Identification of Inhibitors of Yellow Fever Virus Replication Using a Replicon-Based High-Throughput Assay[∇]

Chinmay G. Patkar,¹† Martha Larsen,² Michael Owston,¹ Janet L. Smith,² and Richard J. Kuhn^{1*}

Markey Center for Structural Biology, Department of Biological Sciences, 915 W. State Street, Purdue University, West Lafayette, Indiana 47907-2054,¹ and Life Sciences Institute, University of Michigan, 210 Washtenaw Avenue, Ann Arbor, Michigan 48109-2216²

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Flaviviruses cause severe disease in humans and are a public health priority worldwide. However, no effective therapies or drugs are commercially available yet. Several flavivirus replicon-based assays amenable to high-throughput screening of inhibitors have been reported recently. We developed and performed a replicon-based high-throughput assay for screening small-molecule inhibitors of yellow fever virus (YFV) replication. This assay utilized packaged pseudoinfectious particles containing a YFV replicon that expresses *Renilla* luciferase in a replication-dependent manner. Several small-molecule compounds with inhibitory activity at micromolar concentrations were identified in the high-throughput screen. These compounds were subsequently tested for their inhibitory activities against YFV replication and propagation in low-throughput assays. Furthermore, YFV mutants that escaped inhibition by two of the compounds were isolated, and in both cases, the mutations were mapped to the NS4B coding region, suggesting a novel inhibitory target for these compounds. This study opens up new avenues for pursuing the nonenzymatic nonstructural proteins as targets for antivirals against YFV and other flaviviruses.

The genus *Flavivirus*, belonging to the family *Flaviviridae*, comprises more than 70 members, including the prototype yellow fever virus (YFV), the four serotypes of dengue virus (DENV1, DENV2, DENV3, and DENV4), West Nile virus (WNV), Kunjin virus (KUN), tick-borne encephalitis virus (TBEV), and Japanese encephalitis virus (JEV) (16). These arthropod-borne pathogens are causative agents of severe and sometimes fatal disease in humans and have a considerable socioeconomic effect worldwide. The four serotypes of DENV (DENV1 to DENV4) have become the most important arthropod-borne human viruses. Each year there are an estimated 50 to 100 million DENV infections, with 500,000 cases of dengue hemorrhagic fever that require hospitalization and approximately 25,000 deaths, mainly of children (9). The development of a highly effective vaccine strain in the 1930s and mosquito eradication programs essentially eliminated YFV outbreaks in North America; however, YFV continues to be a major threat in many developing countries. There are still an estimated 200,000 asymptomatic cases and about 30,000 deaths annually from YFV, chiefly in Africa and South America (29), with a recent outbreak (in February 2008) in Paraguay. Furthermore, reductions in mosquito control programs and growing urbanization over the past several decades have led to a resurgence of the distribution of mosquito vectors for flaviviruses. The recent introduction and spread in the United States of WNV, a virus previously restricted to the Eastern Hemisphere, has

further highlighted the public health challenges posed by flaviviruses (15).

Flaviviruses are small, spherical, enveloped viruses containing an approximately 11,000 nucleotide, positive-sense RNA genome. The genomic RNA has a type I cap at its 5' end to allow translation similar to cellular mRNAs but lacks a polyadenine tract at the 3' end (16). The viral RNA contains a single long open reading frame, which when translated gives rise to a long polyprotein that is co- and posttranslationally processed by viral and cellular proteases into three structural proteins (capsid [C], premembrane [prM; the precursor form of M], and envelope [E]) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (16). The nonstructural proteins are responsible for various enzymatic activities, including RNA-dependent RNA polymerase (NS5), helicase (NS3), and protease (NS2B-NS3), and together they form the replicase complex, which functions primarily to carry out viral genome replication (16). The structural proteins, along with the host-derived lipid bilayer and a single copy of the RNA genome, assemble to form the flavivirus virion. The virion is approximately 50 nm in diameter, with a relatively smooth outer surface that is constructed from 180 copies of both the major E protein and the small M protein (14, 16, 20). The E and M proteins are anchored to the underlying viral membrane through C-terminal transmembrane domains. At the center of the virion resides the nucleocapsid core, which is composed of C protein in complex with the viral genome.

The reemergence of flaviviruses as a significant public health concern has regenerated interest in the development of new vaccine strains and antiviral agents. Recent progress in the understanding of the molecular biology and immunology of flaviviruses has paved the way for the development of novel therapeutics against flaviviruses. Several therapies, including ribavirin, alpha interferon, 6-azauridine, mycophenolic acid,

* Corresponding author. Mailing address: Markey Center for Structural Biology, Department of Biological Sciences, 915 W. State St., Purdue University, West Lafayette, IN 47907-2054. Phone: (765) 494-1164. Fax: (765) 496-1189. E-mail: kuhn@purdue.edu.

† Present address: UMass Medical School, 55 Lake Avenue North, Worcester, MA 01655.

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and glycyrrhizin, have been tested for their antiviral activities with varying degrees of success (1, 2, 3, 27). Ribavirin (1- β -D-ribofuranosyl-1*H*-1,2,4-triazole-3-carboximide), a nucleoside analog, has been shown to be active against many DNA and RNA viruses, including flaviviruses, but the effective concentration in cell culture is very high, and it is largely ineffective in animal models (12, 18, 27). Alpha interferon has been shown to have broad antiviral activity in vitro and immunostimulatory effects in vivo and has been considered as a possible therapy for flaviviral encephalitis (4, 26). 6-Azauridine and glycyrrhizin have inhibitory activity against flavivirus replication; however, they are nonspecific and display low selective indices (2). Thus, antiviral agents with more potency must be identified. To that end, there has been an ongoing search for small-molecule inhibitors using high-throughput screening (HTS) assays, and novel inhibitors of WNV replication have been identified using a HTS assay and a subgenomic replicon screen (8, 22).

In this report, efforts toward the discovery of inhibitors of YFV replication using a replicon-based HTS assay are described. Previously, we have reported the development of a YFV replicon construct, YF-R.luc2A-RP, which expressed a *Renilla* luciferase gene in a replication-dependent manner (10). Furthermore, we demonstrated that YF-R.luc2A-RP could be packaged into pseudoinfectious particles (PIPs) by supplying the YFV structural proteins using a Sindbis virus (SINV) helper construct, SIN-CprME (10). Utilizing PIPs, an HTS assay was developed and performed at the Center for Chemical Genomics (CCG) facility at the University of Michigan to identify small-molecule compounds that inhibited YFV genome replication (based on reductions in luciferase levels). Primary and secondary screens revealed several compounds with activity in the micromolar range, and compounds that demonstrated a 50% effective concentration (EC₅₀) of <1 μ M were selected for further analysis. Additional assays were performed to confirm the inhibition of YFV replicon RNA replication as well as virus propagation. Further investigations led to the isolation of escape mutants for two of the compounds. The mutations selected in the presence of these two compounds mapped to NS4B, revealing a novel target for inhibition of YFV replication.

