Available online at www.sciencedirect.com
SCIENCE DIRECT
Antiviral Research 70 (2006) 39–50
Antiviral Research
www.elsevier.com/locate/antiviral

Discovery of small molecule inhibitors of West Nile virus using a high-throughput sub-genomic replicon screen

Baohua Gu [a,*], Serguey Ouzunov [a], Liguan Wang [a], Peter Mason [b,c], Nigel Bourne [c,d], Andy Cuconati [e], Timothy M. Block [a,e]

[a] *Drexel Institute for Biotechnology and Virology Research, Drexel University, College of Medicine, 700 E. Butler Avenue, Doylestown, PA 18901, Unite States*
[b] *Department of Pathology, University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555, Unite States*
[c] *Sealy Center for Vaccine Development, University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555, Unite States*
[d] *Department of Pediatrics, University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555, Unite States*
[e] *Institute for Hepatitis and Virology Research, 700 E. Butler Avenue, Doylestown, PA 18901, Unite States*

Received 27 September 2005; accepted 9 January 2006

Abstract

West Nile virus (WNV) is a positive-sense, single-stranded RNA virus of the family Flaviviridae. WNV persistently infects insect cells, but can causes acute cytopathic infection of mammalian cells and is an etiologic agent of viral encephalitis in humans. By using a cell line expressing a WNV subgenomic replicon [Rossi, S.L., Zhao, Q., O'Donnell, V.K., Mason, P.W., 2005. Adaptation of West Nile virus replicons to cells in culture and use of replicon-bearing cells to probe antiviral action. Virology 331 (2), 457–470], we developed a high-throughput assay and used it to screen a library of small molecule compounds for inhibitors of WNV replication in the absence of live virus. Here we report the identification of novel small molecule inhibitors for WNV replicon replication. We demonstrate that the compounds inhibited WNV replication-dependent luciferase expression in the replicon cells and reduced WNV viral protein accumulation and viral RNA copy number in the replicon cells. Two classes of compounds with multiple hits, parazolotrahydrothophenes and pyrozolopyrimidines, showed preliminary structure–activity relationships. In WNV infection assays, one pyrozolopyrimidine compound was confirmed to have antiviral activity. These compounds should be valuable for developing anti-WNV therapeutic drugs as well as research tools to study the mechanism of WNV replication.

Keywords: High-throughput screen (HTS); West Nile virus; Small molecule inhibitor

1. Introduction

West Nile virus (WNV) is a mosquito-borne virus that was introduced into the US in 1999 (Anderson et al., 1999; Briese et al., 1999; Lanciotti et al., 1999) and has subsequently spread across the country producing tens of thousands of human infections that have resulted in several hundred deaths. Thus WNV is a significant public health concern for which there is currently no effective vaccine or antiviral drug therapy (Granwehr et al., 2004). Currently, the only available treatment is supportive, and the only existing means of prevention is mosquito control, which has had limited success. WNV is also considered to be an agent of bioterror concern (Fauci et al., 2005); thus safe and effective therapeutics are urgently needed.

WNV is a positive-sense, single-strand RNA virus that is a member of the *Flavivirus* genus of the Flaviviridae (Brinton, 2002). Its genome is 11 kilobases in length and contains both a 5′- and 3′-non-translated region (NTR). The genome encodes a single polyprotein, which is proteolytically processed by viral and cellular proteases into 10 functional proteins (Chambers et al., 1990). The genes for structural proteins, the capsid (C), membrane (M; which exists in cells as its precursor, prM), and envelope (E) are encoded in the 5′ third of the genome, while those for the nonstructural proteins (NS), NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Chambers et al., 1990) are encoded by the remainder of the genome. The virus can infect many cell types and produces plaques. However, the requirement that infectious virus work be carried out under BL3 conditions

* Corresponding author. Tel.: +1 215 489 4937; fax: +1 215 489 4920.
E-mail address: baohua.gu@drexel.edu (B. Gu).

0166-3542/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2006.01.005

makes it more difficult to use live virus assays in HTS to identify antiviral compounds.

Recently, replication competent subgenomic WNV replicon RNAs have been described (Puig-Basagoiti et al., 2005; Rossi et al., 2005; Shi et al., 2002). These replicon RNAs contain the 5′-NTR, a portion of the C-coding region, a polyprotein encoding NS1–NS5, and the 3′-NTR. Viral RNA replication requires that the viral RNA-dependent RNA polymerase, NS5, in conjunction with other viral NS proteins and possibly cell factors (Brinton, 2001), synthesize a minus strand RNA from the RNA template. The minus strand RNAs, in replicative form, in turn serve as templates for the synthesis of new genomic RNAs which also serve as the only viral mRNA in the infected cell.

A stable BHK cell line replicating WNV replicons expressing a firefly luciferase (Luc) gene co-expressed from a polyprotein containing the selectable marker gene, neomycin phosphotransferase (NPT) has been recently described (Rossi et al., 2005). Here we report the utilization of this cell line in a 96-well format high-throughput screen (HTS), and report on our initial success in using this assay to identify compounds with antiviral activity from a screen of a library of >35,000 compounds. From this screen we have identified several classes of small molecules that reduced the Luc activity, as well as WNV protein and RNA synthesis in the WNV replicon cells. In addition, the anti-viral activity of one compound was confirmed by direct demonstration of its ability to reduce WNV replication in BHK cells.

2. Materials and methods

2.1. Cell and media

The BHK 26.5 cell line stably expressing a WNV replicon encoding Luc has been described previously (Rossi et al., 2005). These cells were maintained in modified Eagle's media (MEM) with 10% FBS, penicillin/streptomycin/plasmacin (InvivoGen. San Diego, CA), and 400 μg/ml G418 and used between passages 35 and 40 to ensure reproducibility. HTS assay media contained only 3% FBS to reduce cell growth and the amount of protein that could potentially bind to test compounds, thus reducing their activity.

2.2. Chemical library

The chemical library included compounds from the collections of Asinex Inc. (Moscow, Russia), Chembridge Inc. (San Diego, CA) and Maybridge Inc. (Cornwall, UK). The compounds were selected by computational means for diversity, solubility and drug-like qualities, eliminating highly reactive groups and species known to exhibit non-specific biological effects, and exhibiting an average molecular weight of approximately 350 Da. Library stock plates ("mother" plates) contained compounds dissolved in tissue-culture grade dimethylsulfoxide (DMSO) at a concentration of 10 mM. Dilution ("daughter") plates were produced by 10-fold dilution of the compounds from the mother plates in DMSO. Both mother and daughter plates were sealed with plastic film and stored at −20 °C.

