The Identification of Novel Small Molecule Inhibitors of West Nile Virus Infection

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ABSTRACT

West Nile virus (WNV) has spread throughout the United States and Canada and now annually causes a clinical spectrum of human disease ranging from a self-limiting acute febrile illness to acute flaccid paralysis and lethal encephalitis. No therapy or vaccine is currently approved for use in humans. Using high throughput screening assays that included a luciferase expressing WNV subgenomic replicon and an NS1 capture ELISA, we evaluated a chemical library of over 80,000 compounds for their capacity to inhibit WNV replication. We identified ten compounds with strong inhibitory activity against genetically diverse WNV and Kunjin (KUNV) virus isolates. Many of the inhibitory compounds belonged to a chemical family of secondary sulfonamides, and have not been described previously to inhibit WNV or other related or unrelated viruses. Several of these compounds inhibited WNV infection in the sub-micromolar range, had selectivity indices of greater than ten, and inhibited replication of other flaviviruses, including Dengue (DENV) and yellow fever (YFV) viruses. One of the most promising compounds, AP30451, specifically blocked translation of a yellow fever virus replicon but not a Sindbis virus replicon or an internal ribosome entry site (IRES) containing mRNA. Overall, these compounds comprise a novel class of promising inhibitors for therapy against WNV and other flavivirus infections in humans.
INTRODUCTION

West Nile virus (WNV) is a single-stranded positive polarity RNA Flavivirus that cycles enzootically between Culex species of mosquitoes and birds but also infects and causes disease in humans, horses, and other vertebrate species. It is related to other viruses that cause human disease including DENV, YFV, and Japanese (JEV), St. Louis, and tick-borne encephalitis viruses. Historically, WNV caused sporadic outbreaks of a mild febrile illness in regions of Africa, the Middle East, Asia, and Australia. However, in the last decade, the ecology and epidemiology of infection changed. New outbreaks in parts of Eastern Europe and North America were associated with higher rates of severe neurological disease (37, 41, 73). WNV has spread to all 48 continental United States as well as to Canada, Mexico, Caribbean, and more recently South America (62). The more severe symptoms of WNV infection occur in the elderly and immunocompromised, although serious illness has been observed across all age ranges. The fatality to case ratio of recent WNV outbreaks is 4-14%, and it can rise to 10-19% in hospitalized cases. Annual outbreaks occur in the United States (34), with ~ 24,000 human cases diagnosed between 1999 and 2006 (http://www.cdc.gov/ncidod/dvbid/westnile/surv&control.htm#maps), and an estimated 730,000 undiagnosed infections in 2003 alone (12). No vaccine or specific therapy for WNV is currently approved for humans.

Recent animal studies suggest that administration of anti-WNV antibodies may be therapeutic. Three groups have demonstrated that immune human γ-globulin partially protects mice against WNV-induced mortality even when therapy was delayed up to five days after infection (6, 27, 46). Small numbers of human patients have received immune γ-globulin therapy against WNV infection, and case reports (36, 84) have documented clinical improvement in humans with neurological WNV infection. Human monoclonal antibodies (mAb), humanized
mAbs, and antibody fragments against the WNV envelope protein have been recently developed (33, 72, 87). These reagents have high neutralizing activity in vitro and provide equivalent or superior protection in vivo in mice and hamsters compared with γ-globulin (33, 65, 72, 87). One possible limitation of mAb therapy, however, is the rapid emergence of escape mutants that could compromise inhibitory activity (53).

Several well-characterized antiviral agents have been tested for inhibitory activity against WNV. Pretreatment of cells in vitro with IFN-α potently inhibits flavivirus infection including WNV (2, 15, 21, 22, 28), and mice that lack IFN-α/β receptors are highly susceptible to lethal WNV infection (48, 79). However, the inhibitory effect of IFN is markedly attenuated once viral replication has begun (22, 55) because flavivirus non-structural proteins block IFN signaling (7, 44, 55-57, 68, 69). Pretreatment of rodents with IFN-α inhibited St. Louis encephalitis virus infection and decreased WNV viral load and mortality (11, 64), and treatment with IFN-α after infection reduced complications in human St. Louis encephalitis virus cases. In an uncontrolled study, a small number of human cases of WNV encephalitis were successfully treated with IFN-α (47, 76, 83). Consequently, a randomized non-blinded clinical trial of IFN α2b has been initiated for WNV infection (http://nyhq.org/posting/rahal.html).

The cellular enzyme inosine monophosphate dehydrogenase (IMPDH) has been a target of antiviral development. IMPDH catalyzes an essential step in the de novo biosynthesis of guanine nucleotides. Ribavirin (1-β-D-ribofuranosyl-1H-1,2,4-triazole-3-carboximide) is as a guanosine analogue that competitively inhibits IMPDH resulting in depletion of intracellular GTP pools (52). While the mechanism of its broad-spectrum antiviral activity is uncertain, low pools of GTP may interfere with the guanylylation step of RNA capping and inhibit viral RNA polymerases (42). Alternatively, phosphorylated ribavirin may incorporate directly into the
nascent RNA strand leading to loss of viral genome integrity by virtue of an error catastrophe effect (16, 18). Although ribavirin has inhibitory activity against WNV infection in vitro (2, 18, 45) animal experiments have not been promising. Treatment of WNV-infected hamsters with ribavirin resulted in increased mortality (64). Moreover, in a WNV outbreak in 2000, an elevated 41% mortality rate was observed among 37 patients that received ribavirin therapy (13).

