Triaryl Pyrazoline Compound Inhibits Flavivirus RNA Replication

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Triaryl pyrazoline {[5-(4-chloro-phenyl)-3-thiophen-2-yl-4,5-dihydro-pyrazol-1-yl]-phenyl-methanone} inhibits flavivirus infection in cell culture. The inhibitor was identified through high-throughput screening of a compound library using a luciferase-expressing West Nile (WN) virus infection assay. The compound inhibited an epidemic strain of WN virus without detectable cytotoxicity (a 50% effective concentration of 28 μ M and a compound concentration of ≥ 300 μ M required to reduce 50% cell viability). Besides WN virus, the compound also inhibited other flaviviruses (dengue, yellow fever, and St. Louis encephalitis viruses), an alphavirus (Western equine encephalitis virus), a coronavirus (mouse hepatitis virus), and a rhabdovirus (vesicular stomatitis virus). However, the compound did not suppress an orthomyxovirus (influenza virus) or a retrovirus (human immunodeficiency virus type 1). Mode-of-action analyses in WN virus showed that the compound did not inhibit viral entry or virion assembly but specifically suppressed viral RNA synthesis. To examine the mechanism of inhibition of dengue virus, we developed two replicon systems for dengue type 1 virus: (i) a stable cell line that harbored replicons containing a luciferase reporter and a neomycin phosphotransferase selection marker and (ii) a luciferase-expressing replicon that could differentiate between viral translation and RNA replication. Analyses of the compound in the dengue type 1 virus replicon systems showed that it weakly suppressed viral translation but significantly inhibited viral RNA synthesis. Overall, the results demonstrate that triaryl pyrazoline exerts a broad spectrum of antiflavivirus activity through potent inhibition of viral RNA replication. This novel inhibitor could be developed for potential treatment of flavivirus infection.

The family *Flaviviridae* contains three genera, the flaviviruses, the pestiviruses, and the hepatitis C viruses. Many members within the *Flavivirus* genus are arthropod-borne pathogens that cause significant human morbidity and mortality. The global emerging flaviviruses include the four serotypes of dengue (DEN), yellow fever (YF), West Nile (WN), Japanese encephalitis (JE), and tick-borne encephalitis viruses (5). The World Health Organization estimated annual human cases of more than 50 million, 200,000, and 50,000 for DEN (52), YF (54), and JE (53) viral infections, respectively. The recent epidemics of WN virus in the United States have caused thousands of human cases, representing the largest meningoencephalitis outbreak in the Western Hemisphere and the largest WN virus outbreak ever reported (6). Human vaccines are currently available only for YF, JE, and tick-borne encephalitis viruses, and no effective antiviral therapy has been developed for treatment of flavivirus infections. Therefore, it is a public health priority to develop and improve vaccines and antiviral agents for prevention and treatment of flavivirus infections.

Inhibitors of any steps essential for the viral life cycle could potentially be developed for treatment of flavivirus infection. Flaviviruses enter host cells through receptor-mediated endo-

cytosis. The virions are approximately 50 nm in diameter (23) and contain a single-stranded, plus-sense RNA genome of about 11 kb in length (7). The genomic RNA contains a 5' untranslated region (UTR), a single open reading frame (ORF), and a 3' UTR. Upon uncoating from the nucleocapsid, the ORF of the viral genome encodes a polyprotein that is co- and posttranslationally processed by viral and cellular proteases into 10 N-terminal mature proteins, designated as capsid (C), premembrane (prM) or membrane (M), envelope (E), nonstructural protein 1 (NS1), NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (7). Among those, C, prM/M, and E are structural proteins which are involved primarily in viral particle formation. The NS proteins are responsible for viral RNA replication but may also function in viral assembly (24, 28) and evasion of host immune response (16, 27, 35). NS3 is a multifunctional protein with activities of a serine protease (with NS2B as a cofactor) (14), 5' RNA triphosphatase (50), nucleoside triphosphatase (NTPase) (50), and RNA helicase (49). NS5 has the functions of an RNA-dependent RNA polymerase (47) and 2'O-methyltransferase, which is involved in the methylation of the 5' RNA cap structure (13, 22). Replication complexes form at the endoplasmic reticulum (ER) membrane and transcribe genomic plus-sense RNA into a complementary minus-sense RNA, which in turn serves as the template for the synthesis of more plus-sense RNA (7). The plus-sense RNA is then packaged by viral C protein to form a progeny nucleocapsid that is enclosed in an envelope consisting of a host-derived lipid bilayer and viral prM/M and E proteins.