2.3. HTS assay to detect reduction in Luc activity and cytotoxicity of library compounds

BHK 26.5 cells were plated in 96-well plates at 10,000 cells/well in MEM plus 3% FBS. Twenty-four hours later, compounds were added to a final concentration of 10 μM and 1% DMSO using a Biomek NX Robotic liquid handler (Beckman Coulter, Fullerton, CA). Each 96-well plate included untreated wells, 1% DMSO-treated wells, and 2.5 μg/ml mycophenolic acid (MPA) (Sigma, St. Louis, MO) treated wells. Following 24 h incubation the media was removed and Luc activity measured by adding 100 μl/well of a 1:1 mixture of Steady Glo reagent (Promega, Madson, WI) and culture medium. After 5 min incubation, the luminescence was read on a TopCount (Perkin-Elmer, Boston, MA). Cytotoxicity was measured for primary hit compounds by MTT assay using 96-well plates incubated in parallel with a set of plates used for confirmatory Luc assays (as described above). For the MTT assay, the cell medium was removed and the monolayers were incubated with 100 μl/well of 0.5 mg/ml MTT for 6 h at 37 °C. The MTT solution was then aspirated and 100 μl/well of the 10% SDS (in 0.01N HCl) was added, followed by spectrophotometric quantitation (570 nm) of the insoluble MTT reaction product.

2.4. WNV yield reduction assay

To measure activity against live WNV, BHK cells were plated in 96-well plates at a concentration of 12,000 cells/well. One day later the cells were infected with WNV for 1 h at an MOI of 0.05. The cells were then washed once and refed with fresh DMEM containing dilutions of the test compound. Plates were then incubated at 37 °C for 48 h, the supernatant collected and the WNV produced titered. For virus titration, Vero cells were plated in 96-well plates at 8000 cells/well and incubated overnight. The Vero cell monolayer were then infected for 1 h with various dilutions of the WNV supernatant, overlaid with media containing 0.6% tragacanth (ICN, CA) and incubated at 37 °C for 48 h. The culture media was then aspirated; the plate was rinsed, air-dried, and fixed with 50 μl/well acetone/methanol (50:50). Viral foci were detected for enumeration by immunostaining as described previously (Rossi et al., 2005).

2.5. Western blot analysis

Total cell lysates were harvested from BHK 26.5 cells in 1× SDS sample buffer. The lysates were heated at 70 °C for 10 min in the presence of DTT before electrophoresis on a 10% Tris–glycine SDS polyacrylamide gel (Invitrogen) in 1× Tris–glycine buffer. The proteins were transferred to PVDF membranes (Invitrogen). Following the transfer, the membranes were rinsed once with TBS containing 0.5% Tween-20 (TBS-Tween) and blocked in TBS-Tween containing 5% non-fat milk for 1 h. After washing with PBS-Tween, the membranes were incubated with a polyclonal mouse-anti-WNV ascitic fluid (Rossi et al., 2005) at 1:3000 dilution for 1 h at 25 °C. Followed by washing and incubation with a

horseradish peroxidase (HRP)-conjugated, goat anti-mouse IgG (Amersham) diluted 1:5000. The blots were then washed in PBS-Tween, and treated with Super Signal Chemiluminescent Reagent (Pierce, Rockford, IL) according to the manufacturer's protocol and exposed to X-ray film. For controls, the blots were stripped and re-probed with an antibody to beta-actin (Chemicon, Temecula, CA), which was detected as described above.

2.6. Northern blot analysis

Total cellular RNA was extracted using the RNeasy Kit (Qiagen, Valencia, CA). Northern blot analysis was performed as described previously (Gu et al., 2003). Briefly, 5 μg total RNA was electrophoresed through a 1.0% agarose gel containing 2.2 M formaldehyde, transferred to a nylon membrane and immobilized by UV cross-linking (Stratagene, La Jolla, CA). After pre-hybridization in 5 ml of QuickHyb (Amersham, Piscataway, NJ) solution for 30 min, hybridization was carried out using alpha-[32P] dCTP-labeled probe made by random primer labeling of a 2.7 kb NS5 DNA fragment and a human beta-actin DNA at 65 °C. The membrane was washed twice in 2× SSC/0.1% SDS for 10 min at room temperature and twice in 0.1× SSC/0.1% SDS for 15 min at 68 °C. Membranes were exposed to Molecular Imager FX phosphoimager (BioRad, Hercules, CA) and the radiographic signals were collected and quantitated.

3. Results

3.1. HTS optimization

Stable cell lines with autonomously replicating sub-genomic WNV genomes have been established in several laboratories (see Section 1). Viral RNA replication in these cells involves multiple viral encoded proteins which form the replication complex in conjunction with cellular factors, and thus these cells provide multiple targets that could be disrupted by small molecule-inhibitors of viral replication. We have recently described a cell line (BHK 26.5) that stably expresses a WNV replicon that encodes a Luc reporter gene inserted in the 3′ nontranslated region (Fig. 1A). It allows viral replication to be monitored by simply measuring the Luc activity in the cells. To optimize the use of these BHK 26.5 cells in a Luc-based HTS assay, we evaluated the effects of a number of variables. To examine the effects of cell density, BHK 26.5 cells were seeded at densities ranging from 1500 to 60,000 cells/well. As shown in Fig. 1B, the Luc signal ranged from 5000 to 180,000 RLU. The signal correlated closely with cell number with a slope of approximately one at lower cell densities (Fig. 1B). We were able to consistently produced easily measurable Luc signals (30,000 RLU) at a density of 10,000 cell/well, so this density was selected for the HTS assay. Furthermore, at this density the cells did not become overly confluent during the assay, a condition in which replicon copy number might be adversely affected (Pietschmann et al.,