MATERIALS AND METHODS

Cell culture and viruses. Baby hamster kidney cells (BHK-15) obtained from the American Type Culture Collection were maintained in minimal essential medium (MEM) (Life Technologies) supplemented with 10% fetal bovine serum (FBS). Parental virus or mutant YFV stocks were generated by standard procedures from either parental virus or mutated pYF23, a plasmid derived from a full-length cDNA clone of YFV 17D (pACNR/FLYF).

Virus infection and plaque assays. For virus infection, virus stocks (diluted in medium to obtain appropriate titers) were adsorbed on a monolayer of BHK-15 cells for 1 h at room temperature with gentle rocking. Cells were then overlaid with the appropriate volume of MEM with 5% FBS, either with or without compounds. For plaque assays, 10-fold serial dilutions of culture supernatants were made in phosphate-buffered saline (PBS) containing 1% FBS. BHK-15 cells at ~90% confluence were inoculated with the dilutions for 1 h at room temperature and were then overlaid with MEM containing 5% FBS and 1% agarose. Cells were incubated at 37°C for 3 days; then they were stained with 2% crystal violet, and the plaque phenotypes were analyzed. For plaque assays in the presence of compounds, compounds at appropriate concentrations were added directly to the agarose overlay. For virus growth analysis, BHK-15 cells were infected with either parental or mutant viruses at a multiplicity of infection (MOI) of 1 and were overlaid with medium containing compounds at various dilutions. Aliquots of culture supernatants were harvested at various time points, and the virus titers were determined by a standard plaque assay.

Plasmids and cloning procedures. The YF-R.luc2A-RP replicon of YFV was derived from YF23. Details of cloning are described elsewhere (10). SINV-based replicons expressing the YFV structural proteins were constructed from pToto64, a full-length cDNA clone of SINV, and the construction of the SIN-CprME and SIN-DSCprME helper replicon constructs has been described by Jones et al. (10). The SIN-Luc replicon was generated by the deletion of the coding region of the SINV structural proteins in pToto64 and the addition of a luciferase gene that was transcribed using the SINV subgenomic promoter. pRL-CMV, a plasmid designed for the expression of *Renilla* luciferase in mammalian cells, was obtained from Promega, Inc. The K128R NS4B mutation was engineered in YF23 and YF-R.luc2A-RP using standard PCR techniques. Mutagenized plasmid DNAs were sequenced to confirm the presence of the mutation.

RNA transcription and transfection. RNA transcripts of YF23, YF-R.luc2A-RP constructs, and the SIN-CprME replicon were generated by in vitro transcription using SP6 RNA polymerase (Amersham Biosciences) from DNA templates linearized by digestion with the XhoI (for YFV plasmids) or SacI (for the SINV replicon) restriction enzyme and were subsequently purified using GFX columns (Amersham Biosciences). For DEAE-dextran transfection, BHK-15 cells were treated with DEAE-dextran for 1 h at 37°C, after which the DEAE-dextran was aspirated and RNA diluted in PBS was added to the cells. Cells were kept at room temperature for 30 min and then overlaid with MEM containing 5% FBS and 1% agarose. For electroporation of BHK-15 cells, subconfluent monolayers of cells grown in T-75 culture flasks (~1 $\times$ 10⁶ cells) were harvested by trypsinization and washed twice with PBS before final resuspension in 400 μ l PBS. The resulting cells were combined with ~10 μ g of in vitro-transcribed RNA, placed in a 2-mm-gap cuvette (Bio-Rad), and electroporated (two pulses at settings of 1.5 kV, 25 μ F, and 200 Ω) using a GenePulser II apparatus (Bio-Rad). Following a 5-min recovery at room temperature, cells were resuspended in MEM supplemented with 10% FBS and were incubated at 37°C in the presence of 5% CO₂.

Generation of PIPs. BHK-15 cells were electroporated with ~10 μ g of in vitro-transcribed YF-R.luc2A-RP replicon RNA, and resuspended cells were plated in a T-75 flask and incubated at 37°C for 24 h. To initiate the production of PIPs, the electroporated cells were subjected to a second round of electroporation using SIN-CprME transcripts and were then incubated at 37°C. Cell supernatants from the cotransfected cells were harvested at 12 to 15 h following the second transfection. In order to check the efficiency of PIP production, 100 μ l of supernatant was used to infect naïve BHK-15 cells; cell extracts were taken from these cells at 24 h postinfection; and luciferase assays were performed.

Luciferase assays. For the high-throughput assay, 50 μ l of EnduRen substrate (Promega) diluted 1:1,000 according to the manufacturer's recommendations was added directly to cells in 384-plate wells. Plates were incubated at 37°C for an additional 2 h, and luciferase activity was measured using PHERAstar (BMGLabTech). To determine *Renilla* luciferase activity in the follow-up low-throughput experiments, cells were washed with PBS and lysed using *Renilla* lysis buffer, and lysates were stored at -80°C. Prior to the assay, frozen extracts were thawed and then homogenized by brief vortexing. Luciferase activity was initiated by the addition of 10 μ l of cytoplasmic extracts to 50 μ l of *Renilla* luciferase substrate (Promega). Luciferase activity was detected using the TD-20/20 luminometer (Turner Designs) and was measured in relative light units.