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Several categories of inhibitors for flaviviruses have been reported. The first includes inhibitors of nucleoside triphosphate synthesis, including mycophenolic acid (MPA), ribavirin, and 6-azauridine (34). Among these, ribavirin has been in clinical use for treatment of hepatitis C virus infection. Various mechanisms of action for ribavirin have been suggested (17). Inhibition of IMP dehydrogenase was recently shown to be the predominant mechanism of ribavirin-mediated inhibition of flavivirus infections (26). Besides inhibition of nucleoside triphosphate synthesis, some nucleoside analogues suppress viral replication by direct incorporation into viral RNA chains (10, 33, 38). In the second category are inhibitors of helicase and protease activities of NS3. A series of nucleoside analogues were reported to inhibit flavivirus NTPase/helicase activity (3, 56). The protease activity of DEN virus NS3 was found to be inhibited by peptide-like, α -keto amide backbone compounds mimicking the conserved cleavage sites (basic residues at the P2 and P1 positions and a residue with a small side chain at the P1' position) (25). Third are inhibitors of host α -glucosidase that suppress virion secretion and infectivity. Castanospermine and deoxynojirimycin are α -glucosidase inhibitors that could disrupt folding of the DEN virus envelope protein by preventing the removal of the terminal glucose residue on the N-linked glycan (8, 55). Castanospermine was recently shown to be efficacious in a mouse model of DEN virus infection (51). Fourth is passive immunity using intravenous immunoglobulin (Ig). Passive administration of monoclonal antibodies was reported to limit the encephalitis caused by St. Louis encephalitis (SLE) (31), JE (20, 41), YF (4, 43), and WN (46) viruses. Humanized monoclonal antibodies against WN virus E protein were recently shown to have therapeutic efficacy in mice, even when administered as a single dose 5 days after infection (37). The final category involves nucleic acid-based antiviral therapy. RNA silencing was shown to suppress DEN-2 virus (1) and WN virus (32) in tissue culture. Recently, antisense phosphorodiamidate morpholino oligomers were reported to potently inhibit WN and DEN viral infections in tissue culture (11, 21).

The goal of this study was to identify new inhibitors of flavivirus infection. We have identified triaryl pyrazoline, which inhibits multiple flaviviruses without detectable cytotoxicity. Mode-of-action analyses indicated that the compound inhibits both WN and DEN type 1 (DEN-1) virus infections through significant suppression of viral RNA synthesis. In addition, we demonstrate that DEN-1 virus replicon systems are suitable for high-throughput screening (HTS) for inhibitors of DEN virus.

MATERIALS AND METHODS

Cells and viruses. Vero and BHK-21 cells were maintained in Dulbecco modified Eagle medium with 10% fetal bovine serum (FBS) in 5% CO₂ at 37°C. A BHK-21 cell line containing a luciferase-expressing WN virus replicon was maintained with 1 mg/ml G418 in the culture medium (30). WN virus was derived from a full-length infectious cDNA clone of an epidemic strain (45). A full-length WN virus containing a luciferase reporter was previously constructed from an infectious cDNA clone by inserting an encephalomyocarditis virus internal ribosomal entry site (EMCV IRES)-luciferase fragment into the 3' UTR of the WN virus genome (11). WN (strain 3356), YF (17D vaccine strain), DEN-2 (strain New Guinea), SLE (strain Kern217.3.1.1), and western equine encephalitis (WEE; strain Cova 746) viruses were generously provided by Laura Kramer at Wadsworth Center, Albany, NY. Vesicular stomatitis virus (VSV; New Jersey serotype) and mouse hepatitis virus (MHV; strain A59) were also used for antiviral assays. A laboratory strain of influenza virus A/WSN/33 was rescued by

reverse genetics (36) and propagated by infecting 10-day-old embryonated chicken eggs. Allantoic fluid containing virus was harvested at 72 h postinoculation, and the titer was determined by plaque assay.