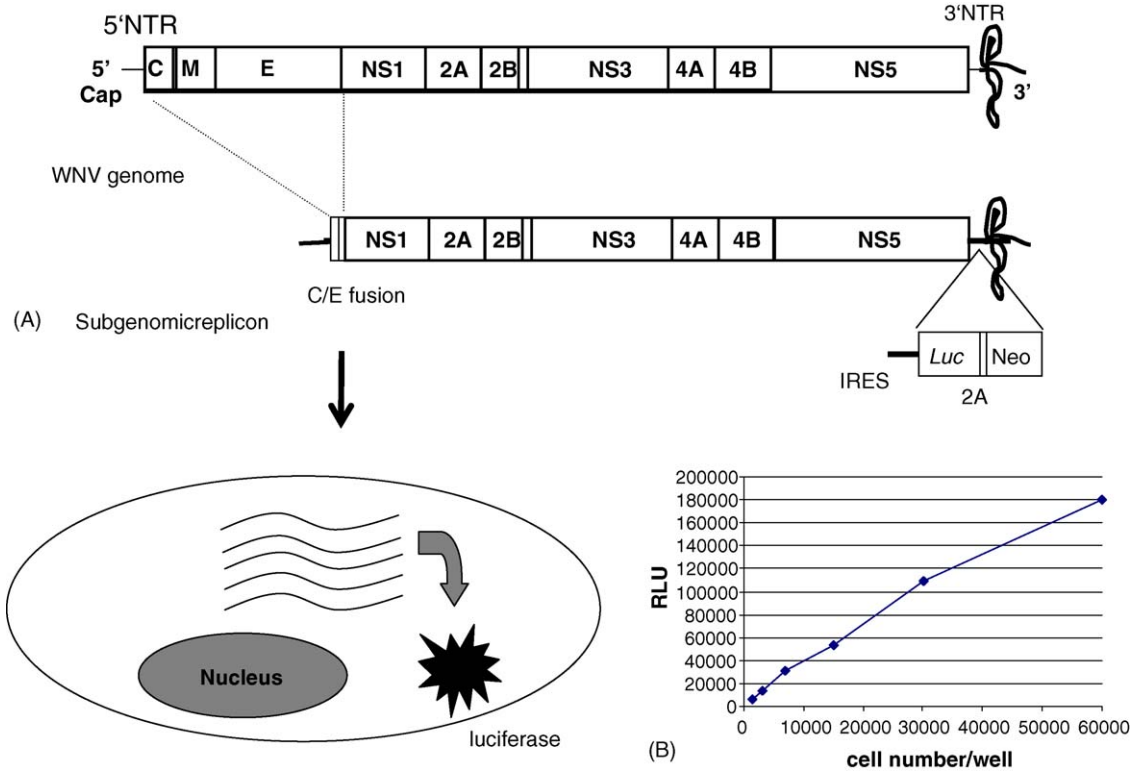


Fig. 1. (A) Schematic representation of the WNV replicon used for HTS. The WNV and the subgenomic replicon genomes are depicted with the genes (non-structural gene NS1, 2A, 2B, 3, 4A, 4B and 5) shown as boxes. The WNV structural genes (capsid (C), membrane (M) and envelope (E)) were deleted in the replicons. Luc and the Neo^r gene under the control of EMCV IRES were inserted at the 3′-NTR of the replicon. (B) Detection of Luc in 96-well plates of BHK 26.5 cells demonstrating the linear relationship between cell number and Luc activity.

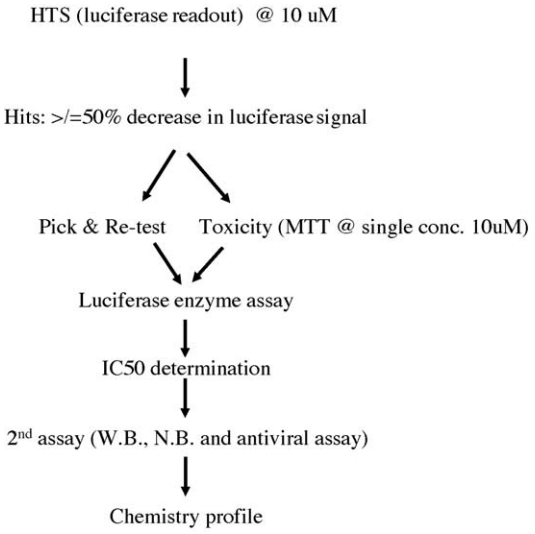


Fig. 2. Flow chart of the HTS. Compounds were tested in the HTS assay at single concentration of 10 μM in single wells. The initial candidate compounds were selected based on 50% decrease in Luc activity. The initial candidate compounds were re-tested in duplicate 96 wells plates manually for their activity and cytotoxicity. The positive compounds from the re-test were examined to rule out the possibility of inhibiting Luc enzyme activity before assayed to determine their IC50. Western blot (WB) and Northern blot (NB) assays were conducted to confirm the final candidate compounds.

2001). We next examined the effect of cell passage on Luc activity and showed that the Luc activity was stable for at least 10 passages (data not shown). All HTS assays were performed with cells that were within 5 passages from the original frozen stocks (passage 35–40 from colony selection). We also examined the impact of DMSO concentration; these studies revealed that the cells tolerated 1% DMSO, but there was a slight decrease in the Luc signal when 2% DMSO was used (data not shown). Therefore, in the HTS assay the DMSO concentration was limited to 1%.

Minimal assay signal variation and consistent high signal to background ratio is important for an HTS assay. We, therefore, determined Z', a statistical measurement of the distance between the standard deviations for the signal versus the noise of an assay, by treating multiple samples with mycophenolic acid (MPA), a known albeit nonspecific, WNV inhibitor (Morrey et al., 2002). Briefly, 40 wells of a 96-well plate were treated with 2.5 μg/ml MPA in 1% final DMSO, and 40 wells were treated with 1% DMSO only. After 24 h, the Luc was measured. The Z' calculated by the equation: $1 - (3 \times \text{S.D.}_{\text{DMSO}} + 3 \times \text{S.D.}_{\text{MPA}})/(\text{mean}_{\text{DMSO}} - \text{mean}_{\text{MPA}})$ was approximately 0.5, indicating that the assay is reliable (Zhang et al., 1999). The assay consistently produced a signal-to-background ratio of around 8-to-1. Based on these preliminary studies, we developed an HTS paradigm shown in flow-chart form in Fig. 2.

3.2. HTS screening to identify WNV inhibitors

We screened over 35,000 compounds in the HTS assay at a single concentration of 10 μM in single wells. Employing a 50% reduction in Luc as the criterion for further evaluation approximately 1100 compounds were identified (~3% hit rate). This was considerably higher than expected, suggesting that false positives from assay variation and/or cytotoxic compounds were included. Nevertheless, these compounds were picked out from the "daughter" plates and activity re-evaluated at 10 μM concentration manually. Duplicate 96-well plates were prepared for side-by-side Luc assay and toxicity determination to rule out obvious toxic compounds. Upon completion of these assays, most compounds were disqualified as either false positives (they did not reduce Luc activity in this re-test) or toxic to cells. As a result, 25 preliminary candidate compounds were selected for further analysis and triage.

3.3. Luc enzyme inhibitors were ruled out

Although the initial HTS identified compounds that reduced Luc activity without causing cytotoxicity in the WNV replicon BHK 26.5 cell line, it was conceivable that some of these inhibitors could inhibit the Luc enzyme itself rather than viral RNA replication. To examine this possibility, all 25 preliminary candidate compounds were tested in an in vitro Luc enzyme assay by incubating the compounds with cell lysates prepared from WNV replicon cells. Under such conditions, replication of WNV did not contribute to Luc activity and thus inhibitors of the Luc enzyme could be identified. Fig. 3A shows that as expected, MPA and DMSO did not reduce Luc activity in this assay. However, the luminescence signal was dramatically reduced by two of the candidate compounds, 52-F2 and 124-F3 indicating that they were active against the Luc enzyme rather than the WNV replicon. Consistent with these data, 52-F2 failed to reduce WNV NS3 protein level (Fig. 3B).

3.4. IC50 determination of the hit compounds

In an attempt to quantify and rank the activity of the 23 remaining candidate compounds, a serial dilution of each compound was prepared and assayed with the BHK 26.5 cells to determine the IC50 for the Luc readout. Fig. 4 shows the dose–response curves, which revealed that a number of compounds were active only at the highest, 10 μM concentration indicating that they are less potent. At the 10 μM highest concentrations tested, only three or four compounds produced IC90 values; due to the limited amount of each compound available we were not able to extend testing to determine IC90's for all the compounds. Although several compounds had IC50s between 2 and 5 μM, it is difficult to clearly rank the compounds based on these results. Therefore, all 23 compounds were carried forward in order not to miss out potential inhibitors.