HTS using YFV PIPs. BHK-15 cells in a 100-mm-diameter culture plate were infected with 2 ml of YFV PIPs at an MOI of approximately 1. At 2 h postinfection, cells were trypsinized and resuspended in medium, and 30 μ l of resuspended cells was added to 384-well culture plates (so as to obtain approximately 2,000 to 3,000 cells per well) with compounds in 30 μ l of medium. For the primary screening, wells were prepared by addition of 0.2 μ l of compounds at a concentration of 1 to 1.5 mM (final compound concentration, 5 to 10 μ M using the HDR [high-density replication] tool on the liquid handling Biomek FX [Beckman]). The compound libraries at the CCG comprise more than 34,000 compounds obtained from commercial suppliers and maintained at -20°C in dimethyl sulfoxide (DMSO). Plates were then incubated at 37°C in the presence of 5% CO₂. At 30 h postinfection, the medium was aspirated to 10 μ l with an ELx405 plate washer (BioTek); 10 μ l medium containing 0.6 μ M EnduRen (Promega) was added to each well in a 384-well plate; and luciferase activity was measured. Ribavirin (50 μ M) was used as a positive control for inhibition, and DMSO (0.5%)-treated cells were used as a negative control. Compounds that showed >50% inhibition, based on luciferase activity, were tested in a dose-response assay. For the dose-response assay, ninefold serial dilutions of the compounds, ranging from 100 μ M to 10 nM, were employed, and similar tests were performed. Hill curves were plotted using luciferase activity for each of the compound dilutions, and EC₅₀s were calculated by nonlinear regression analysis using Prism (GraphPad Software, Inc.). One percent DMSO was utilized as a negative control, and 50 μ M ribavirin served as a positive control, for inhibition.

Cytotoxicity assay. Monolayers of BHK-15 cells at 90% confluence in a 96-well plate were treated with compounds (dissolved in DMSO) serially diluted in MEM containing 5% FBS and were incubated at 37°C. At 30 h posttreatment, compounds were aspirated, and cells were washed with MEM and overlaid with 100 μ l per well of appropriately diluted substrate from the Quick Cell Proliferation assay kit (BioVision Inc.). Cells were incubated at 37°C for an additional 1 h, and the optical density at 450 nm was determined using a SpectraMax plate reader (Molecular Devices). Optical densities at 450 nm for each of the compound dilutions were compared to those obtained for cells treated with DMSO (1%) alone, and 50% cytotoxic concentrations (CC_{50} s) were calculated using Prism.

RNA extraction and RT-PCR. BHK-15 cells were infected with YFV (either the parental virus or a mutant), overlaid with a medium (MEM containing 5% FBS) containing compounds at various concentrations, and incubated at 37°C. For RNA extraction, at 24 h postinfection, cells were washed three times with PBS and lysed with lysis buffer (buffer RLN, RNeasy minikit; Qiagen) to obtain cytoplasmic extracts. Cytoplasmic RNA was then extracted according to the manufacturer's instructions. Reverse transcription-PCRs (RT-PCRs) were set up using the cMaster RTplusPCR system (Eppendorf) by the one-step RT-PCR method according to the manufacturer's recommendations. RT-PCRs were set up using a set of YFV genome-specific primers that spanned the entire YFV cDNA using the following conditions: a one-step RT consisting of one cycle at 45°C for 1 h and one cycle at 95°C for 5 min, and a three-step PCR procedure consisting of 15 s at 95°C, 30 s at 52°C, and 2 min at 68°C for 30 cycles. RT-PCR fragments were purified using GFX columns and were subsequently sequenced. Quantitative real-time RT-PCR (qRT-PCR) was performed using a SYBR green qRT-PCR kit (Invitrogen) for the quantification of viral RNA as well as cellular 18S rRNA according to the manufacturer's instructions. For qRT-PCR of the viral RNA, the following primers were used: 5'-ATGACCACGAAGACATG CTT/+ and 5'-CAGTGATCCGCACAGCTTGT/-. For qRT-PCR of the 18S rRNA, the following primers were used: 5'-TCAAGAACGAAAGTCGGAGG/+ and 5'-GGACATCTAAGGGCATCACA/-. Reactions with 25- μ l reaction mixtures were set up, and qRT-PCRs of both viral RNA and 18S rRNA were carried out under the same conditions by using an ABI Prism 7300 sequence detection system (Applied Biosystems), with a one-step RT consisting of one cycle at 50°C for 20 min and one cycle at 95°C for 5 min, and a two-step PCR procedure consisting of 15 s at 95°C and 1 min at 60°C for 40 cycles.

Immunofluorescence assay. BHK-15 cells were grown on plastic coverslips, infected with YFV, overlaid with a medium (MEM containing 5% FBS) containing compounds at various concentrations, and incubated at 37°C. At 24 h postinfection, cells were fixed with methanol at room temperature for 15 min. Following fixation, cells were washed with PBS and incubated with a 10-mg/ml bovine serum albumin (BSA) solution in PBS. Cells were then incubated with the primary antibody for 45 min at room temperature with gentle rocking. A primary monoclonal antibody against YFV E (Chemicon) was diluted 1:100 in 10 mg/ml BSA solution. Following incubation with the primary antibody, cells were washed with PBS and incubated with a 1:200 dilution of a Texas red-conjugated antimouse monoclonal antibody (Chemicon) in a 10-mg/ml BSA solution. Following incubation with the secondary antibody, cells were washed with PBS, and coverslips were mounted on a slide, cell side down, in Fluorosave reagent (Gibco), for examination by fluorescence microscopy. Fluorescent images were visualized using an epifluorescent microscope equipped with a digital charge-coupled device camera (Nikon).

Revertant viruses. BHK-15 cells were infected with YFV at an MOI of 0.1, overlaid with medium containing CCG-3394 at a concentration of 10 μ M, and incubated at 37°C. At 4 days postinfection, culture supernatants (labeled as P1) were harvested, and plaque assays were performed in the presence of CCG-3394 (10 μ M). The plaque phenotypes obtained for P1 supernatants were compared with those obtained for parental YFV in the presence of the compound. Plaques obtained for P1 supernatants were small, similar in size to those obtained for parental YFV in the presence of the compound, suggesting a lack of revertant viruses in the P1 supernatants. Naïve BHK-15 cells were then infected with 250 μ l of the P1 supernatants and were subsequently overlaid with medium containing CCG-3394 at a concentration of 10 μ M and then incubated at 37°C. At 4 days postinfection, P2 supernatants were harvested and used for plaque phenotype analysis. This procedure was repeated for four passages (P4 supernatants). Plaque phenotype analysis of P4 supernatants obtained from CCG-3394-treated cells revealed the presence of plaques larger than those obtained for parental YFV in the presence of the compound, suggesting the presence in the P4 supernatants of viruses that had escaped inhibition by CCG-3394. A similar procedure was used to obtain a virus resistant to compound CCG-4088, but the virus was grown with the compound at concentrations of 1 μ M and 7.5 μ M. In the presence of CCG-4088 at 7.5 μ M, but not 1 μ M, large plaques mixed with

small plaques were present in the P2 and P3 supernatants, whereas the P4 supernatant had all large plaques. The plaques were then amplified using standard procedures to obtain virus stocks. A monolayer of BHK-15 cells in a 35-mm-diameter plate was infected with 250 μ l of the revertant virus stock, and viral RNA was extracted at 24 h postinfection. RT-PCR was performed as described above to obtain fragments representing the entire virus genome, which were subsequently sequenced to identify changes in the revertant virus genome.