Construction of DEN-1 virus reporting replicons. Three types of reporting replicons were prepared for DEN-1 virus Western Pacific 74 strain (GenBank accession no. U88535) (40). The type 1 replicon contained a *Renilla* luciferase (Rluc) gene fused in frame with the ORF of the genome at a position where the structural genes from nucleotides (nt) 209 to 2326 were deleted. An overlap extension approach was used to prepare a DNA fragment containing the following sequence: BamHI/AscI/T7 promoter/DEN-1(nt 1 to 208)/NotI/Rluc/BsiWI/DEN-1(nt 2327 to 2712). A full-length infectious clone of a DEN-1 virus strain (40) was used as a template for amplifying DEN-1 virus sequences. The PCR product was digested with BamHI and XhoI (nt 2606 to 2611 of DEN-1 virus) and cloned into a pBR322-based plasmid (44). Next, the XhoI-to-SacII fragment representing the rest of the DEN-1 virus genome was cleaved off from the full-length DEN-1 virus clone and ligated into the pBR322-based intermediate plasmid described above, resulting in DEN-1 Rluc-Rep plasmid.

The type 2 replicon was constructed by insertion of a foot-and-mouth disease virus (FMDV) 2A sequence (42) immediately downstream of the Rluc reporter in DEN-1 Rluc-Rep. The FMDV 2A sequence was designed to cleave the polypeptide at the N terminus of the residual E fragment in the replicon. Complementary oligonucleotides representing the FMDV 2A sequence (42) containing a BsiWI site on both ends were annealed in a hybridization buffer (10 mM NaCl and 10 mM Tris, pH 7.5) by being heated at 95°C for 3 min, followed by slow cooling to room temperature. After treatment with T4 polynucleotide kinase (to phosphorylate the 5' ends of the annealed oligonucleotides for subsequent DNA ligation) (New England Biolabs, Beverly, MA) in T4 DNA ligase buffer at 37°C for 1 h, the annealed oligonucleotides were purified through a gel purification column (QIAGEN, Valencia, CA) and inserted into the unique BsiWI site of plasmid DEN-1 Rluc-Rep, resulting in DEN-1 Rluc-2A-Rep.

The type 3 replicon was prepared to generate a stable cell line containing a persistently replicating DEN-1 virus replicon. A reporting cassette containing the following sequence was directly PCR amplified from a replicon pLN-BR of bovine viral diarrhea virus (18): Ubi (ubiquitin)-Neo (neomycin phosphotransferase)-EMCV IRES. The bovine viral diarrhea virus replicon was generously provided by Weidong Zhong from Valeant Pharmaceutical International Inc., Costa Mesa, CA. The reporting cassette was then cloned into DEN-1 Rluc-Rep at the unique BsiWI site, resulting in plasmid DEN-1 Rluc-Neo-Rep. For all constructs, DNA sequencing was performed to ensure that no mutations had occurred during PCR amplification. Standard procedures were performed for PCR and DNA cloning, with modifications as previously described (45).

Characterization of Rluc-expressing replicon of DEN-1 virus. Plasmid DNA for various DEN-1 virus replicons was linearized with SacII and subjected to in vitro transcription using a T7 mMESSAGE mMACHINE kit (Ambion, Austin, TX) as previously described (29). The replication kinetics of each replicon was examined by transfection of BHK-21 cells, followed by an Rluc assay at indicated time points posttransfection (p.t.). RNA replication was also monitored by expression of viral proteins using an indirect immunofluorescence analysis (IFA). The IFA was performed using DEN-1 virus immune mouse ascites fluid (ATCC, Manassas, VA) and goat anti-mouse IgG conjugated with Texas Red as primary and secondary antibodies, respectively (44).

Establishment of stable cell lines containing a luciferase-expressing replicon of DEN-1 virus. Vero cells were transfected with DEN-1 Rluc-Neo-Rep RNA and selected under G418 (1 mg/ml) from day 1 p.t. After 3 weeks of selection, individual foci were detached using sterile cloning disks (Bel-Art Products, Pequannock, NJ) soaked with trypsin. The Rluc-Neo-Rep-containing Vero cells (absorbed onto the disks) were then transferred into a 24-well plate, amplified, and subjected to an Rluc assay and IFA.