3.5. Confirmation of the WNV replicon inhibitor compounds

The candidate compounds were next further assessed in secondary assays to ascertain that they inhibited the WNV replicon. First, in the Western blot analysis shown in Fig. 5, total

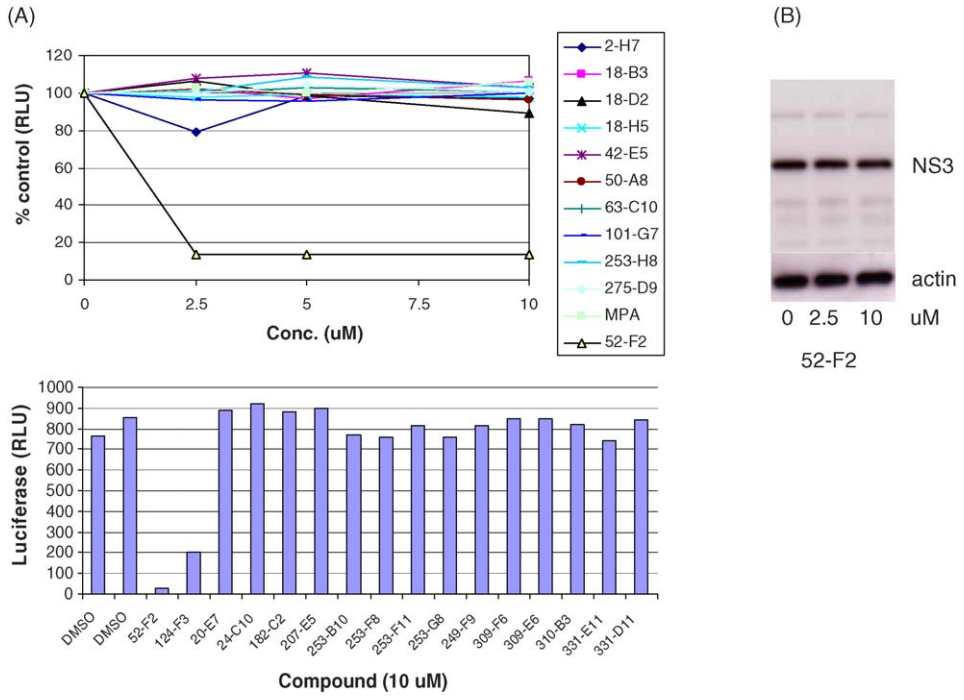


Fig. 3. (A) In vitro Luc enzyme assay to assess inhibitory activity against Luc enzyme. In two separate experiments, compounds were either tested with 3, 5, 10 μM (top panel) or only 10 μM concentrations (bottom panel). The cell lysates containing Luc enzyme were incubated with the indicated compounds for 30 min before luciferin substrate was added and the luminescence was measured after 5 min. One percent DMSO was used as a negative control. (B) Western blot demonstrating that 52-F2 did not inhibit WNV viral protein accumulation in the WNV replicon cells. 4×10^4 replicon cells in 24-well plates were incubated with the indicated concentrations of 52-F2 for 48 h. Total cell proteins from the treated cells were separated on SDS-PAGE and transferred to PVDF membrane. The blot was probed with a monoclonal antibody against WNV proteins. The same blot was probed for Beta-actin for loading controls.

cell proteins were prepared from WNV replicon cells treated with the hit compounds and the viral protein NS3 was detected with a polyclonal sera produced in mice infected with WNV (Rossi et al., 2005). The same blots were stripped and probed for beta-actin to show equal loading. As shown in Fig. 5, all of the compounds had minimal effect on the beta-actin level, which is consistent with the MTT data showing that these compounds are not cytotoxic at the 10 μM concentration used. The control compound, MPA, reduced WNV NS3 protein level at the 2.5 μg/ml concentration used. The Western blot data demonstrate that the tested compounds all reduced WNV NS3 protein level in replicon cells after 48 h drug treatment. A dose dependency is also clearly seen with several compounds, e.g.18-D2 and 331-D11 (structures shown in Table 1). However, a few compounds reduced viral protein only at the 10 μM, the highest concentration used. Examples include 310-D3 and 309-F6 (Table 1). Comparison of these Western blot data with the IC50 data determined with the Luc assay, demonstrated a reasonably good correlation between Luc reduction and viral protein reduction.

Secondly, to demonstrate that the compounds can inhibit WNV replicon RNA replication, we utilized Northern blots to analyze the WNV RNA level in BHK 26.5 cells treated with a subset of the compounds. 18-B3 and 275-F9 represent two different classes of compounds (Table 1). In this experiment, a WNV NS5 probe was used to detect viral RNA and a probe for human beta-actin was included as loading control. The specificity of the NS5 probe is shown by the inability of Huh-7 cell RNA to produce a hybridization signal (Fig. 6, lane 7). Fig. 6 shows that after 48 h treatment, WNV RNA was reduced more than five-fold in cells treated with 2.5 μg/ml MPA (Fig. 6, comparing lane 1 with lanes 2 and 3), whereas compounds 18-B3 and 275-F9 reduced WNV RNA by 1.5 and two-fold at 10 μM concentration, respectively (Fig. 6, lanes 4 and 5). Consistent with its inability to reduce WNV protein level (Fig. 3C), the Luc inhibitor, 52-F2, did not reduce the WNV RNA level (Fig. 6, lane 6). These data indicate that the candidate compounds that inhibited WNV replicon protein synthesis also prevented replicon RNA replication.

Although the compounds selected from the WNV replicon screen were shown to reduce replicon protein and RNA synthesis, the data from the Western and Northern blots also showed that these compounds had limited potency indicating that they might require further improvement in by chemical modification to become potent virus inhibitors Nevertheless, several of these original compounds selected from the WNV replicon based screen were evaluated in an antiviral assay against WNV live virus. Five compounds (18-D2, 275-D9, 309-F6, 331-D11, 331-E11) from three structure-related classes were retrieved from the mother plate and tested in WNV yield reduction assay. Fig. 7 shows that one of the compounds (309-F6; a pyrozolopyrimidine) had good activity in this assay with the WNV titer being reduced by approximately 90% at 20 μM. In parallel toxicity assessment, only slight cell toxicity was observed at 20 μM. 309-F6 had an estimated selective index of more than 8.