RESULTS

HTS assay for discovery of small-molecule inhibitors of YFV genome replication. YFV PIPs were produced by sequential electroporation of YF-R.luc2A-RP and SIN-CprME transcripts as described previously (10) (Fig. 1A and B). Screening of compounds in a high-throughput manner for inhibitors of YFV genome replication was performed at the CCG facility at the University of Michigan. The assay involved infection of a monolayer of BHK-15 cells in 100-mm-diameter plates with YFV PIPs and incubation at 37°C for 2 h. These cells were then trypsinized, resuspended in medium, and aliquoted into 384-well plates already spotted with compounds. Following incubation at 37°C for an additional 28 h, luciferase activity in cells was measured using the *Renilla* luciferase substrate EnduRen (Fig. 1C). For the measurement of luciferase activity, EnduRen is directly added to cells without the tedious need to obtain cell extracts, rendering it particularly useful for the HTS assay. Ribavirin (50 μ M), a known inhibitor of flavivirus genome replication, was used as a positive control for inhibition and to monitor screening reliability, and cells treated with 0.5% DMSO were used as a negative control.

A flow chart of the various stages of the compound-screening process is shown in Fig. 1D. In the primary screening, more than 34,000 compounds were tested at a single concentration of approximately 10 μ M. Five hundred ten compounds that demonstrated reductions in luciferase activity of >50% from that for the ribavirin control were selected. The statistical performance of the HTS assay was evaluated by calculating the coefficient of variation (expressed as a percentage) and the Z factor (32). Additionally, compounds that were found to be hits in other cell-based assays completed at the CCG were eliminated on the premise that they either were nonspecific, were cytotoxic, or directly inhibited luciferase activity; 196 remaining compounds were selected for dose-response analyses. For the dose-response assays, compounds with ninefold dilutions ranging from 10^2 μ M to 10^{-6} μ M were spotted onto 384-well plates, and an inhibition assay was performed. Inhibition curves were plotted with two replicates for each compound concentration, and the EC_{50} s were determined. Twenty-one compounds displayed EC_{50} s of <1 μ M, and larger quantities of six of these compounds were obtained for further biological assays (Table 1).

Compounds were tested for their inhibitory activities against YF-R.luc2A-RP in a low-throughput assay, which differed slightly from the HTS assay. In the low-throughput assay, cells in a 96-well plate were infected with PIPs and then overlaid with medium containing compounds at various dilutions ranging from 50 μ M to 10^{-5} μ M. Cell extracts were taken at 30 h postinfection, and luciferase assays were performed using a standard *Renilla* luciferase substrate. Ribavirin (50 μ M) was used as a control for inhibition, and cells treated with 1% DMSO served as a negative control. Inhibition curves were

FIG. 1. Schematic of constructs used and experimental design of the high-throughput assay. (A) (Top) Schematic representation of the YFV genome, showing regions coding for the structural (open rectangles) and nonstructural (light shaded rectangles) proteins that are translated as a polyprotein. The 5' and 3' nontranslated regions (NTRs) are depicted as solid lines. (Center) Schematic representation of the YFV replicon construct, YF-R.luc2A-RP (10). Light shaded rectangles represent the YFV nonstructural protein coding sequence. The star indicates the presence of the RNA cyclization sequence that is located within the coding sequence for the C protein and so is retained in the replicon. The filled box represents the NS1 signal sequence. R.Luc, *Renilla* luciferase; 2A, a 17-amino-acid autoproteolytic peptide from foot-and-mouth disease virus. (Bottom) Schematic representation of the Sindbis virus replicon construct used to express YFV structural proteins, SIN-CprME (10). The coding sequence for the Sindbis virus nonstructural proteins nsp1 to nsp4 is indicated. The YFV structural proteins (open boxes) are expressed from the Sindbis virus subgenomic promoter (bent arrow). Lines indicate 5' and 3' NTRs. The poly(A) tail at the 3' end is also shown. (B) Schematic for packaging of YF-R.luc2A-RP into YFV PIPs. YFV PIPs were produced by sequential electroporation of YF-R.luc2A-RP and SIN-CprME transcripts. PIPs produced were tested for their efficiency by infecting naive BHK-15 cells and, at 24 h postinfection, measuring luciferase activity in these cells. (C) Schematic of the experimental design of the high-throughput assay. BHK-15 cells were infected with PIPs, trypsinized, and aliquoted into 384-well plates spotted with compounds (10 μ M). At 30 h postinfection, luciferase activity in these cells was measured. (D) Flow chart of the various stages of the compound-screening process leading to the selection of hits.

then plotted and EC₅₀s determined for the six compounds listed in Table 1. Inhibition curves for compounds CCG-4088, CCG-3394, and ribavirin are shown in Fig. 2A. Average EC₅₀s for CCG-4088 and CCG-3394 were 0.4 μ M and 1.48 μ M, respectively. The slight discrepancy between the EC₅₀s obtained by the HTS assay (performed at the CCG, University of Michigan) and the follow-up assays (performed at Purdue University) could be attributed to the minor differences in the assay procedures and/or in stock compounds.

These compounds were subsequently tested in BHK-15 cells at 90% confluence for their cytotoxicity using the Quick Cell Proliferation kit, a standard XTT-based cell cytotoxicity kit (BioVision Inc.), as described in Materials and Methods. Cytotoxicity curves were plotted, and CC₅₀s were determined and used to calculate the selective index (SI) for each compound (Table 1). Compounds CCG-4088 and CCG-3394 displayed SI values of 32.7 and 20.7, respectively, and were selected for further virological assays.

Compounds CCG-4088 and CCG-3394 inhibit YF-R.luc2A-RP replication. Since the compounds were selected in a screen against YFV replicon replication, they could be active in inhibition either of translation of viral proteins, of amplification of the RNA genome, or of luciferase activity itself, any of which would result in a reduction in the luciferase readout. In order to check whether the compounds inhibited viral genome translation, BHK-15 cells were infected with PIPs and overlaid with medium containing either DMSO (1%), CCG-4088 (10 μ M), CCG-3394 (10 μ M), ribavirin (20 μ M), or cycloheximide (1 mg/ml). Cell extracts were taken at 2, 4, 6, 8, 12, 24, and 36 h postinfection, and luciferase activity was measured. Luciferase activity at early time points is indicative of translation of the input viral RNA, while luciferase activity at later time points represents proteins produced from RNA generated by replication. Luciferase readings at early time points were similar for cells treated with DMSO, CCG-4088, or CCG-3394, suggesting that the compounds had no effect on viral genome translation.