Mycophenolic acid (MPA) is a non-nucleoside, non-competitive inhibitor of IMPDH that has broad-spectrum antiviral activity in vitro, including against WNV (24, 66). Unfortunately, MPA had poor efficacy in mice against WNV (B. Geiss and M. Diamond, unpublished results), likely because of its significant immunosuppressive effects. VX-497, a phenyloxazole derivative that is structurally unrelated to ribavirin and MPA, is a potent, reversible, non-competitive inhibitor of IMPDH (61). Although it is has potent antiviral activity against several DNA and RNA viruses, it was relatively inactive against DENV and YFV and has not been tested against WNV.

Novel small molecule inhibitors have been identified that inhibit WNV translation and replication (9, 32, 35, 75). Gu et al. used a cell-based subgenomic replicon screen to identify pyrozolopyrimidine compound with anti-WNV activity, although the selectivity index was only 8. Puig-Basagoiti et al. (75) identified a triaryl pyrazoline compound that inhibited flavivirus RNA replication. This compound inhibited viral replication of an epidemic strain of WNV in Vero cells with an EC\(_{50}\) of 15 µM, and had broad antiviral activity against related (e.g., DENV or YFV) or unrelated (mouse hepatitis and vesicular stomatitis viruses) RNA viruses.

Herein, we describe a screening strategy for the identification of small molecule inhibitors with antiviral activity against WNV and other flaviviruses. We identify several compounds with sub-micromolar activity and minimal cell toxicity. Based on our screening platform, these inhibitors likely target steps after entry and before assembly. One compound,
AP30451, specifically inhibited translation of flavivirus mRNA and blocked WNV replication in several cell types, including primary neuron cultures.
MATERIALS AND METHODS

Cells and viruses. Baby hamster kidney cells (BHK21-15), monkey Vero cells, human Huh-7.5 hepatoma (8), and human A549 lung carcinoma cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) with 10% fetal bovine serum as previously described (20). The lineage I New York isolate (3000.0259, 2000, passage 2) was described previously (25, 26), and the KUNV isolate (CH 16532) was a generous gift of Dr. J. Anderson (New Haven, CT). The DENV-2 strain (16681) is a prototype dengue hemorrhagic fever isolate from Thailand and has been described previously (77).

WNV lineage I replicon. A plasmid containing the cDNA of a lineage I WNV subgenomic replicon that expresses renilla luciferase was generated from an infectious cDNA clone of the New York 1999 strain (provided by R. Kinney, Centers for Disease Control, Fort Collins, CO). Briefly, pWN.CA26-hRUPac was engineered by deleting WNV nucleotides 181-2379 and fusing the first 31 amino acids of the capsid protein to the renilla luciferase gene, the coding sequences of the ubiquitin autocleavage peptide, and the puromycin-resistance conferring gene (pac). The DNA template was prepared by linearizing pWN.CA26-hRUPac with Xba I restriction endonuclease followed by phenol:chloroform extraction and ethanol precipitation. Replicon RNA was generated using the Amplicap T7 DNA-dependent RNA polymerase High Yield Message Maker kit (Epicenter Technologies, Madison WI). RNA transcripts were electroporated into BHK-21 cells and stable clones of replicon-expressing cells (BHK-WNV-Rep) were isolated after selection with 5 μg/ml of puromycin (Sigma-Aldrich, St. Louis MO).

Inhibitor screening with the WNV replicon. A library of over 80,000 small molecules was obtained from commercial suppliers. Compounds were maintained at -80°C in DMSO at a stock concentration of 10 mM. Primary screens were performed at a single concentration (25
µM) of compound in 1% DMSO in 96-well plates containing 2.5 x 10^5 BHK-WNV-Rep cells. Twenty-four hours after treatment, cells were lysed and a luciferase assay was performed. Compounds that showed greater than 50% inhibition were tested in quadruplicate with an extended dose range (0.75 µM to 75 µM) and an EC_{50} was determined. In parallel, these compounds were also tested for cell toxicity using an ATP metabolic assay (Celltiter-Glo, Promega, Madison, WI) to obtain the concentration that is cytotoxic for 50% of cells (CC_{50}). The selectivity index (SI; CC_{50}/EC_{50}) was determined and used to prioritize lead compounds.

*Inhibitor screening by NS1 ELISA.* Lead compounds from the replicon screen were tested in a secondary screen for their inhibitory activity on KUNV infection of Vero cells. KUNV is a lineage I strain of WNV that can be manipulated at biosafety level (BSL)-2, which facilitated high-throughput screening. As NS1 is a secreted non-structural glycoprotein that accumulates in supernatants of flavivirus-infected cells, we developed a highly quantitative capture ELISA using previously described anti-NS1 mAbs (3-NS1 and 17-NS1) (14). Vero cells were incubated with different doses of lead compounds, infected with Kunjin virus at a multiplicity of infection (MOI) of 0.01, and supernatants were harvested 48 hours after infection. Microtiter plates were coated with 17-NS1 (10 µg/ml) overnight at 4°C and blocked for one hour at 37°C in 150 mM NaCl, 25 mM Tris-HCl pH 7.5, 1% bovine serum albumin (BSA), 3% horse serum, 0.05% NP-40, and 0.025% NaN_3. Subsequently, supernatants from Kunjin infected Vero cells were treated with NP-40 (final concentration 0.1% to inactivate virus) and added to wells for one hour at room temperature. Plates were rinsed four times with wash buffer (150 mM NaCl, 25 mM Tris-HCl pH 7.5, 1% BSA, 0.025% NP-40, and 0.025% NaN_3) and incubated sequentially for one hour with biotinylated 3-NS1 (2 µg/ml in wash buffer), streptavidin-HRP (2 µg/ml in wash buffer), and tetramethylbenzidine (TMB) substrate. After stopping the reaction...
with 1N H$_2$SO$_4$, the optical density was measured at 450 nm on a BMG plate reader (BMG Labtech, Durham, NC).