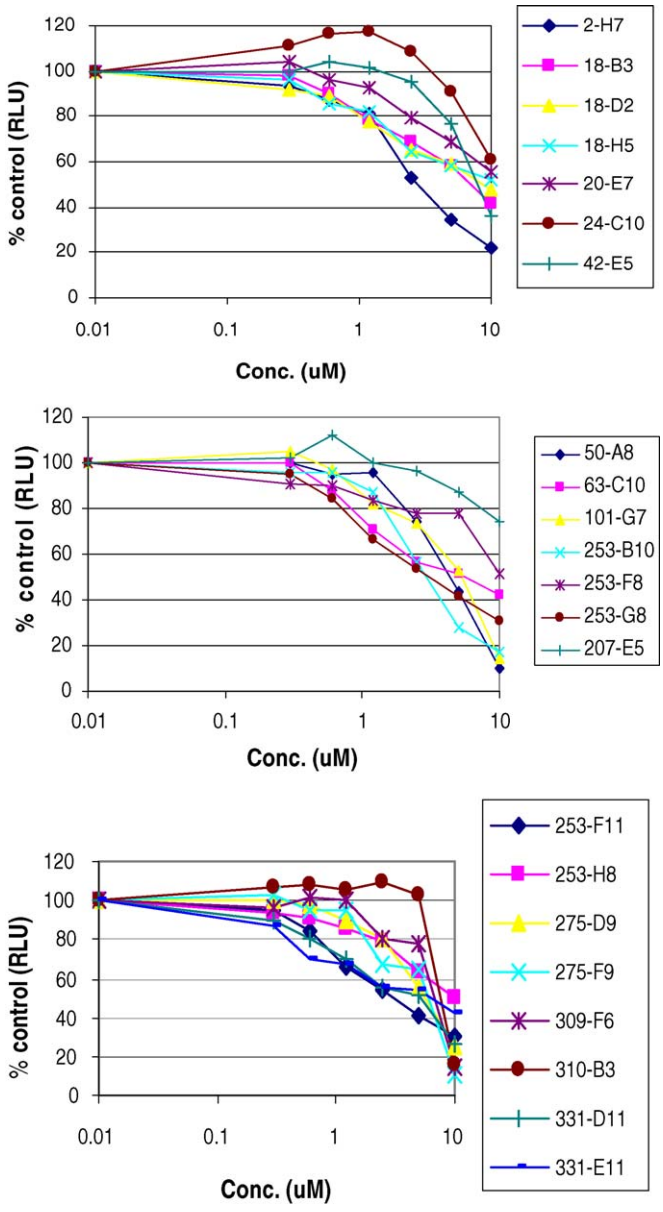


Fig. 4. IC50 determination for the candidate compounds. 1×10^4 BHK 26.5 cells in 96 wells were plated 24 h before the addition of various concentrations of the indicated compounds as described in Section 2. After 24 h, the Luc activity in the cells was quantitated by using Steady Glo reagent. On the *Y*-axis, the luminescence signal relative to control wells treated with 1% DMSO was shown. On the *X*-axis, the concentration of the compounds is shown in log scale.

4. Discussion

In this report, we describe the development and use of a WNV replicon-based HTS screen and the discovery of compounds with potential antiviral activity from a library of >35,000 compounds. The assay used a preliminary screening concentration of 10 μM and detected antiviral activity by measuring levels of Luc, which was encoded in the WNV replicon. Preliminary candidate compounds were defined by their ability to reduce Luc activity by more than 50%. These preliminary candidates were validated by retesting, and demonstrating an absence of cytotoxic activity, which would have also reduced Luc activity in the replicon-bearing cells. Activity of this "refined" set of hits against WNV replication was confirmed by demonstrating that the compounds did not directly inhibit Luc enzymatic activity, and a demonstration that the compounds reduced the levels of WNV RNA and protein in replicon-bearing cells. Several compounds were tested in a live virus yield reduction assay and an active compound was identified demonstrating the usefulness of our replicon-based HTS assay.

Using the replicon assay, we identified 23 compounds that met our criteria of selective inhibition of WNV RNA and/or protein accumulation. It is pointed out that we set the criteria relatively low for the purpose of obtaining less potent compounds to progress to live WNV antiviral assay since it is not clear how replicon inhibitors can translate into virus inhibitors. The 23 compounds are summarized in Table 1. It is our intention that the reported structures could generate interest so similar compounds will be tested in WNV assays. As shown in Table 1, some of the compounds share common structural features that can be used to group them into 11 different classes. Some compounds, 24-C10, 42-E5, 50-A8, 63-C10, 101-G7, and 207-E5 are chemically attractive (chemically stable, non-reactive, and easy to synthesize derivatives) and appear to be well suited for further analysis. However, two compounds, 2-H7 and 182-C2, have simple structures and lack uniqueness and practically accessible functional groups for further modification.

Candidate compounds 331-D11 and 331-E11 have similar chemical structures, but their hydroxyamidine group is potentially reactive and may pose potential stability problems. Furthermore, these two compounds are highly polar and thus may display problematic pharmacokinetics. Therefore, these two compounds are less attractive for further development. They did not inhibit significantly WNV production.

Of particular interest is the group of parazolotrahydrothophene (pyrozoloHTH) compounds that include 18-B3, 18-D2, 18-H5, 20-E7, 253-B10, 253-F8, 253-F11, 253-G8, and 253-H8 (Table 1). This group of compounds all has a pyrozoloHTH core structure with modification at the aryl phenol ring group and the 5-position amide group. The IC50 of these compounds ranged from 2 to 10 μM in the replicon cell. In addition, a few similar compounds exist in our library that were not active in the HTS assays (not shown), indicating that there is a preliminary structure–activity relationship (SAR). One representative compound, 18-B3, inhibited the WNV RNA replication at 10 μM (Fig. 6), confirming the value of this class of compounds. However, when tested in WNV infection assays, 18-D2 did not show significant activity against the virus. The difference in activity in the replicon and virus infection assays indicates that the replicon system might be more sensitive to this group of compounds. Testing of the other compounds in this group in the virus assay is needed for better understanding.

Four pyrozolopyrimidine compounds (275-D9, 275-F9, 309-F6 and 310-B3) appear to be worthy of further development.