TABLE 1. Chemical structures and inhibition data of YFV lead compounds

Compound	Structure	EC ₅₀ (μM) by:		SI ^c
		HTS assay ^a	Follow-up dose-response analysis ^b	
CCG-4088		0.13 ± 0.2	0.4 ± 0.2	32.7
CCG-3394		0.16 ± 0.33	1.48 ± 0.42	20.7
CCG-3925		0.43 ± 0.32	1.9 ± 0.32	6.2
CCG-7648		0.64 ± 1.37	12.6 ± 11.3	5.3
CCG-6269		0.18 ± 0.33	0.32 ± 0.15	4.7
C CCG-3374		0.41 ± 0.11	2.3 ± 0.04	1.8

^a One experiment was performed in duplicate.^b Three experiments were performed in triplicate.^c Calculated as the CC₅₀ divided by the EC₅₀ obtained by follow-up dose-response analyses.

(Fig. 2B). Additionally, as shown in Fig. 2B, at later time points luciferase activity was dramatically lower for CCG-4088- and CCG-3394-treated cells than for DMSO-treated cells, suggesting that these compounds likely inhibited viral genome replication. This was confirmed by the observation that the inhibition profiles for CCG-4088 and CCG-3394 were similar to those obtained for ribavirin, which was used as a control for the inhibition of replication. As expected, luciferase activity in cells treated with cycloheximide (a known inhibitor of translation) was abolished at early time points as well as at late time points. Furthermore, a similar inhibition assay was performed on cells transfected either with the SIN-Luc replicon (a Sindbis virus replicon expressing luciferase in a replication-dependent manner) or with pRL-CMV (a mammalian expression vector expressing the luciferase gene), and no reduction of luciferase activity was observed (data not shown). These observations suggested that CCG-4088 and CCG-3394 acted specifically on the YFV replicon and did not affect YF-R.luc2A-RP RNA translation or protein

expression but inhibited some subsequent step, presumably RNA replication.

In order to directly verify whether CCG-4088 and CCG-3394 inhibited YF-R.luc2A-RP RNA amplification, amounts of viral RNA in infected cells were determined in the presence of compounds. BHK-15 cells were infected with PIPs and overlaid with medium containing either DMSO (1%) or CCG-4088 or CCG-3394 at concentrations ranging from 10 μM to 0.01 μM, and cytoplasmic RNA was isolated from these cells. Absolute viral RNA amounts were determined using qRT-PCR with virus-specific oligonucleotide sequences. The amounts of viral RNA were normalized to amounts of cellular 18S rRNA (used as an internal control), and amounts of RNA in cells treated with compounds were compared to those in DMSO-treated cells. As shown in Fig. 2C, more than 20-fold and 15-fold reductions in RNA amounts were obtained with 10 μM concentrations of CCG-4088 and CCG-3394, respectively. Levels of repression were progressively reduced at lower

FIG. 2. Analysis of the inhibitory activities of compounds CCG-4088 and CCG-3394 using YF-R.luc2A-RP. (A) EC_{50} curves of CCG-4088 and CCG-3394 for the inhibition of YF-R.luc2A-RP replication. The EC_{50} curve of ribavirin, used as a positive control for inhibition of YF-R.luc2A-RP replication, is also shown. Results are representative of three independent experiments carried out in triplicate. Luciferase activity is expressed in relative light units (RLU). (B) Effects of CCG-4088 and CCG-3394 on viral translation. BHK-15 cells infected with PIPs were treated with either DMSO (1%), CCG-4088 (10 μ M), CCG-3394 (10 μ M), ribavirin (30 μ M), or cycloheximide (1 mg/ml). Extracts were taken at 2, 4, 6, 8, 12, 24, and 36 h postinfection, and luciferase activity was measured. The results are averages for three experiments. (C) Effects of CCG-4088 and CCG-3394 on viral RNA amplification. BHK-15 cells infected with PIPs were treated with DMSO (1%), CCG-4088, or CCG-3394 at concentrations of 10 μ M, 1 μ M, 0.1 μ M, and 0.01 μ M. Cytoplasmic RNA was harvested, and viral RNA amounts were determined by qRT-PCR. Viral RNA amounts were normalized to cellular 18S rRNA amounts (also determined by qRT-PCR) and compared with viral RNA amounts in DMSO-treated cells. Reductions (n -fold) in RNA amounts in cells treated with CCG-4088 and CCG-3394 are shown. The dashed line indicates RNA amounts in DMSO-treated cells (no reduction). The results are averages for three experiments. Error bars indicate standard deviations. (D) Effect of the time of addition of compound CCG-4088 or CCG-3394 on YF-R.luc2A-RP replication. BHK-15 cells were infected with PIPs and treated with various dilutions of CCG-4088 (left) or CCG-3394 (right) at 0, 1, 2, 3, 6, and 12 h postinfection. Cell extracts were taken at 24 h after the addition of the compound, and luciferase activity was measured. Luciferase activity in compound-treated cells was compared to that in cells treated with 1% DMSO, and the percentage of activity was plotted. The results are averages for three experiments carried out in duplicate. Error bars indicate standard deviations.

concentrations of the compounds, with approximately fourfold and twofold repression for CCG-4088 and CCG-3394, respectively, at 1 μ M concentrations, suggesting that these compounds were, directly or indirectly, inhibiting some step in YFV RNA replication.

