lu, J., Wu, C.L., Yi, L.N., Xie, X., Shi, X.D., Fang, S.S., Zan, H., Kung, H.F., He, M.L., 2011. Three fatal cases of pandemic 2009 influenza A virus infection in Shenzhen are associated with cytokine storm. Respir. Physiol. Neurobiol. 175 (1), 185–187.

Connor, R.I., Chen, B.K., Choe, S., Landau, N.R., 1995. Vpr is required for efficient replication of human immunodeficiency virus type-1 in mononuclear phagocytes. Virology 206 (2), 935–944.

D'Cruz, O.J., Venkatachalam, T.K., Mao, C., Qazi, S., Uckun, F.M., 2002. Structural requirements for potent anti-human immunodeficiency virus (HIV) and sperm-immobilizing activities of cyclohexenyl thiourea and urea non-nucleoside inhibitors of HIV-1 reverse transcriptase. Biol. Reprod. 67 (6), 1959–1974.

de Chassey, B., Meyniel-Schicklin, L., Aublin-Gex, A., Andre, P., Lotteau, V., 2012. New horizons for antiviral drug discovery from virus–host protein interaction networks. Curr Opin Virol 2 (5), 606–613.

Dougherty, A.M., Guo, H., Westby, G., Liu, Y., Simsek, E., Guo, J.T., Mehta, A., Norton, P., Gu, B., Block, T., Cuconati, A., 2007. A substituted tetrahydro-tetrazolo-pyrimidine is a specific and novel inhibitor of hepatitis B virus surface antigen secretion. Antimicrob. Agents Chemother. 51 (12), 4427–4437.

Du, Y., Ye, H., Gill, T., Wang, L., Guo, F., Cuconati, A., Guo, J.T., Block, T.M., Chang, J., Xu, X., 2013. *N*-Alkyldeoxynojirimycin derivatives with novel terminal tertiary amide substitution for treatment of bovine viral diarrhea virus (BVDV), Dengue, and Tacaribe virus infections. Bioorg. Med. Chem. Lett. 23 (7), 2172–2176.

Fung, J., Lai, C.L., Seto, W.K., Yuen, M.F., 2011. Nucleoside/nucleotide analogues in the treatment of chronic hepatitis B. J. Antimicrob. Chemother. 66 (12), 2715–2725.

Guo, F., Mead, J., Aliya, N., Wang, L., Cuconati, A., Wei, L., Li, K., Block, T.M., Guo, J.T., Chang, J., 2012. RO 90–7501 enhances TLR3 and RLR agonist induced antiviral response. PLoS One 7 (10), e42583.

Guo, H., Pan, X., Mao, R., Zhang, X., Wang, L., Lu, X., Chang, J., Guo, J.T., Passic, S., Krebs, F.C., Wigdahl, B., Warren, T.K., Retterer, C.J., Bavari, S., Xu, X., Cuconati, A., Block, T.M., 2011. Alkylated porphyrins have broad antiviral activity against hepadnaviruses, flaviviruses, filoviruses, and arenaviruses. Antimicrob. Agents Chemother. 55 (2), 478–486.

Guo, J.T., Bichko, V.V., Seeger, C., 2001. Effect of alpha interferon on the hepatitis C virus replicon. J. Virol. 75 (18), 8516–8523.

Habjan, M., Andersson, I., Klingstrom, J., Schumann, M., Martin, A., Zimmermann, P., Wagner, V., Pichlmair, A., Schneider, U., Muhlberger, E., Mirazimi, A., Weber, F., 2008. Processing of genome 5' termini as a strategy of negative-strand RNA viruses to avoid RIG-I-dependent interferon induction. PLoS One 3 (4), e2032.

He, J., Choe, S., Walker, R., Di Marzio, P., Morgan, D.O., Landau, N.R., 1995. Human immunodeficiency virus type 1 viral protein R (Vpr) arrests cells in the G2 phase of the cell cycle by inhibiting p34cdc2 activity. J. Virol. 69 (11), 6705–6711.