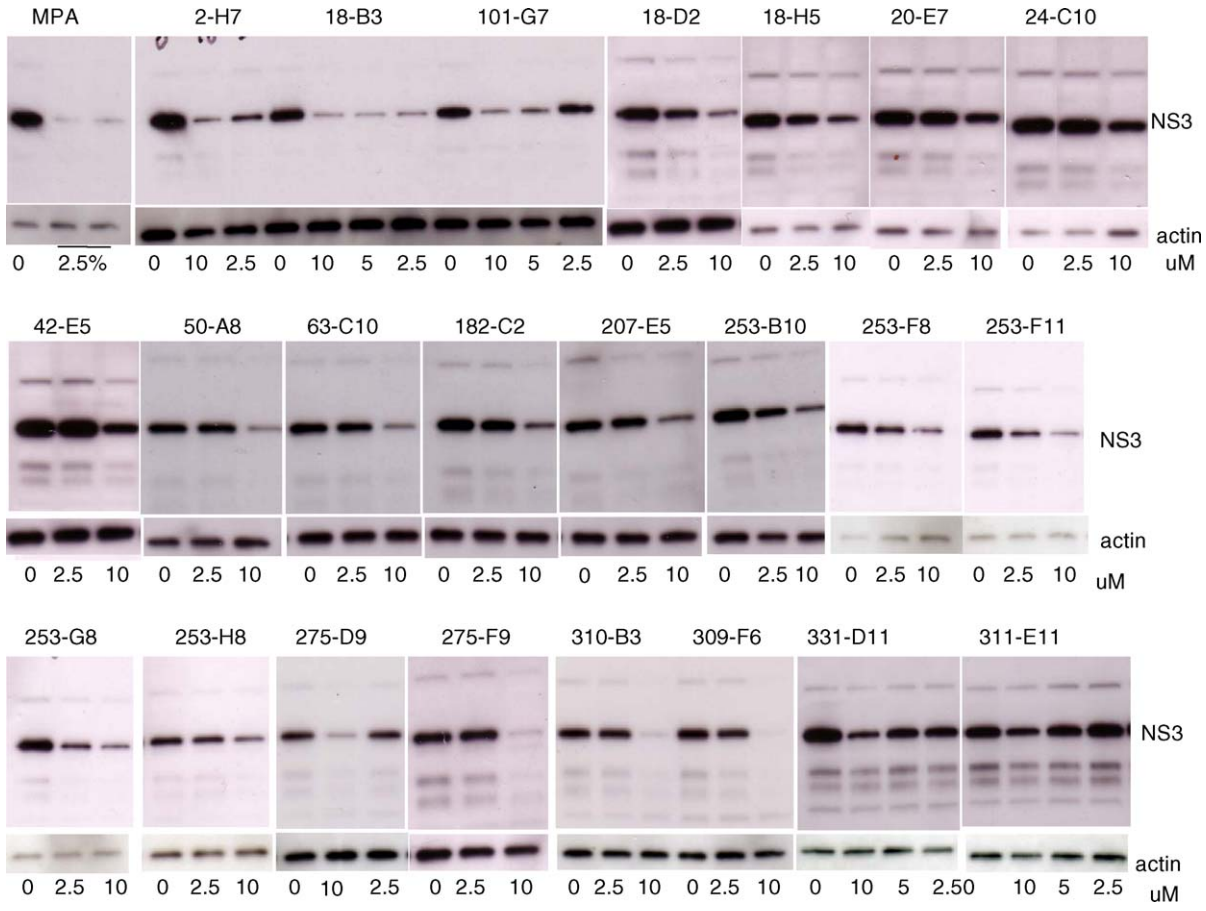


Fig. 5. Western blot analysis of the WNV protein from compound-treated replicon cells. 4×10^4 cells were seeded in 24-well plates 24 h before the various concentrations of compounds were added. After 48 h of drug treatment, SDS-Sample buffer were added to the wells to lyse the cells. The protein samples were separated on SDS-PAGE and transferred to PVDF membrane. Viral protein was detected by probing with a monoclonal antibody against WNV. The same membrane was stripped and probed for beta-actin with the signals shown at the bottom of the blot. 2.5 μg/ml MPA was used as positive control in the experiment.

Although our candidate pyrozolopyrimidine compounds were generally less potent than the PyrozoloHTH compounds in the replicon assays presented here, one pyrozolopyrimidine compound, 275-F9, indeed reduced WNV RNA level in our Northern blot analysis (Fig. 6). Most importantly, the compound, 309-F6, inhibited WNV viral production in live virus infection assays (Fig. 7). This result demonstrates that selecting compounds even with less activity from the replicon system and testing them in the virus assay is useful. The chemistry of 309-F6 is attractive from a synthesis/modification point of view. Since the similar compound, 275-D9, had no detectable activity in the WNV infection assay, the information can facilitate SAR studies.

At the present, the mechanisms of action for our candidate compounds are not known. Whether the compounds are targeting a viral protein or a cellular process that is involved in WNV replication is not clear. To address this issue, we are attempting to develop virus resistance to the most potent candidates in order to find out whether a specific viral protein is targeted by their action. From the western blot data (Fig. 5), it is tempting to speculate that none of the compounds inhibited the NS3 protease activity, since inhibition of NS3 would be expected to alter WNV polyprotein processing and there are no visible compound-induced changes in viral protein migration on these blots. Other possible targets include various aspects of RNA replication. To assess some of these aspects in in vitro, we are establishing WNV polymerase and RNA helicase assays to investigate whether the compounds can inhibit these enzymatic activities.

Although we have identified several candidate antiviral compounds that can inhibit the WNV replication-dependent Luc activity or reduce WNV viral protein accumulation or inhibit WN virus production, it is possible that the observed activity is due to contaminants in the test compounds in the original library. Re-synthesis and re-testing of the compounds, along with homologues will be needed to insure the identity of the agents responsible for the activity we have detected.

Several small molecules, in particular, nucleoside analogs have been reported to be active in controlling flavivirus infections (Morrey et al., 2002), supporting the contention that antiviral drugs could be useful in combating the emerging public health threat produced by WNV infection. Although several of these nucleoside analogues display higher activity in replicon assays than our candidate compounds, our candidate compounds

Table 1
Summary of HTS compounds with activity in WNV replicon cells

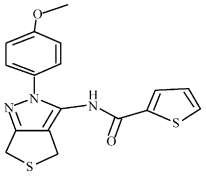
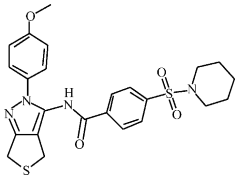
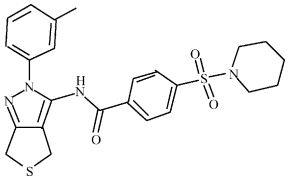
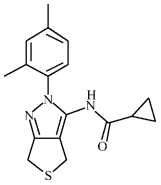
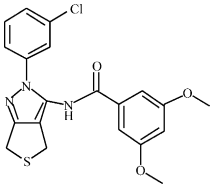
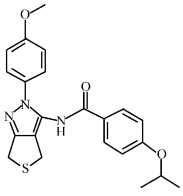
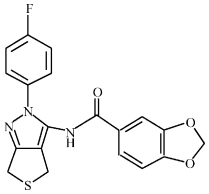
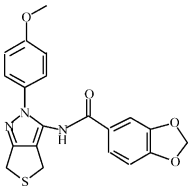
Compound[a]	Structure	Chemical name	Fold decrease (HTS)[b]	Fold decrease (re-test)[c]	IC50 (μM)[d]	CC50 (μM)[e]
18-B3		Thiophene-2-carboxylic acid [2-(4-methoxy-phenyl)-2,6-dihydro-4H-thieno[3,4-c]pyrazol-3-yl]-amide	3	2	5	>30
18-D2		*N*-[2-(4-Methoxy-phenyl)-2,6-dihydro-4H-thieno[3,4-c]pyrazol-3-yl]-4-(piperidine-1-sulfonyl)-benzamide	3	2.8	7.5	>30
18-H5		4-(Piperidine-1-sulfonyl)-*N*-(2-*m*-tolyl-2,6-dihydro-4H-thieno[3,4-c]pyrazol-3-yl)-benzamide	2	2	7.5	>30
20-E7		Cyclopropanecarboxylic acid [2-(2,4-dimethyl-phenyl)-2,6-dihydro-4H-thieno[3,4-c]pyrazol-3-yl]-amide	2.3	2.4	10	>10
253-B10		*N*-[2-(3-Chloro-phenyl)-2,6-dihydro-4H-thieno[3,4-c]pyrazol-3-yl]-3,5-dimethoxy-benzamide	2	2	3	>30
253-F8		4-Isopropoxy-*N*-[2-(4-methoxy-phenyl)-2,6-dihydro-4H-thieno[3,4-c]pyrazol-3-yl]-benzamide	2.5	2	10	>30
253-F11		Benzo[1,3]dioxole-5-carboxylic acid [2-(4-fluoro-phenyl)-2,6-dihydro-4H-thieno[3,4-c]pyrazol-3-yl]-amide	3	2	10	>30
253-G8		Benzo[1,3]dioxole-5-carboxylic acid [2-(4-methoxy-phenyl)-2,6-dihydro-4H-thieno[3,4-c]pyrazol-3-yl]-amide	2	2	2.5	>30