**Inhibitor screening with virulent New York strain of WNV.** Compounds that were inhibitory in both the replicon and KUNV NS1 assays were tested for their ability to block infection of WNV 3000.0259, a virulent strain of WNV isolated in New York in 2000 and previously characterized in our laboratory (23). Initially, Vero cells were infected with WNV at an MOI of 0.01 at 37°C in the presence of increasing doses of inhibitor in 0.5% DMSO solution. One day later, cells were harvested, fixed, permeabilized, stained for intracellular E protein using an anti-WNV mAb (Alexa 647-conjugated E16 (72)), and processed by flow cytometry. Similar studies were performed in BHK21 cells with the 16681 strain of DENV-2, except that an MOI of 0.01 was used and cells were harvested at 72 hours after infection and stained with an anti-DENV-2 mAb (3H5-1 (31)).

**Neuron cultures and infection studies.** Cortical neurons were prepared from embryonic day 15 (E15) wild type C57BL/6 mouse embryos as described (49). Cells were seeded at a density of 5 x 10$^5$ cells/well in poly-D-lysine/laminin coated 24-well plates, cultured in DMEM containing 5% heat-inactivated horse serum and 5% heat inactivated FBS, and maintained in NeuroBASAL medium supplemented with B27 (Invitrogen) and L-glutamine. For infection experiments, cortical neurons cultured for 3 to 4 days were infected at an MOI of 0.1 for 1 hour at 37°C followed by serial washing with PBS to remove free virus and addition of compounds AP30451, AP18417, AP34456 (50 μM or 16 μM), or DMSO as a vehicle control. The concentration of DMSO used (0.5%) in these studies did not cause significant toxicity in neurons compared to the medium control. For virus production experiments, supernatants were harvested at 24 hours post infection and viral titers were determined by plaque assay on BHK21 cells.
For immunostaining experiments, cortical neurons were infected with WNV at an MOI of 0.1 and treated with compounds. WNV-infected and control uninfected neurons were fixed at 48 hours post infection with 4% paraformaldehyde (PFA) in PBS at 4°C for 15 minutes. Cells were permeabilized in PBS with 0.2% Triton X-100, blocked (5% normal goat serum and 0.2% Triton X-100), and stained with rat anti-WNV immune serum and a mouse antibody against the neuronal marker NeuN (Chemicon International, Temecula, CA), or a control mouse IgG. Cells were then incubated with Alexa-488-conjugated anti-mouse IgG (Invitrogen) and Cy3 conjugated anti-rat IgG (Jackson Laboratories, West Grove, PA) and the nucleic acid stain TO-PRO-3 (Invitrogen). Neurons were visualized using a Zeiss 510 Meta LSM confocal microscope.

Neuronal survival studies were performed as previously described (80). Cortical neurons were treated with 50 µM or 16 µM of the compounds AP30451, AP18417, AP34456, or DMSO as a vehicle control. Neuron survival was assessed 24 hours after treatment using the CellTiter-Blue Cell Viability Assay as per the manufacturer’s instructions (Promega). The data were normalized to DMSO-treated neurons harvested at the same time point.

**Transient reporter gene assays.** Transient expression of reporter genes was used to determine the mechanism of compound-mediated inhibition. A YFV replicon (YF-hRupac) was engineered by deleting nucleotides 27-755 of the 17D strain of YFV and fusing the first 26 amino acids of the capsid protein to the renilla luciferase gene, the coding sequences of the ubiquitin autocleavage peptide, and the puromycin-resistance gene (pac). The Sindbis virus replicon was described previously and contains the luciferase gene under control of the subgenomic promoter (1). The encephalomyocarditis (EMCV) internal ribosome entry site (IRES)-dependent lacZ mRNA was generated from the pTM1 plasmid by inserting the lacZ open reading frame encoding the β-galactosidase protein into the multiple cloning site to create
pTM1lacZ (67). The m7G-capped firefly luciferase mRNA was generated from the Luciferase T7 control DNA plasmid purchased from Promega (Madison WI).

DNA template for all reporter constructs was prepared as described above for the WNV replicon, with the following differences. Replicon mRNA was generated using the mMessage mMachine High Yield Capped RNA Transcription Kit (Ambion, Austin TX). RNA transcripts (2 to 4 µg) were electroporated into 5 x 10⁶ BHK-21 cells. Subsequently, 2x10⁶ cells were seeded into 96 well plates, immediately treated with compounds or controls in 1% DMSO, and assayed for reporter gene activity at 2, 4 and 6 hours for early translation, and 24, 30 and 48 hours for late, replication-dependent translation. Renilla luciferase, firefly luciferase, and β-galactosidase activity were detected using commercial assay systems from Promega Corporation (Madison, WI) and Applied Biosystems (Foster City, CA). Similarly seeded and treated 96 well plates were used to measure cell viability in parallel using the CellTiter Glo luminescent assay kit (Promega), which determines the relative cell metabolic activity by measuring the amount of ATP using a luciferase-based reporter gene system. Data from eight wells per treatment were averaged and calculated as transient reporter relative light units (RLU) per ATP-dependent luciferase relative light units to normalize for cell number. Experiments were repeated several times on independent days.