It was of interest to investigate the effect of the time of

compound addition on viral RNA replication. For this purpose, BHK-15 cells were infected with PIPs and overlaid with medium. Subsequently, a medium containing either DMSO (1%) or various dilutions of the compounds was added to the infected cells at either 0, 1, 2, 3, 6, or 12 h postinfection. Cell extracts were taken at 24 h after the addition of compounds,

FIG. 3. Effects of compounds CCG-4088 and CCG-3394 on YFV propagation. (A) Inhibition of YFV propagation in the presence of CCG-4088 and CCG-3394. BHK-15 cells were infected with YFV at an MOI of 0.1 and were treated with various concentrations of CCG-4088 or CCG-3394. An immunofluorescence assay was performed at 40 h postinfection to detect E protein-expressing cells as a measure of virus spread. Infected cells treated with DMSO (1%) were used as a control. (B) Effects of CCG-4088 and CCG-3394 on virus release using cumulative growth curves. BHK-15 cells were infected with YFV at an MOI of 1 and were treated with various concentrations of CCG-4088 or CCG-3394. Culture supernatants were harvested at 12, 24, 36, and 48 h postinfection, and titers of infectious virus released were calculated by plaque assays. Infected cells treated with DMSO (1%) were used as a control. The results are averages for two experiments.

and luciferase assays were performed. Interestingly, addition of CCG-4088 or CCG-3394 to infected cells at times up to 3 h postinfection resulted in inhibitory activity almost similar to that for addition of compounds right after PIP infection (0 h) (Fig. 2D). However, a dramatic reduction in inhibition levels was observed upon addition of compounds later than 3 h postinfection. This suggests that perhaps these compounds inhibit some early step in the RNA replication cycle and that once replication has advanced beyond a certain point, the efficacy of compounds (especially at lower concentrations) decreases.

Compounds CCG-4088 and CCG-3394 inhibit YFV growth.

The abilities of CCG-4088 and CCG-3394 to inhibit YFV propagation were also investigated. BHK-15 cells were infected with YFV at a low MOI (0.1). Cells were then overlaid with medium containing either DMSO (1%) or compounds at various dilutions, and at 40 h postinfection, cells were fixed, and an immunofluorescence assay was performed using an anti-E antibody. In the DMSO-treated sample, almost all cells expressed E protein, suggesting that there was efficient virus release followed by reinfection (Fig. 3A). However, very few of the cells

treated with either CCG-4088 or CCG-3394 were positive, especially at the 10 μ M and 1 μ M concentrations, suggesting inhibition of the spread of YFV in the presence of the compounds.

To characterize the growth properties of YFV in the presence of the compounds, cumulative growth curves were performed. BHK-15 cells were infected with YFV and overlaid with medium containing either DMSO (1%) or compounds at various dilutions, and culture supernatants was harvested at different time points postinfection. The titer of infectious virus released was then calculated by a standard plaque assay using BHK-15 cells. As shown in Fig. 3B, the amounts of infectious virus released from cells treated with compounds at concentrations of 10 μ M and 1 μ M were at least 1 to 2 log units lower than those from DMSO-treated cells at all the time points assayed. The inhibition of virus release was progressively reduced at lower concentrations of the compounds, in agreement with the results obtained using the YF-R.luc2A-RP replicon.

Additionally, a known number of PFU was used to infect a monolayer of BHK-15 cells, which were then overlaid with agarose containing either DMSO (1%) or compounds at a 10

μM concentration. Although the numbers of plaques obtained were similar in DMSO- and compound-treated cells, the plaques obtained in compound-treated cells were very small compared to those obtained in DMSO-treated cells (data not shown), presumably as a consequence of the reduction in the level of replication in the presence of the compounds.

Isolation and characterization of CCG-3394-resistant YFV.

In order to isolate mutant viruses that escape compound inhibition and to facilitate the identification of the site of action of CCG-4088 and CCG-3394, YFV was passaged several times in the presence of either CCG-4088 or CCG-3394. At each passage, the culture medium was harvested, and the viruses released were analyzed by a plaque assay in the presence of the compound. The fourth passage in the presence of CCG-3394 yielded viruses that displayed a large-plaque phenotype in the presence of CCG-3394, as opposed to small plaques that were obtained for supernatants taken from previous passages, indicating the emergence of escape mutants in passage 4. Individual virus clones were then isolated and amplified, and viral RNA was completely sequenced. Sequencing revealed the presence of only one point mutation, a change from A to G at nucleotide 7270 that resulted in a lysine-to-arginine change at amino acid 128 of NS4B (K128R). Plaque assays of the second and third passages of YFV in the presence of compound CCG-4088 at 7.5 μM , but not 1 μM , revealed a mixture of large and small plaques. The plaques of the virus from passage 4 in 7.5 μM CCG-4088 were all large. Sequencing of this virus revealed two point mutations. The first mutation was the same A-to-G change at nucleotide 7270, and the second mutation was an A-to-G change at nucleotide 9178 that resulted in a glutamine-to-proline change at amino acid 514 of NS5 (Q514P).

To obtain direct evidence that the K128R mutation was responsible for the CCG-3394-resistant phenotype, the point mutation was engineered into the YFV infectious cDNA clone. K128R RNA transcripts were transfected into BHK-15 cells using DEAE-dextran, and the cells were incubated at 37°C. At 3 days posttransfection, plaques were picked and amplified, and a K128R virus stock was generated. Virus propagation assays were then performed as described previously in order to check whether the K128R virus can escape CCG-3394 inhibition. Briefly, BHK-15 cells were infected with either the parental (wild-type [WT]) or the K128R virus at a low MOI and were then overlaid with medium containing CCG-3394; at 40 h postinfection, an immunofluorescence assay was performed to determine the number of virus-infected cells. As expected, very few positive cells were obtained for WT-infected cells in the presence of CCG-3394. However, the numbers of positive K128R virus-infected cells treated with CCG-3394, although slightly lower than those of positive DMSO-treated cells, were at least 20-fold higher than those of positive WT-infected cells treated with the same CCG-3394 concentration, suggesting that the K128R substitution indeed conferred CCG-3394 resistance (Fig. 4A). In order to test whether the K128R virus also escapes CCG-4088 inhibition, a similar experiment was performed in the presence of CCG-4088. Again, the numbers of E protein-positive-K128R virus-infected cells treated with CCG-4088 were more than 20-fold higher than the numbers of positive WT-infected cells treated with the same CCG-4088 concentration, suggesting that the K128R change also conferred CCG-4088 resistance (Fig. 4A).

To characterize the growth properties of the K128R virus in the presence of the compounds, cumulative growth curves were performed as described above, and the titer of infectious virus released was calculated by a standard plaque assay. As shown in Fig. 4B, the growth kinetics of parental (WT) and K128R viruses were similar in DMSO-treated samples. However, larger amounts of K128R virus than of WT virus were released at all time points from cells treated with either CCG-4088 or CCG-3394.

In order to confirm the resistance of K128R to CCG-4088 and CCG-3394, the K128R mutation was engineered into YF-R.luc2A-RP, and luciferase activity in the presence of compounds was measured. Luciferase activity for the K128R replicon was 10- to 20-fold higher (depending on the concentration of the compound) than that for the WT replicon in the presence of CCG-4088 or CCG-3394, confirming previous observations with the infectious virus (Fig. 4C). Additionally, ribavirin and compound CCG-6269 (another compound that was active in the high-throughput assay) were found to inhibit WT and K128R replicons to similar levels, indicating that the K128R mutation specifically confers resistance to inhibition by CCG-4088 and CCG-3394. It should be noted that the resistance is not absolute; there is still some inhibition of the K128R virus in the presence of the compounds.