Table 1 (*Continued*)

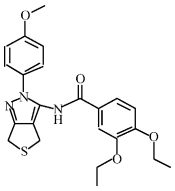
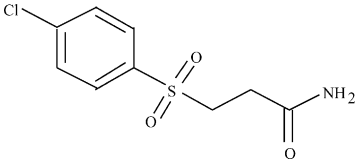
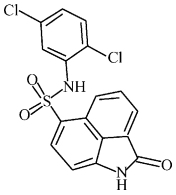
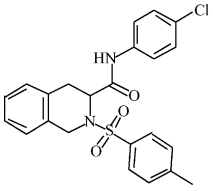
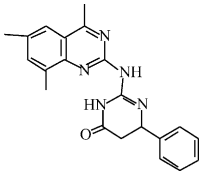
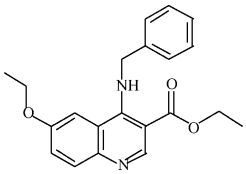
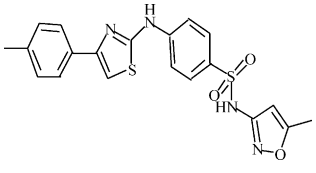
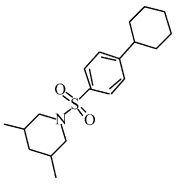
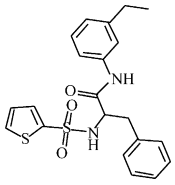
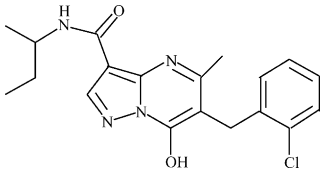
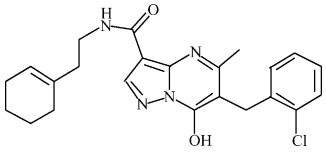
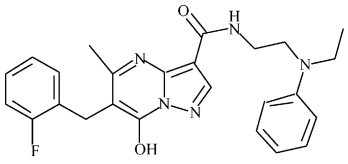
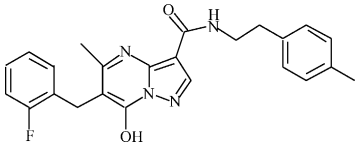
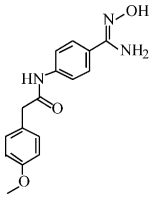
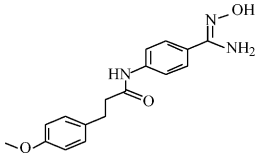
Compound[a]	Structure	Chemical name	Fold decrease (HTS)[b]	Fold decrease (re-test)[c]	IC50 (μM)[d]	CC50 (μM)[e]
253-H8		3,4-Diethoxy-*N*-[2-(4-methoxy-phenyl)-2,6-dihydro-4H-thieno[3,4-c]pyrazol-3-yl]-benzamide	2	1.5	10	>30
2-H7		3-(4-Chloro-benzenesulfonyl)-propionamide	4.5	2.5	2.5	>20
24-C10		2-Oxo-1,2-dihydro-benzo[cd]indole-6-sulfonic acid (2,5-dichloro-phenyl)-amide	2.5	1.8	10	>10
42-E5		2-(Toluene-4-sulfonyl)-1,2,3,4-tetrahydro-isoquinoline-3-carboxylic acid (4-chloro-phenyl)-amide	7	1.7	7.5	>10
50-A8		6-Phenyl-2-(4,6,8-trimethyl-quinazolin-2-ylamino)-5,6-dihydro-3H-pyrimidin-4-one	4.5	2.3	5	>10
63-C10		4-Benzylamino-6-ethoxy-quinoline-3-carboxylic acid ethyl ester	3.5	ND	5	>20
101-G7		*N*-(5-Methyl-isoxazol-3-yl)-4-(4-*p*-tolyl-thiazol-2-ylamino)-benzenesulfonamide	6	2	5	>10
182-C2		1-(4-Cyclohexyl-benzenesulfonyl)-3,5-dimethyl-piperidine	5	1.4	ND	>10

Table 1 (*Continued*)

Compound[a]	Structure	Chemical name	Fold decrease (HTS)[b]	Fold decrease (re-test)[c]	IC50 (μM)[d]	CC50 (μM)[e]
207-E5		*N*-(3-Ethyl-phenyl)-3-phenyl-2-(thiophene-2-sulfonylamino)-propionamide	5	2	10	>10
275-D9		6-(2-Chloro-benzyl)-7-hydroxy-5-methyl-pyrazolo[1,5-a]pyrimidine-3-carboxylic acid sec-butylamide	10	2.9	7.5	>10
275-F9		6-(2-Chloro-benzyl)-7-hydroxy-5-methyl-pyrazolo[1,5-a]pyrimidine-3-carboxylic acid (2-cyclohex-1-enyl-ethyl)-amide	27	4.5	7.5	>10
309-F6		6-(2-Fluoro-benzyl)-7-hydroxy-5-methyl-pyrazolo[1,5-a]pyrimidine-3-carboxylic acid [2-(ethyl-phenyl-amino)-ethyl]-amide	7	6	10	>10
310-B3		6-(2-Fluoro-benzyl)-7-hydroxy-5-methyl-pyrazolo[1,5-a]pyrimidine-3-carboxylic acid (2-*p*-tolyl-ethyl)-amide	17	4	10	>10
331-D11		*N*-[4-(*N*-Hydroxycarbamimidoyl)-phenyl]-2-(4-methoxy-phenyl)-acetamide	4	2	5	>30
331-E11		*N*-[4-(*N*-Hydroxycarbamimidoyl)-phenyl]-3-(4-methoxy-phenyl)-propionamide	3	1.5	5	>30

[a] The name of the compound corresponds to the position of the compound in our 96-well compound library.
[b] The decrease in Luc in compound-treated wells compared to DMSO control wells in initial HTS single-well assays.
[c] The decrease in Luc in compound-treated wells compared to DMSO wells in manual re-test.
[d] IC50: concentration of the compound that produces 50% decrease in Luc. The highest concentration of compounds used in IC50 determination is 10 μM.
[e] CC50: concentration of compound that produced 50% decrease in MTT signals. Most of the compounds were tested up to only 10 μM; a few were up to 30 μM.