**Statistical Analysis.** An unpaired T-test was used to determine statistically significant differences. All data were analyzed using Prism software (GraphPadPrism4, San Diego, CA).
RESULTS

A stable cell line that constitutively replicates a WNV subgenomic replicon. A plasmid (pWN.CA26-hRUPac.N) was constructed that contained a subgenomic WNV replicon with a renilla luciferase gene and a puromycin acetylase selectable marker (Fig 1A). After generating full-length 7-methyl-guanosine (m^7G)-capped RNA transcripts *in vitro* using a T7 DNA-dependent RNA polymerase, BHK cells were transfected by electroporation and a stable cell line that constitutively propagated a WNV replicon was isolated after selection with puromycin. Replication was verified by re-introduction of total RNA extracted from the stable cell line into naïve BHK cells and observing a replication-dependent increase in renilla expression (data not shown). One high-expressing clonal cell line, BHK/WN-CA26-hRUPacN3-2, was chosen for further studies. Luciferase expression from this cell line was linear over a wide range of cell numbers, with >100 relative luciferase units (RLU) per cell (Fig 1B). Luciferase expression from these cells was also tested following treatment with ribavirin, an established *in vitro* inhibitor of flavivirus replication (Fig 1C). The EC50 and CC50 for ribavirin were 6.85 µM and 87 µM respectively, in good agreement with previous studies (24).

Primary antiviral screen using the WNV replicon. We tested the performance of the WNV replicon cells in a primary screening format in 96-well plates. The assay was performed with 10^4 cells per well and luciferase was measured 24 hours after compound addition. The assay had an average signal of 259,168± 55625 RLU with a signal-to-noise of 1,103. Ribavirin was used to monitor screening reliability, and the WN-CA26hRUPac replicon assay was tested for quality control and statistical performance by calculating the percent coefficient of variation (% CV), and the ‘screening window coefficient’ or Z factor (90). The Z factor is defined as the ratio of the separation of two values to the signal range of the assay and is a representation of the
The dynamic range and variability of a particular assay. A trial screen of 70 plates was performed and the results analyzed. All % CVs were less than 14%, and the Z value was greater than 0.9, which indicated excellent performance (90).

A flow diagram of the primary screening program is shown (Fig 1D). Over 80,000 compounds were tested at a single dose of 25 µM. 1,355 of these compounds showed a greater than 80% reduction in luciferase signal. These compounds were subsequently tested in complete dose response analyses for inhibitory (EC$_{50}$) and cytotoxicity (CC$_{50}$) with four replicates of each concentration (0.25 to 75 µM). Notably, 242 of 1,355 compounds had EC$_{50}$ values of ≤ 10 µM and a CC$_{50}$ to EC$_{50}$ ratio (i.e. selectivity index, SI) of greater than 10. These compounds were designated replicon hits and were subsequently tested in secondary virological assays. The EC$_{50}$ and CC$_{50}$ curves of four example compounds with inhibitory activity against WNV replicon propagation are shown (Fig 2A-D).

**Secondary virological screens.** Compounds with favorable inhibitory profiles in the replicon screen were evaluated in virus-based assays. As a secondary screen, compounds were tested for their ability to inhibit KUNV, a lineage I Australian WNV strain that is closely related (97.6% amino acid sequence identity) to North American WNV isolates yet can be used under biosafety level (BSL)-2 conditions. For high-throughput screening, we measured the level of NS1, a secreted non-structural viral glycoprotein, in the supernatant of KUNV-infected Vero cells as a marker of infection using an NS1 capture ELISA. This assay was developed with our previously characterized anti-NS1 mAbs (14) and is based on published experiments (54, 59, 89). For validation purposes, we demonstrated the effect of a known inhibitor, IFN-α, on production of KUNV NS1 at 48 hours after infection (Fig 3A). Five concentrations (0.5 to 75 µM) of 242 compounds were evaluated using the NS1 ELISA; forty of these showed 50%
inhibition in the low micromolar range. These compounds were then tested for a complete dose
response (EC$_{50}$ and CC$_{50}$) using this assay. Ten compounds exhibited an EC$_{50}$ <10 µM and a SI
>10: four representative inhibition profiles are shown (Fig 3B-E). These compounds fell into
four compound classes (Table 1). One of them, AP30451, exhibited an excellent EC$_{50}$ of 60 nM
and an SI of 533. As seven different compounds with potent inhibitory activity in two different
assays on distinct cell types were secondary sulfonamides (Table 2), this class of compounds
became the focus of further testing.