In order to determine if the Q514P mutation in NS5 conferred resistance to compound CCG-4088, the individual Q514P mutation and the two mutations together (K128R and Q514P) were engineered into the YFV cDNA infectious clone. Standard plaque assays were performed both in the presence and in the absence of compound CCG-4088. The dual mutation (K128R Q514P) and the single NS4B mutation (K128R) demonstrated parental-virus (large) plaque sizes in the absence of CCG-4088 and in the presence of CCG-4088. Parental YFV demonstrated small plaques in the presence of CCG-4088. The Q514P mutation by itself demonstrated medium-sized plaques in the absence of CCG-4088 and small plaques in the presence of the compound. Virus initially produced with the single Q514P mutation reverted to the parental sequence by the second passage, suggesting that this mutation is less stable than the K128R or dual mutation.

Compounds CCG-4088 and CCG-3394 act together to inhibit YF-R.luc2A-RP replication. Unexpectedly, the K128R mutation resulted in resistance to both compounds CCG-4088 and CCG-3394, suggesting that CCG-4088 and CCG-3394 could be targeting a common site, perhaps the region around K128 in NS4B. Thus, it was of interest to investigate whether CCG-4088 and CCG-3394 act additively for inhibition. Additivity in this context is defined as an overall effect of the two compounds in combination that is higher than the effective inhibition obtained using either of the two compounds singly.

Briefly, BHK-15 cells were infected with PIPs and overlaid with medium containing either CCG-4088 or CCG-3394 or both together at various concentrations, and luciferase activities at 30 h postinfection were compared. For accurate interpretation of results, it was surmised that the total effective concentration of compounds mixed together would have to be similar to the effective concentrations of compounds added individually (based on luciferase activity). Thus, in order to achieve equivalent effective concentrations of each compound, CCG-4088 and CCG-3394 were combined such that the final

FIG. 4. Analysis of the NS4B K128R revertant. (A) Effects of CCG-4088 and CCG-3394 on K128R virus infection. BHK-15 cells were infected with either the parental (WT) or the K128R virus at an MOI of 0.1 and were treated with various concentrations of CCG-4088 or CCG-3394. An immunofluorescence assay was performed at 40 h postinfection to detect E protein-expressing cells as a measure of virus spread. Infected cells treated with DMSO (1%) were used as a control. (B) Effects of compounds on K128R virus release using cumulative growth curves. BHK-15 cells were infected with either the parental (WT) or the K128R virus at an MOI of 1 and were treated with either DMSO (1%), CCG-4088 (10 μ M), or CCG-3394 (10 μ M). Culture supernatants were harvested at 12, 24, 36, and 48 h postinfection, and titers of infectious virus released were calculated by standard plaque assays. The results are averages for two experiments. (C) Effects of compounds on K128R replicon replication. BHK-15 cells were infected with either parental (WT) or K128R PIPs and were treated with either DMSO (1%) or various concentrations of CCG-4088, CCG-3394, or CCG-6269. Cell extracts were taken at 24 h postinfection, and luciferase activity was measured. Luciferase activity in DMSO-treated cells was set to 100%, and the percentage of luciferase activity in compound-treated cells was subsequently calculated and plotted. The results are averages for three experiments carried out in duplicate. Error bars indicate standard deviations.

concentrations of each compound in the mixture were either 1 μ M and 10 μ M, 0.1 μ M and 1 μ M, or 0.01 μ M and 0.1 μ M, respectively. Also, it was inferred that the effective concentration of compounds when added individually would have to be similar to the total effective concentration of compounds when mixed together. Therefore, the concentration of CCG-4088 when added alone was either 2 μ M, 0.2 μ M, or 0.02 μ M, and that of CCG-3394 alone was either 20 μ M, 2 μ M, or 0.2 μ M. As shown in Fig. 5, the luciferase activity in cells treated with a mixture of CCG-4088 and CCG-3394 was significantly lower at all concentrations tested than the luciferase activity in cells treated with either CCG-4088 ($P \leq 0.02$) or CCG-3394 ($P \leq 0.01$), suggesting that CCG-4088 and CCG-3394 act additively for inhibition of YF-R.luc2A-RP replication. The lack of resistance of the K128R mutant to CCG-6269 inhibition (see the preceding section) implied that the target site (or inhibitory mechanism) for CCG-6269 is probably different from that for CCG-4088 or CCG-3394. Therefore, it was reasoned that CCG-6269 would act additively when combined with either CCG-4088 or CCG-3394 and thus would serve as a control for displaying this additive activity. Indeed, luciferase activity in cells treated with a

combination of CCG-6269 and CCG-4088 or a combination of CCG-6269 and CCG-3394 was lower than that in cells treated with either CCG-6269, CCG-4088, or CCG-3394 alone (Fig. 5).

DISCUSSION

Recent advances in the molecular biology of flaviviruses have led to the development of various reagents and assays that are amenable to high-throughput compound screens in order to discover antiviral compounds. HTS assays for screening inhibitors of the enzymatic activity of flaviviral proteins, specifically the protease and helicase, are useful and have been reported previously (5, 19). However, cell-based HTS methods offer the potential advantage of including antiviral targets that are not amenable to biochemical screening methods. For flaviviruses, two types of cell-based HTS assays have been reported; one method involves the treatment of cell lines harboring persistently replicating flavivirus genomes expressing a reporter gene (either a green fluorescent protein gene or a luciferase gene) with small-molecule compounds, and the second method involves infecting cells with PIPs (which contain a

FIG. 5. Compounds CCG-4088 and CCG-3394 act together for the inhibition of YF-R.luc2A-RP replication. BHK-15 cells were infected with PIPs and then treated with either DMSO (1%) or various concentrations of CCG-4088, CCG-3394, or CCG-6269, either alone or in combination with each other, as indicated. Cell extracts were taken at 24 h postinfection, and luciferase activity was measured. Luciferase activity in DMSO-treated cells was set to 100%, and the percentage of luciferase activity in compound-treated cells was subsequently calculated and plotted. Results are averages for two experiments carried out in duplicate. Error bars indicate standard deviations.

replicon genome expressing a specific reporter gene) and then treating the infected cells with compounds (8, 22, 24). In both cases, the selection of hits is based on the reduction in reporter gene expression as a readout for genome replication.