Jiang, D., Weidner, J.M., Qing, M., Pan, X.B., Guo, H., Xu, C., Zhang, X., Birk, A., Chang, J., Shi, P.Y., Block, T.M., Guo, J.T., 2010. Identification of five interferon-induced cellular proteins that inhibit west nile virus and dengue virus infections. J. Virol. 84 (16), 8332–8341.

Jilek, B.L., Zarr, M., Sampah, M.E., Rabi, S.A., Bullen, C.K., Lai, J., Shen, L., Siliciano, R.F., 2012. A quantitative basis for antiretroviral therapy for HIV-1 infection. Nat. Med. 18 (3), 446–451.

Li, K., Chen, Z., Kato, N., Gale Jr., M., Lemon, S.M., 2005. Distinct poly(I-C) and virus-activated signaling pathways leading to interferon-beta production in hepatocytes. J. Biol. Chem. 280 (17), 16739–16747.

Loo, Y.M., Gale Jr., M., 2011. Immune signaling by RIG-I-like receptors. Immunity 34 (5), 680–692.

Ma-Lauer, Y., Lei, J., Hilgenfeld, R., von Brunn, A., 2012. Virus–host interactomes–antiviral drug discovery. Curr. Opin. Virol. 2 (5), 614–621.

Mibayashi, M., Martinez-Sobrido, L., Loo, Y.M., Cardenas, W.B., Gale Jr., M., Garcia-Sastre, A., 2007. Inhibition of retinoic acid-inducible gene I-mediated induction of beta interferon by the NS1 protein of influenza A virus. J. Virol. 81 (2), 514–524.

Negishi, H., Osawa, T., Ogami, K., Ouyang, X., Sakaguchi, S., Koshiba, R., Yanai, H., Seko, Y., Shitara, H., Bishop, K., Yonekawa, H., Tamura, T., Kaisho, T., Taya, C., Taniguchi, T., Honda, K., 2008. A critical link between Toll-like receptor 3 and type II interferon signaling pathways in antiviral innate immunity. Proc. Natl. Acad. Sci. U.S.A. 105 (51), 20446–20451.

Ortigoza, M.B., Dibben, O., Maamary, J., Martinez-Gil, L., Leyva-Grado, V.H., Abreu Jr., P., Ayllon, J., Palese, P., Shaw, M.L., 2012. A novel small molecule inhibitor of influenza A viruses that targets polymerase function and indirectly induces interferon. PLoS Pathog. 8 (4), e1002668.

Oslund, K.L., Baumgarth, N., 2011. Influenza-induced innate immunity: regulators of viral replication, respiratory tract pathology & adaptive immunity. Future Virol. 6 (8), 951–962.

Paessler, S., Walker, D.H., 2013. Pathogenesis of the viral hemorrhagic fevers. Annu. Rev. Pathol. 8 (1), 411–440.

Paget, C.J., Kisner, K., Stone, R.L., DeLong, D.C., 1969. Heterocyclic substituted ureas. II. Immunosuppressive and antiviral activity of benzothiazole- and benzoxazoleureas. J. Med. Chem. 12 (6), 1016–1018.

Panda, D., Cherry, S., 2012. Cell-based genomic screening: elucidating virus–host interactions. Curr. Opin. Virol. 2 (6), 778–786.

Pang, T., Cardosa, M.J., Guzman, M.G., 2007. Of cascades and perfect storms: the immunopathogenesis of dengue haemorrhagic fever-dengue shock syndrome (DHF/DSS). Immunol. Cell Biol. 85 (1), 43–45.

Qu, X., Pan, X., Weidner, J., Yu, W., Alonzi, D., Xu, X., Butters, T., Block, T., Guo, J.T., Chang, J., 2011. Inhibitors of Endoplasmic Reticulum {alpha}-Glucosidases Potently Suppress Hepatitis C Virus Virion Assembly and Release. Antimicrob. Agents Chemother. 55 (3), 1036–1044.