**Tertiary screens with a virulent North American WNV isolate.** To confirm the
inhibitory activity of our lead compounds, we tested their ability to inhibit infection in Vero cells
with a virulent North American WNV strain that was isolated from New York in 2000 (25, 26).
Compounds were added to Vero cells at the time of infection, and 24 hours later inhibition was
evaluated by a previously described flow cytometry assay (20) for decreases in WNV envelope
(E) protein expression. Notably, several compounds significantly reduced WNV infection in
Vero cells (Table 3 and Fig 4). Some of the most potent inhibitors in the Vero cell assay were
also tested for their ability to reduce WNV production in primary mouse cortical neurons.
Neurons are the primary target of WNV infection *in vivo* in the central nervous system (CNS) of
humans and other vertebrate animals (17). One compound (AP30451) showed a potent antiviral
effect in neurons at a dose that did not cause significant cell toxicity (Fig 5). In contrast, other
compounds that inhibited infection in Vero cells either had no inhibitory activity (AP18417) or
had toxic effects (AP34456) in neurons.

**Effect of inhibitors on other Flaviviridae family members.** To examine whether
candidate lead compounds inhibited other viruses in the *Flaviviridae* family, we performed
analogous replication assays with previously described DENV-2 (88), YFV (10), and hepatitis C
virus (HCV) (8, 58) replicons that contain luciferase reporter genes. Notably, compound AP30451 strongly inhibited DENV and YFV, members of the Flavivirus genus (Fig 6A and B), with EC50 values in the low micromolar range and specificity indexes above 10. In contrast, little, if any, specific inhibition was observed with HCV, a member of the Hepacivirus genus (Fig 6C). Of note, AP30451 appeared to have slightly greater toxicity in the human Huh 7.5 hepatoma cell lines used in the HCV studies; this was not, however, consistently observed with other human lines including HeLa and 293T cells (data not shown). Because the compound AP30451 was active against DENV-2, we subsequently tested additional inhibitors of WNV infection against DENV-2 by flow cytometric analysis of intracellular viral antigen in BHK21 cells at 72 hours after infection. Notably, several of the compounds tested that inhibited WNV infection in cells also strongly inhibited DENV-2 infection, including compound AP30451 (Fig 7). For AP30451, significant inhibition was observed regardless of whether the compound was added immediately before or after infection (data not shown).

Mechanism of action. We next assessed the mechanism of action of AP30451, our most consistently potent anti-WNV compound in all screening assays. As this antiviral compound was initially identified in the replicon screening assay, we speculated that AP30451 should inhibit translation or replication, and is unlikely to affect viral entry, encapsidation, secretion, or maturation. To further dissect whether AP30451 inhibited flavivirus translation or replication, we examined its effect on luciferase activity at early time points after transfection of infectious replicon RNA. For these studies, we used the YFV replicon for two reasons: (a) AP30451 inhibited YFV replicon propagation (see Fig 6B); (b) the luciferase signal at early time points representing translation of input RNA was significantly higher than that from WNV or DENV-2 replicons, and thus easier to interpret potential drug-mediated inhibition (data not shown).
vitro transcribed m\(^7\)G-capped RNA from the YFV replicon cDNA was transfected into BHK cells, and translation of luciferase was measured in the presence of DMSO diluent, AP30451, ribavirin, or the translation inhibitor cycloheximide (Fig 8A). As expected, cycloheximide virtually abolished replicon translation at early and late times after RNA transfection. Ribavirin, a guanosine analogue and inhibitor of RNA-dependent RNA polymerases (42), had no effect on early translation but did significantly inhibit later translation, likely due to its effect on replication. In contrast, AP30451 blocked both the early and later phases of translation to <10% of DMSO control (P \(\leq\) 0.001). To confirm that the inhibitory effect was specific for flaviviruses, we evaluated the ability of AP30451 to inhibit translation of other viral and non-viral m\(^7\)G-capped and uncapped mRNA. Whereas cycloheximide uniformly blocked mRNA translation, AP30451 did not reduce translation of m\(^7\)G-capped firefly luciferase, or Sindbis virus replicon mRNA, or uncapped mRNA containing an EMCV internal ribosome entry signal (IRES) (Fig 8B-D). For the Sindbis virus replicon, expression of the luciferase gene is under control of the 26S subgenomic promoter and thus is only translated after an initial round of replication of the genomic RNA (82). Overall, AP30451 appeared to inhibit translation of flavivirus RNA specifically.
Currently, there is no approved specific therapy for WNV, or other Flaviviruses. In this report, we performed a primary screen of 80,000 small molecule compounds from a commercial library with a cell-based replicon assay that measured WNV replication as a function of luciferase expression. This primary screen was reproducible, had a robust signal-to-noise ratio, and was amenable to high-throughput applications. Indeed, analogous replicon-based primary screens to identify inhibitors of flavivirus replication have been performed by other groups (35, 74, 75). One limitation of this assay is that candidate inhibitors block translation or replication and not viral entry, encapsidation, secretion, or maturation. Our strategy involved identification of compounds that reduced luciferase activity in lysates of cells followed by a more thorough dose response analysis with multiple data points to rule out cytotoxicity and define the selectivity index. Compounds with favorable inhibitory profiles in the replicon assay were then subjected to three lower-throughput assays using infectious virus. These included the use of a capture ELISA that detected the relative amounts of secreted NS1 in the supernatants of infected cells, and viral infection assays with Vero cells, BHK cells, and primary cortical neurons, the latter a known target of WNV infection in vivo (43, 80, 81, 85). Based on these screens, we identified several compounds whose antiviral profile is sufficiently attractive to justify further drug development efforts, including structure activity relationships, lead optimization, pharmacokinetic studies, toxicokinetic studies, and animal efficacy studies.