In this report, we describe the development and application of a YFV replicon-based HTS assay using PIPs, leading to the discovery of compounds with potent antiviral activity. The use of PIPs as a tool for screening compounds for the inhibition of genome replication is ideal in that PIPs are safe, because no infectious virus is produced and no complications in the analysis arise due to virus spread and reinfection. Briefly, BHK-15 cells were infected with PIPs containing the YF-R.luc2A-RP replicon and were treated with compounds, and at 30 h postinfection, luciferase activity in the infected cells was measured. A reduction in the level of luciferase expression in infected cells was an indicator of the inhibition of replicon replication by the compound. A primary screening with more than 34,000 compounds was performed, and compounds were selected for the secondary screen based on their activity (50% inhibition compared to ribavirin), specificity, and reproducibility. Confirmatory screening was performed using a dose-response assay (compound concentrations ranging from 100 μ M to 10 nM), and compounds with EC_{50} s of <1 μ M were selected and analyzed further in low-throughput assays. Based on our data, the replicon-based HTS assay described here was reproducible and accurate. One advantage of using this PIP-based assay over using a replicon-based cell line is that the possibility exists of adaptive mutations arising in the persistent replicon in the cell line, perhaps resulting in erroneous conclusions in the inhibition assays, and such a possibility is precluded in our PIP-based assay (23, 25). Therefore, the results obtained are more reproducible when compounds are tested for inhibition of full-

length genome or virus. Ribavirin, a known inhibitor of flavivirus replication, displayed dose-dependent inhibition of YFV replication, with an EC_{50} of approximately 20 μ M (as observed previously), further highlighting the accuracy of our HTS assay (1, 2). However, it should be noted that, like all replicon-based assays, this PIP-based assay selects for potential inhibitors of viral genome translation or replication only and not for inhibitors of viral entry, assembly, or release. Furthermore, since compounds were added to cells within 2 h after infection with PIPs, the effects of compounds only at the early stages of replication were analyzed. This was evident in the time-of-addition experiment, where reductions in inhibitory activity were observed upon the addition of compounds at 12 h postinfection, presumably when there were nearly peak levels of genome replication, compared to the addition of compounds immediately after PIP infection (0 h).

Nevertheless, it was shown that compounds CCG-4088 and CCG-3394 efficiently inhibited YFV replication with EC_{50} s of 0.4 μ M and 1.48 μ M, respectively. Both compounds appear to have relatives that are known viral inhibitors; CCG-3394 is a secondary sulfonamide, a class of compounds that has been found to inhibit WNV replication, although the secondary sulfonamides have been reported to inhibit early translation of flavivirus RNA, whereas CCG-3394 did not seem to affect RNA translation in our experiments (22). CCG-4088 has a physical similarity to a compound (C6131864) that has been demonstrated to be an activator of RNase L, an antiviral protein that cleaves single-stranded RNA (28). It will be interesting to determine the mechanisms of action of these compounds and to assess whether RNase L plays any role in their inhibition. Using immunofluorescence assays and cumulative growth curve analysis, it was demonstrated that compounds CCG-4088

and CCG-3394 inhibited the growth of YFV. It should be noted that the efficacy of compounds against YFV was slightly lower than their activity against the YFV replicon. This difference could be a result of virus release and reinfection occurring in virus-infected cells, which is precluded in PIP-infected cells. Finally, we were able to isolate escape mutants to both CCG-3394 and CCG-4088. The mutations were mapped, and both resulted in a change of a lysine at position 128 in NS4B to arginine (K128R), suggesting a common mechanism of inhibition of YFV by CCG-4088 and CCG-3394, involving NS4B. Subsequently, a virus resistant to CCG-4088 that had the same K128R mutation in NS4B was identified. However, this revertant to CCG-4088 also had a mutation in NS5, with a glutamine-to-proline substitution at amino acid 514.

The NS5 Q514P mutation is near the active site of the RNA-dependent RNA polymerase domain of NS5. The mutation by itself did not confer resistance to CCG-4088, as demonstrated by small plaques in the presence of the compound, and the mutant also demonstrated smaller plaques than the parental virus in the absence of the compound. However, the dual (K128R Q514P) mutant is stable and demonstrates parental virus growth characteristics, perhaps suggesting an interaction (protein-protein, or genetic) between NS4B and NS5.

Flavivirus NS4B is a small (248-residue) hydrophobic protein that is indispensable for genome replication (16, 17). Although immunoprecipitation studies using antibodies to double-stranded RNA failed to detect NS4B in the replication complex, deletions or insertions within NS4B have been shown to inhibit the replication of KUN and bovine viral diarrhea virus, an animal pathogen belonging to the family *Flaviviridae* (6, 13). DENV NS4B has also been shown to interact with NS3 and to enhance NS3 helicase activity (30). The NS4B proteins of YFV, WNV, and DENV have been shown to partially block the activation of STAT1/STAT2 and interferon-stimulated response element promoters in cells stimulated with interferon (11, 21). NS4B has also been detected in the nuclear and perinuclear regions of infected cells, but the precise role of the nuclear localization of NS4B is not known (31). NS4B of hepatitis C virus, a member of the family *Flaviviridae*, has also been shown to be sufficient for the induction of morphological changes in the endoplasmic reticulum membrane that are also characteristic and probably important for flavivirus replication (7). Thus, although NS4B is thought to play several important roles in flavivirus replication, its exact functions are unclear. Presumably, based on the K128R escape mutant, the inhibitory activities of CCG-4088 and CCG-3394 are a consequence of affecting one or more functions of NS4B, but it is difficult to identify exactly the target site or mode of action of the compounds. NS4B residue 128 is predicted to be at the end of a transmembrane domain, on the cytoplasmic side of the endoplasmic reticulum membrane, and it is outside of the region determined to be needed for interferon inhibition in DENV (11, 17). Although these data support our conclusion that CCG-4088 and CCG-3394 act additively to inhibit YFV-R.luc2A-RP replication, it is difficult to hypothesize how the K128R mutation results in a virus that can escape inhibition by these compounds.

In summary, we have developed and performed a simple, robust, and reproducible HTS assay for inhibitors of YFV replication. Several novel inhibitors of YFV replication were

identified. The results have been encouraging for further drug development efforts, including hits-to-lead optimization, structure-activity studies, pharmacological analysis, and animal infectivity assays, and these studies are in progress.

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