HTS antiviral assays. For WN virus, three HTS assays (a luciferase-expressing replicon cell line, a virus-like particle [VLP] infection assay, and a reporting full-length viral infection assay) were previously established and validated for antiviral screening in a 96-well format (39). Since the VLP infection assay and the reporting WN virus infection assay involved infectious particles, they were performed in biosafety level 3 containment. The replicon cell line-based assays (for both WN and DEN-1 viruses) were performed in a biosafety level 2 laboratory because no infectious particles were involved. Fifty percent effective concentration (EC₅₀) values were calculated based on the luciferase signal and compound concentration curves using regression analysis (SAS, version 6.12; SAS Institute Inc., Cary, NC).

Screening of a compound library. A small molecular compound library consisting of 200 small molecules with diverse structures was screened to identify WN virus inhibitors. All compounds were dissolved in dimethyl sulfoxide (DMSO) and assayed at a final concentration of 1% DMSO. A full-length

Rluc-expressing WN virus was used to screen the compound library. Briefly, Vero cells were seeded at 8×10^4 cells per well of a 96-well plate. Six hours after cells were seeded, they were infected with the Rluc-expressing virus (multiplicity of infection [MOI] of 0.1) and treated immediately with 30 μ M of compounds. The screening concentration at 30 μ M was empirically selected. The plates were assayed at 24 h postinfection (p.i.) using an Rluc assay kit (Promega, Madison, WI) and a Veritas microplate luminometer (Turner Biosystem Inc., Sunnyvale, CA). Compounds exhibiting greater than 50% inhibition of Rluc activity were assayed for cytotoxicity.

MTT cell proliferation assay. An MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] cell proliferation assay (ATCC) was used to estimate potential cytotoxicity of the compound. Approximately 1×10^4 BHK-21 or Vero cells in 100 μ l medium were seeded per well in a 96-well plate. After 6 h of incubation, 1 μ l of compound dissolved in DMSO was added to cells at the indicated concentrations. After 48 h of incubation, 10 μ l of MTT reagent was added and cells were incubated for another 3.5 h, after which 100 μ l of detergent reagent was added. The plates were swirled gently and left in darkness at room temperature for 4 h. A microtiter plate reader (Molecular Devices Corporation, Sunnyvale, CA) with a 550-nm filter was used to record the absorbance, from which a CC_{50} (compound concentration required to reduce 50% cell viability) value was estimated.

Viral titer reduction assay. Viral titer reduction assays were performed to examine the antiviral activities of triaryl pyrazoline in VSV and WN, YF (17D), DEN-2, SLE, and WEE viruses. Approximately 9×10^5 Vero cells per well were seeded in a 12-well plate. After incubation for 12 h, the cells were infected with individual virus (MOI of 0.1) and treated immediately with the compound at the indicated concentrations. For WN, YF (17D), DEN-2, SLE, and WEE viruses, samples of culture medium were collected at 42 h p.i., stored at -80°C , and subjected to plaque assays on Vero cells. For VSV, culture medium was collected at 16 h p.i.

A double-overlay protocol was followed for plaque assays. For WN virus, approximately 6×10^5 Vero cells per well were seeded in a six-well plate and incubated for 3 days to reach full confluence. Cells were infected with 100 μ l of 10-fold dilutions of the virus for 1 h at 37°C . Afterwards, 3 ml of a first layer containing 0.6% Oxoid agar, basal medium Eagle with 1% FBS, 0.02% DEAE dextran, and 0.13% NaHCO_3 was added to the infected cells. Two days later, 3 ml of a second layer containing 1% Noble agar, basal medium Eagle with 1% FBS, 0.02% DEAE dextran, 0.13% NaHCO_3 , and 0.004% neutral red was added over the first layer. The plates were further incubated for 12 h before plaques were counted. The protocol for WEE virus was identical to that for WN virus. For other viruses, the time interval between the addition of the first and the second layer of agar was 1 day for VSV, 4 days for YF virus and 5 days for DEN-2 and SLE viruses.

For the influenza virus assay, MDCK cells were inoculated with strain A/WSN/33 (MOI of 0.1) in samples of medium (minimal essential medium containing 0.375% bovine serum albumin fraction V [Invitrogen, Carlsbad, CA] and 2 $\mu\text{g}/\text{ml}$ of TPCK [tosylsulfonil phenylalanyl chloromethyl ketone]-treated trypsin) containing 0, 1.2, 3.7, 11, 33, 100, and 300 μM of compound. The inoculum was adsorbed for 1 h at 37°C . Cell monolayers were washed twice with medium and replenished with medium containing various concentrations of the compound. The supernatant was harvested at 48 h p.i., and the 50% tissue culture infective dose was determined by inoculating serial dilutions onto MDCK cell monolayers and monitoring cytopathic effects.