The candidate lead compounds from our screens were classified into four diverse chemical classes. One class, the secondary sulfonamides, which are chemically tractable compounds, encompassed the majority of the candidates. However, this could be a reflection of the particular makeup of our library. Indeed, parallel small molecule inhibitor screens by others...
that used an analogous replicon assay but different chemical libraries identified parazolotrahydrothophenes (35), pyrazolopyrimidines (35), pyrazolines (32, 75) xanthanes, acridines, and quinolines (32). One secondary sulfonamide compound, AP30451, was particularly promising and had antiviral activity not only in continuous cell lines, but also in primary neurons. Compound AP30451 inhibited WNV at low micromolar concentrations in multiple assays, which indicates that it is as potent as any published anti-flavivirus small molecule inhibitor. In general, it is expected that a viable therapeutic antiviral drug should be active at concentrations in the sub-micromolar range, depending on its pharmacokinetic and pharmacodynamic properties. Ongoing studies have shown that secondary sulfonamides are amenable to structure-activity relationship lead optimization, and efforts to improve potency and drug-like properties are underway.

In addition to compounds that have been identified by replicon screening, existing small molecule drugs have been proposed as antiviral agents for WNV. Some of these were evaluated in our screens as controls. Ribavirin has inhibitory activity against WNV infection in cell culture (2, 18, 45); this was confirmed in our replicon-based screen. However, in vivo studies with ribavirin and WNV or other flaviviruses have not been promising, as no clinical benefit (50, 60) or increased mortality (13, 64) was observed. MPA, a non-nucleoside inhibitor of IMPDH, inhibits WNV infection in cells by preventing viral RNA replication (24, 66, 86). We also observed significant inhibition with this compound in vitro. However, this compound also has significant immunosuppressive properties in vivo; increased mortality after WNV infection was observed in mice treated with MPA (B. Geiss and M. Diamond, unpublished results). Taken together, the preclinical data suggests that inhibitors of guanosine biosynthesis, such as ribavirin and MPA, may not be promising therapeutic candidates against WNV infection in vivo.
Since WNV is a neurotropic virus and most symptomatic infections are associated with neuroinvasive disease and infection of neurons (17, 38, 78), it is essential that a therapeutic agent efficiently control WNV infection in neurons. Indeed, our study is the first to demonstrate that a small molecule inhibitor of WNV replication can directly control infection in neurons. Nonetheless, for a small molecule to have antiviral activity against neuronal infection \textit{in vivo}, it must efficiently cross the blood-brain barrier. Studies that evaluate and modify lipophilicity and biodistribution of AP30451, its congeners, or other candidate secondary sulfonamides into different tissue compartments are planned.

Compound AP30451 had broad-spectrum anti-flavivirus activity as it also inhibited DENV infection and YFV replicon propagation. RNA transfection and reporter gene studies suggest that AP30451 blocks the initial phase of translation of infectious viral RNA that occurs prior to viral replication. This was not entirely surprising as that this compound was identified using a replicon screening assay, and thus would not be expected to inhibit viral entry, encapsidation, secretion, or maturation. Inhibition of the early phase of translation by AP30451 distinguishes it from recent studies with the pyrazoline class of inhibitors, which apparently block RNA synthesis (32). Nonetheless, it remains possible that AP30451 could have independent inhibitory effects on RNA replication. Interestingly, the inhibition of translation appeared specific for flavivirus replicon mRNA, as no significant reduction of translation was observed by AP30451 with an $\text{m}^7\text{G}$-capped mRNA, an $\text{m}^7\text{G}$-capped Sindbis virus replicon mRNA, or an IRES-regulated transcript. Thus, AP30451 does not appear to be a general inhibitor of cellular or viral mRNA translation, which, in part, may explain its favorable selectivity profile. Accordingly, AP30451 reduced flavivirus replicon propagation but did not specifically inhibit replication of HCV, a distantly related \textit{Flaviviridae} family member that uses
a unique IRES-dependent translation mechanism (29). AP30451 is also distinguished from
broad-spectrum antiviral compounds such as 2-amino-8-(beta-D-ribofuranosyl) imidazo [1,2-a]-
s-triazine-4-one (ZX-2401), which inhibits infection by several types of RNA viruses, including
*Flaviviridae* family members (71). At present, it remains unclear how AP30451 specifically
inhibits translation of m⁷G-capped flavivirus but not other capped or uncapped cellular or viral
mRNA. Studies are underway to define whether AP30451 affects viral RNA stability or the
initiation or elongation phases of translation.