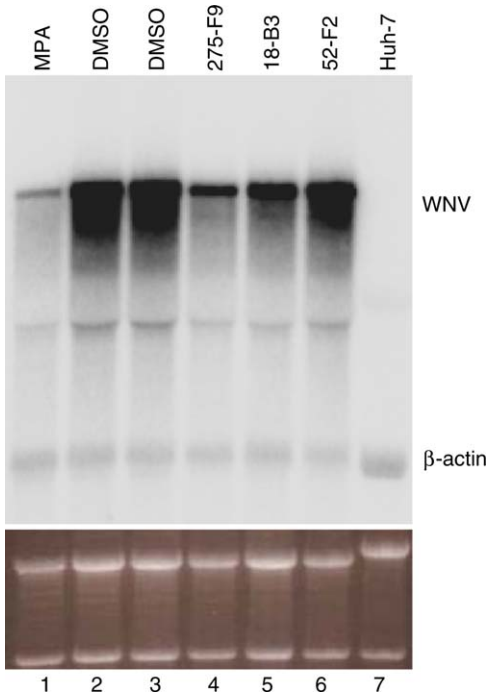


Fig. 6. Northern blot analysis of compound treated WNV replicon cells. 4×10^4 cells were seeded in 24-well plates. After 24 h, the cells were treated with 1% DMSO alone (lanes 2 and 3), 2.5 μg/ml MPA (lane 1) or 10 μM of the compounds 275-F6 (lane 4), 18-B3 (lane 5) or 52-F2 (lane 6). After 48 h, total RNA were isolated, separated on denaturing agrose gels and transferred to nylon membrane. Total RNA from a non-replicon cell (Huh-7) was loaded in lane 7 as negative control. A picture of the gel is shown at the bottom of the figure. The membrane was probed simultaneously with [^{32}P]-dCTP-labeled WNV NS5 and human beta-actin probes. The signal was quantified using a BioRad phosphoimager and WNV RNA level was calculated using beta-actin as reference.

may serve as the starting material to generate a "lead" compound in the rational design of more potent anti-WNV inhibitors. The compounds that we have detected with our HTS assay do not appear to have structural similarity to nucleosides, and thus may

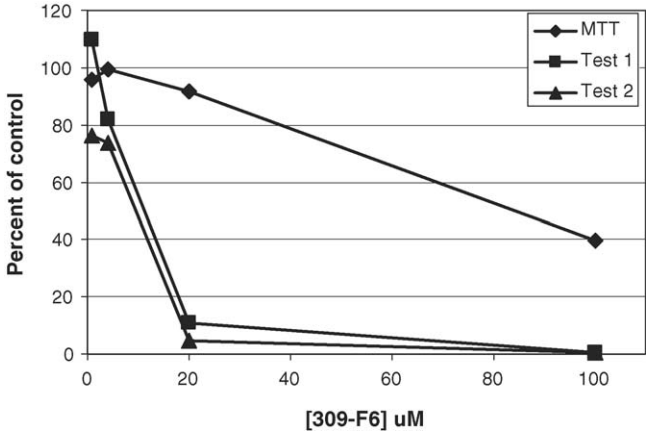


Fig. 7. 309-F6 inhibited WN virus production. Antiviral assay for the compounds was conducted as described in Section 2 on BHK cells. The virus produced in the presence of 1% DMSO or various concentrations of compounds was tittered on Vero cells. The amount of virus relative to the 1% DMSO control was plotted on the *Y*-axis; the concentration of the compound 309-F6 is shown in μM on the *X*-axis. Data from two independently treated wells of are shown.

be useful to develop as drugs that could be delivered in a cocktail with nucleoside inhibitors. Furthermore, the compounds we have identified may be useful research tools in dissecting the molecular mechanisms of WNV replication and viral host interaction.

Acknowledgements

We thank Mr. G. Westby and Ms. A. Dougherty for helping with the compounds library in the high-throughput screen assay, Dr. J.-T. Guo for helping the Northern blot analysis, Mr. N. Dewsbury for WNV yield reduction assays, and Dr. A. Mehta and P. Norton for discussions. We thank Mr. G. Amparo for analyzing the chemistry of the hits. Development of the replicon system was supported by a grant from NIAID (PWM) through the Western Regional Center of Excellence for Biodefense and Emerging Infectious Disease Research (U54 AI057156). This work was supported by the grants AI061441 and AI061441 from the National Institute of Health; the Hepatitis B Foundation and an appropriation from the Commonwealth of Pennsylvania.

References

Anderson, J.F., Andreadis, T.G., Vossbrinck, C.R., Tirrell, S., Wakem, E.M., French, R.A., Garmendia, A.E., Van Kruiningen, H.J., 1999. Isolation of West Nile virus from mosquitoes, crows, and a Cooper's hawk in Connecticut. Science 286 (5448), 2331–2333.

Briese, T., Jia, X.Y., Huang, C., Grady, L.J., Lipkin, W.I., 1999. Identification of a Kunjin/West Nile-like flavivirus in brains of patients with New York encephalitis. Lancet 354 (9186), 1261–1262.

Brinton, M.A., 2001. Host factors involved in West Nile virus replication. Ann. NY Acad. Sci. 951, 207–219.

Brinton, M.A., 2002. The molecular biology of West Nile Virus: a new invader of the western hemisphere. Annu. Rev. Microbiol. 56, 371–402.

Chambers, T.J., Hahn, C.S., Galler, R., Rice, C.M., 1990. Flavivirus genome organization, expression, and replication. Annu. Rev. Microbiol. 44, 649–688.

Fauci, A.S., Touchette, N.A., Folkers, G.K., 2005. Emerging infectious diseases: a 10-year perspective from the National Institute of Allergy and Infectious Diseases. Emerg. Infect. Dis. 11 (4), 519–525.

Granwehr, B.P., Lillibridge, K.M., Higgs, S., Mason, P.W., Aronson, J.F., Campbell, G.A., Barrett, A.D., 2004. West Nile virus: where are we now? Lancet Infect. Dis. 4 (9), 547–556.

Gu, B., Gates, A.T., Isken, O., Behrens, S.E., Sarisky, R.T., 2003. Replication studies using genotype 1a subgenomic hepatitis C virus replicons. J. Virol. 77 (9), 5352–5359.

Lanciotti, R.S., Roehrig, J.T., Deubel, V., Smith, J., Parker, M., Steele, K., Crise, B., Volpe, K.E., Crabtree, M.B., Scherret, J.H., Hall, R.A., MacKenzie, J.S., Cropp, C.B., Panigrahy, B., Ostlund, E., Schmitt, B., Malkinson, M., Banet, C., Weissman, J., Komar, N., Savage, H.M., Stone, W., McNamara, T., Gubler, D.J., 1999. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. Science 286 (5448), 2333–2337.

Morrey, J.D., Smee, D.F., Sidwell, R.W., Tseng, C., 2002. Identification of active antiviral compounds against a New York isolate of West Nile virus. Antiviral Res. 55 (1), 107–116.

Pietschmann, T., Lohmann, V., Rutter, G., Kurpanek, K., Bartenschlager, R., 2001. Characterization of cell lines carrying self-replicating hepatitis C virus RNAs. J. Virol. 75 (3), 1252–1264.

Puig-Basagoiti, F., Deas, T.S., Ren, P., Tilgner, M., Ferguson, D.M., Shi, P.Y., 2005. High-throughput assays using a luciferase-expressing

replicon, virus-like particles, and full-length virus for West Nile virus drug discovery. Antimicrob. Agents Chemother. 49 (12), 4980–4988.

Rossi, S.L., Zhao, Q., O'Donnell, V.K., Mason, P.W., 2005. Adaptation of West Nile virus replicons to cells in culture and use of replicon-bearing cells to probe antiviral action. Virology 331 (2), 457–470.

Shi, P.Y., Tilgner, M., Lo, M.K., 2002. Construction and characterization of subgenomic replicons of New York strain of West Nile virus. Virology 296 (2), 219–233.

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.