For the human immunodeficiency virus (HIV) assay, U373-MAGI-CXCR4 cells (2.5×10^5), indicator cell lines stably transfected with a β -galactosidase reporter gene under the control of the HIV-1 long terminal repeat (48), were infected in triplicate with 250 50% tissue culture infective doses of HIV-1_{LAV/HTLV-3B} in a 12-well plate. HIV-1-uninfected cells were included as a background control. After incubation at 37°C for 2 h, the cells were washed three times with phosphate-buffered saline (PBS), and 0.25-ml samples of fresh medium containing 0, 1.1, 3.7, 11, 33, and 100 μM of compound were added. At day 7 p.i., the cells were washed three times with PBS. Cell lysates were harvested by resuspending the cells in 100 μ l reporter lysis buffer. Lysates were assayed for reporter gene expression, as a marker of productive HIV-1 infection, by using a β -galactosidase enzyme assay system (Promega).

For the MHV assay, mouse 17 clone 1 cells were infected with MHV strain A59 at a multiplicity of 0.1 PFU per cell in the presence of 0, 3.7, 11, 33, and 100 μM of compound. The culture supernatants were harvested at 20 h p.i. and titers of virus were determined on mouse L2 cells using plaque assays as previously described (19).

Time-of-drug-addition assay. A time-of-addition experiment was performed to estimate the step of the viral life cycle that was suppressed by triaryl pyrazoline. Approximately 9×10^5 Vero cells per well were seeded in a 12-well plate, incubated for 12 h for cell attachment, and synchronously infected with WN virus. The infection was carried out at an MOI of 5 for 1 h, followed by three

FIG. 1. Identification of triaryl pyrazoline as an inhibitor of a full-length luciferase-expressing WN virus. (A) A full-length WN virus containing a luciferase reporter (FL Rluc-WN) (11) was used to infect Vero cells at an MOI of 0.1. The infected cells were incubated with a compound library at the indicated concentrations and assayed for luciferase activity at 24 h p.i. Values above each bar indicate percentages of luciferase activity derived from the compound-treated infections versus the luciferase signal derived from the mock-treated infection. MPA was included as a positive control. Error bars indicate standard deviations. (B) Structure of triaryl pyrazoline, an inhibitor identified from the screening.

rounds of PBS washes to remove unabsorbed virus. At different time points p.i., triaryl pyrazoline was added to the infected cells at 100 μM . Samples of culture medium were collected at 24 h p.i., stored at -80°C , and subjected to plaque assay as described above. As a negative control, DMSO was added to infected cells at a final concentration of 1% at 0 and 20 h p.i. to estimate its effect on viral production.

Transient replicon assay. A transient replicon assay was used to quantify compound-mediated inhibition of viral translation and suppression of RNA replication. For WN virus, replicon RNA (10 μg) was electroporated into BHK-21 cells (8×10^6) as previously described (29). The transfected cells were suspended in 25 ml of Dulbecco modified Eagle medium with 10% FBS. Cell suspension (5×10^5 cells in 167 μl per well) was added to 12-well plates, immediately treated with 30 or 100 μM of compound, and assayed for luciferase activity at 2 and 4 h p.t. (representing viral translation) and 72 h p.t. (representing RNA replication). A similar protocol was performed for the DEN-1 virus Rluc-expressing replicon, except that a time point at 48 h p.t. was used to quantify viral RNA replication (see details in Results).

RESULTS

Identification of triaryl pyrazoline as an inhibitor of WN virus. We used a full-length WN virus containing an Rluc gene (FL Rluc-WNV) (Fig. 1A) to screen a compound library with diverse structures. The structures of the compound library have been described elsewhere (15). One major advantage of using the FL Rluc-WNV infection as a primary screening is that the assay includes the complete infection cycle and can identify potential inhibitors of any step of the viral life cycle. Vero cells infected with FL Rluc-WNV (MOI of 0.1) were

incubated with compounds (30 μ M and 1% DMSO) in a 96-well format. Antiviral activities of potential inhibitors were indicated by suppression of Rluc signals at 24 h p.i. The screening results showed that a triaryl pyrazoline compound inhibited Rluc activity by 57% compared with the mock-treated infection (with 1% DMSO) (Fig. 1). As a positive control, MPA (a known WN virus inhibitor) (34) was tested at 1 μ M, yielding an Rluc signal suppression of 87%. The results indicated that triaryl pyrazoline is an inhibitor of WN virus.