Combination drug therapy against rapidly evolving pathogens has become a mainstay of
treatment of a number of viruses, including HCV and HIV (5, 39) to enhance potency and
decrease emergence of resistant variants. For WNV, novel antiviral strategies with biologic
agents have been developed and include type I IFN (2, 15, 47, 76, 83), immune γ-globulin (6,
27), human or humanized neutralizing monoclonal antibodies (33, 72, 87), peptide inhibitors (3,
40), and oligonucleotide-based platforms, including small interfering RNA (4, 19, 30, 51, 63).
Combination therapy *in vitro* with type I IFN and ZX-2401, a broad-spectrum nucleoside with
anti-flavivirus activity, resulted in moderate synergy of inhibition (71). Synergy studies will be
important to perform with compounds like AP30451 and existing candidate biological
therapeutics against WNV. Indeed, as members of our group have recently developed an
inhibitory humanized mAb that blocks virus entry at a post-attachment step with significant
therapeutic activity in vivo (65, 70, 72), we plan to test our lead compounds, in combination with
these agents in animals models of WNV infection.
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We would like to acknowledge the contribution of members of the Diamond laboratory and Apath to this work. We also thank Dr. Richard Kinney (CDC, Fort Collins) for the WNV and DENV infectious cDNA plasmids. This work was supported by the NIH (U01/AI538870) to M.S.D. and P.D.O.
FIGURE LEGENDS

Figure 1. Functional activity of the WNV subgenomic replicon A. Scheme of the WNV replicon. Beginning from the cDNA of the 1999 New York strain infectious clone of WNV, most of capsid (C), and all of the pre-membrane (prM) and envelope (E) genes were deleted, and a renilla luciferase (Ren) reporter gene and puromycin acetylase gene (Pac) were inserted as a fusion protein linked by a ubiquitin protease cleavage site (Ub) to allow for the expression of individual selectable marker and reporter proteins. The cDNA contains a T7 RNA polymerase promoter to allow for in vitro transcription of replicon RNA. The non-structural proteins (NS1-NS5) are translated via the EMCV IRES. B. Linearity of luciferase activity in BHK cells expressing the WNV replicon. C. Luciferase expression from BHK-WNV replicon cells after treatment with ribavirin. The concentration that inhibits replication 50% (EC50) and the concentration that causes 50% cytotoxicity (CC50) for ribavirin were 6.85 µM and 87 µM, respectively. Results are representative of several independent experiments performed in quadruplicate. D. Flow diagram of the several stages in the drug screening process.

Figure 2. Activity and selectivity of four compounds in the WNV replicon assay. The antiviral activity and cytotoxic effect of compounds were assessed at the indicated concentrations in BHK cells by measuring replication-dependent luciferase expression and ATP quantification assay, respectively. Activity in both assays is expressed as a percent of no-drug controls. Depicted are the EC50 and CC50 curves of four chemical inhibitors from our libraries: (A) AP26549, (B) AP12430, (C) AP18417, and (D) AP30451. Results are representative of several independent experiments performed in quadruplicate.

Figure 3. NS1 capture ELISA for assessing antiviral activity against KUNV infection. A. Effect of IFN-α on NS1 production from KUNV virus-infected cells 48 hours after...
infection (MOI 0.01). B-D. Antiviral activity of different compounds from the chemical libraries. Solid curves show the dose response of WNV replication as measured by decrease in detection of secreted NS1 in supernatants of Vero cells by ELISA. Dashed curves show the cytotoxic effect of compounds in Vero cells at the indicated concentrations using an ATP quantification assay. Results are representative of several independent experiments performed in quadruplicate.

**Figure 4. Effect of candidate inhibitors on infection by a virulent North American strain of WNV.** Vero cells were infected with WNV New York 2000 at an MOI of 0.01. One hour later, DMSO vehicle or indicated inhibitors or MPA was added. One day later, cells were harvested and stained with anti-DENV-3 (negative control) or anti-WNV E mAbs and processed by flow cytometry. Histograms are shown, and the M1 gate indicates the percentage of WNV positive cells for each compound. Results are representative of at least three independent experiments performed in duplicate.

**Figure 5. Compound AP30451 inhibits WNV replication in neurons.** Primary cortical neurons were generated from wild type mice and treated with the indicated compounds or the vehicle control DMSO. A. Neurons were treated with compounds AP30451, AP18417, AP34456 or DMSO and survival was evaluated on 24 hours post addition by a florescence-based viability assay. B. Neurons were infected at an MOI of 0.1 and treated with compounds AP30451, AP18417, AP34456 or DMSO one hour after infection. Virus production was determined 24 hours after infection by plaque assay. C-E. Uninfected and WNV-infected neurons were stained for WNV antigen (α-WNV, red) and co-stained for the neuronal marker NeuN (α-NeuN, green) or a mouse IgG control antibody (Ms IgG) and the nuclear stain TO-PRO-3 (blue). Representative images are shown of uninfected neurons (C), infected neurons treated with DMSO (D), and infected neurons treated with 50 µM466 AP30451 (E). Cells that show staining
for WNV antigen and NeuN are denoted with white arrows. The nuclear stain filter was only included in the merged image.

Figure 6. Anti-flavivirus activity of compound AP30451. BHK (A, B) or Huh-7.5 (C) cells expressing Dengue (A), Yellow fever (B) or hepatitis C (C) subgenomic replicons were treated with increasing concentrations of lead compound AP30451. Antiviral activity of AP30451 was assessed by measuring replication-dependent luciferase expression. Cytotoxic effect of compounds was measured at the indicated concentrations using an ATP quantification assay. Activity in both assays is expressed as a percent of no drug controls. Results are representative of several independent experiments performed in quadruplicate.

Figure 7. Inhibition of DENV-2 infection with lead compounds. BHK cells were incubated for 30 minutes with inhibitors prior to infections with DENV-2 (MOI 0.01). Three days later, cells were harvested, permeabilized, stained with anti-DENV-2 mAb, and processed by flow cytometry. The data are an average of three independent experiments performed in triplicate, and error bars indicate standard deviations. The asterisks indicate statistically significant differences (P < 0.05) compared to the 0.5% DMSO vehicle control.