Rajsbaum, R., Albrecht, R.A., Wang, M.K., Maharaj, N.P., Versteeg, G.A., Nistal-Villan, E., Garcia-Sastre, A., Gack, M.U., 2012. Species-specific inhibition of RIG-I

ubiquitination and IFN induction by the influenza A virus NS1 protein. PLoS Pathog. 8 (11), e1003059.
Siu, K.L., Kok, K.H., Ng, M.H., Poon, V.K., Yuen, K.Y., Zheng, B.J., Jin, D.Y., 2009. Severe acute respiratory syndrome coronavirus M protein inhibits type I interferon production by impeding the formation of TRAF3.TANK.TBK1/IKKepsilon complex. J. Biol. Chem. 284 (24), 16202–16209.
Takeuchi, O., Akira, S., 2008. MDA5/RIG-I and virus recognition. Curr. Opin. Immunol. 20 (1), 17–22.
Takeuchi, O., Akira, S., 2010. Pattern recognition receptors and inflammation. Cell 140 (6), 805–820.
Theron, M., Huang, K.J., Chen, Y.W., Liu, C.C., Lei, H.Y., 2005. A probable role for IFN-gamma in the development of a lung immunopathology in SARS. Cytokine 32 (1), 30–38.
Tong, Y., Zhu, Y., Xia, X., Liu, Y., Feng, Y., Hua, X., Chen, Z., Ding, H., Gao, L., Wang, Y., Feitelson, M.A., Zhao, P., Qi, Z.T., 2011. Tupaia CD81, SR-BI, claudin-1, and occludin support hepatitis C virus infection. J. Virol. 85 (6), 2793–2802.
Wang, T., Town, T., Alexopoulou, L., Anderson, J.F., Fikrig, E., Flavell, R.A., 2004. Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. Nat. Med. 10 (12), 1366–1373.
Wauquier, N., Becquart, P., Padilla, C., Baize, S., Leroy, E.M., 2010. Human fatal zaire ebola virus infection is associated with an aberrant innate immunity and with massive lymphocyte apoptosis. PLoS Negl. Trop. Dis. 4 (10), e837.
Yang, P.L., Gao, M., Lin, K., Liu, Q., Villareal, V.A., 2011. Anti-HCV drugs in the pipeline. Curr. Opin. Virol. 1 (6), 607–616.
Yoneyama, M., Kikuchi, M., Matsumoto, K., Imaizumi, T., Miyagishi, M., Taira, K., Foy, E., Loo, Y.M., Gale Jr., M., Akira, S., Yonehara, S., Kato, A., Fujita, T., 2005. Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. J. Immunol. 175 (5), 2851–2858.
Yu, W., Gill, T., Wang, L., Du, Y., Ye, H., Qu, X., Guo, J.T., Cuconati, A., Zhao, K., Block, T.M., Xu, X., Chang, J., 2012. Design, synthesis, and biological evaluation of *N*-alkylated deoxynojirimycin (DNJ) derivatives for the treatment of dengue virus infection. J. Med. Chem. 55 (13), 6061–6075.
Zhang, J.H., Chung, T.D., Oldenburg, K.R., 1999. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. J. Biomol. Screen. 4 (2), 67–73.
Zhou, Z., Wang, N., Woodson, S.E., Dong, Q., Wang, J., Liang, Y., Rijnbrand, R., Wei, L., Nichols, J.E., Guo, J.T., Holbrook, M.R., Lemon, S.M., Li, K., 2011. Antiviral activities of ISG20 in positive-strand RNA virus infections. Virology 409 (2), 175–188.
Zhu, Q., Guo, J.T., Seeger, C., 2003. Replication of hepatitis C virus subgenomes in nonhepatic epithelial and mouse hepatoma cells. J. Virol. 77 (17), 9204–9210.