Figure 8. Effect of WNV inhibitors on viral translation and replication. (A) YFV replicon, (B) Sindbis virus replicon, (C) firefly luciferase, and (D) IRES-β-gal mRNA were generated in vitro after T7 DNA-dependent RNA transcription and transfected by electroporation into BHK21 cells. Immediately after transfection, diluent (1% DMSO), ribavirin (18 µM), AP30451 (15 µM), or cycloheximide (1 mg/ml) were added. Parallel sets of cells were harvested at the indicated times and reporter activity or ATP levels were measured. Data is expressed as reporter relative light units (RLU) per ATP-dependent relative light units to normalize for cell number. One experiment of four performed in octuplicate is shown, and error bars indicate
standard deviations. Note the break in the x- and y-axis to facilitate visualization of both the early and later phases of translation.
### TABLE 1. Chemical classes and virological data of WNV lead compounds.

<table>
<thead>
<tr>
<th>Compound Class</th>
<th>Lead Structure</th>
<th>AP#</th>
<th>WNV Replicon EC50 (µM)</th>
<th>SI</th>
<th>NS1 ELISA EC50 (µM)</th>
<th>SI</th>
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<tbody>
<tr>
<td>5H-Cyclopenta [b]pyridine</td>
<td><img src="image" alt="5H-Cyclopenta [b]pyridine" /></td>
<td>12430</td>
<td>3.41</td>
<td>20.94</td>
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<td>56.82</td>
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<td>Tert-Sulfonamide</td>
<td><img src="image" alt="Tert-Sulfonamide" /></td>
<td>18417</td>
<td>1.23</td>
<td>81.3</td>
<td>1.7</td>
<td>66.96</td>
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<tr>
<td>Thienylpyrimidine</td>
<td><img src="image" alt="Thienylpyrimidine" /></td>
<td>26549</td>
<td>7.94</td>
<td>12.59</td>
<td>2.3</td>
<td>17.4</td>
</tr>
<tr>
<td>Sec-Sulfonamides</td>
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<td>See Table 2</td>
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</table>

The lead compound classes, structures and, EC_{50} and SI (CC_{50}/EC_{50}) derived from replicon and viral assays are shown.
Table 2: Secondary sulfonamide compounds and their virological data.

<table>
<thead>
<tr>
<th>AP#</th>
<th>Structure</th>
<th>WNV Replicon</th>
<th>NS1 ELISA</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>EC50 (µM)</td>
<td>EC50 (µM)</td>
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<td></td>
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<td>SI</td>
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<tr>
<td>69634</td>
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<tr>
<td>31612</td>
<td><img src="image3" alt="Chemical Structure" /></td>
<td>0.85</td>
<td>83.94</td>
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</table>

The secondary sulfonamide structures, EC$_{50}$ and SI (CC$_{50}$/EC$_{50}$) derived from replicon and viral assays are listed.
### Table 3. Inhibition of WNV Infection in Vero Cells

<table>
<thead>
<tr>
<th>Controls</th>
<th>% positive cells</th>
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<tbody>
<tr>
<td>WNV - Media</td>
<td>89 ± 7</td>
</tr>
<tr>
<td>WNV – 0.5% DMSO</td>
<td>88 ± 7</td>
</tr>
<tr>
<td>MPA (10 µg/ml)</td>
<td>24 ± 12**</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Compounds</th>
<th>% positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50 µM 16.6 µM 5.6 µM</td>
</tr>
<tr>
<td>AP30451</td>
<td>1 ± 2** 40 ± 8** 80 ± 7</td>
</tr>
<tr>
<td>AP79018</td>
<td>20 ± 4** 83 ± 3 87 ± 5</td>
</tr>
<tr>
<td>AP74347</td>
<td>74 ± 26 78 ± 20 88 ± 4</td>
</tr>
<tr>
<td>AP45511</td>
<td>60 ± 6** 84 ± 9 84 ± 7</td>
</tr>
<tr>
<td>AP12430</td>
<td>17 ± 9** 49 ± 17** 62 ± 20</td>
</tr>
<tr>
<td>AP69634</td>
<td>5 ± 2** 20 ± 5** 83 ± 6</td>
</tr>
<tr>
<td>AP18417</td>
<td>5 ± 3** 6 ± 3** 61 ± 29</td>
</tr>
<tr>
<td>AP26549</td>
<td>19 ± 5** 61 ± 8** 86 ± 4</td>
</tr>
<tr>
<td>AP34456</td>
<td>0.5 ± 0.3** 8 ± 5** 72 ± 9</td>
</tr>
<tr>
<td>AP31612</td>
<td>39 ± 41 83 ± 10 85 ± 5</td>
</tr>
</tbody>
</table>

Vero cells were infected with the New York strain of WNV for one hour at 37°C. Subsequently, cells were washed and inhibitors were added at the indicated concentrations. One day later, cells were harvested, permeabilized, stained with anti-WNV E mAb, and processed by flow cytometry. The data are an average of three independent experiments performed in duplicate. The asterisks (**) indicate statistically significant differences (P < 0.05) compared to the 0.5% DMSO vehicle control. MPA indicates treatment with mycophenolic acid.


Figure 3
Figure 4
Figure 6

(A) Dengue virus

(B) Yellow fever virus

(C) Hepatitis C virus
Figure 7
Figure 8