Inhibition of an epidemic strain of WN virus without cytotoxicity. To verify the primary screening results, we assayed triaryl pyrazoline in an authentic viral titer reduction assay. Vero cells were infected with an epidemic strain of WN virus (MOI of 0.1), immediately treated with the compound, and assayed for viral yields in culture medium at 42 h p.i. The compound inhibited viral titer in a dose-responsive manner (Fig. 2A). At a concentration of 100 μ M or higher, the compound reduced viral titer by >90%. The EC_{50} value was estimated to be 28 μ M. Next, we performed an MTT assay to exclude the possibility that the observed antiviral activity was due to compound-mediated cytotoxicity. Vero cells were incubated with the compound for 48 h, and cell viability was measured by cellular metabolism of MTT tetrazolium salt (Fig. 2B). No reduction of cell viability was observed up to 300 μ M. A similar result was obtained when compound incubation was extended to 96 h (data not shown). However, we observed tiny crystals under the microscope in culture medium when the compound concentration reached >300 μ M. The results indicate that the CC_{50} of the compound is ≥ 300 μ M.

A broad spectrum of antiviral activity against flaviviruses and other RNA viruses. To examine whether triaryl pyrazoline

FIG. 2. Inhibition of an epidemic strain of WN virus and cytotoxicity of triaryl pyrazoline. (A) Vero cells were infected with an epidemic strain of WN virus (MOI of 0.1), immediately treated with compound at the indicated concentrations, and assayed for viral titers at 42 h p.i. Values above each data point indicate percentages of viral titer from the compound-treated infections compared with that from the mock-treated infection. Data represent means and standard deviations ($n \geq 3$). (B) Cytotoxicity was examined by incubation of Vero cells with the indicated concentrations of the compound. Cell viability was measured by an MTT assay and presented as a percentage of colorimetric absorbance derived from the compound-treated cells compared with that from the mock-treated cells (with 1% DMSO). Average results from two experiments are shown.

FIG. 3. Antiviral activities of triaryl pyrazoline. Viral titer reduction assays were performed for the indicated viruses to determine the antiviral activities of the compound (see details in Materials and Methods). For MHV, VSV, and DEN-2, YF, SLE, and WEE viruses, viral titer reduction (n -fold; viral titer without treatment divided by viral titer with compound treatment) was indicated when infections were treated with 100 μ M of compound.

inhibits other flaviviruses, we performed viral titer reduction assays with DEN-2, YF (17D), and SLE viruses. Vero cells were infected with each virus (MOI of 0.1), incubated with the compound, and quantified for viral yields in culture medium at 42 h p.i. The compound inhibited all three viruses (Fig. 3). Viral titers were significantly reduced when infected cells were treated with 33 μ M of the compound; increasing the concentration of the compound to 100 μ M did not further suppress viral titers. The tested flaviviruses showed different sensitivities to the compound at 100 μ M concentration, with 184-, 34-, 25-, and 12-fold reduction in viral titers for YF, DEN-2, SLE, and WN viruses, respectively (Fig. 3).

To further test the antiviral spectrum, we analyzed the compound with nonflaviviruses (Fig. 3). The compound inhibited WEE virus (a plus-strand RNA alphavirus), MHV (a plus-strand RNA coronavirus), and VSV (a negative-strand RNA rhabdovirus); at 100 μ M concentration, the compound reduced viral titers of WEE virus, MHV, and VSV by 10-, 12-, and 22-fold, respectively. In contrast, the compound did not inhibit influenza virus (a negative-strand RNA orthomyxovirus), HIV-1 (a retrovirus) (Fig. 3), or herpes simplex virus (a DNA virus) (data not shown). The results suggest that triaryl pyrazoline can inhibit a broad spectrum of RNA viruses with selectivity.

Inhibition of WN virus infection through suppression of RNA replication. To identify the step(s) at which triaryl pyrazoline suppresses WN virus infection, we analyzed the inhibitor in three replicon-based assays. These assays cover different steps of the viral life cycle and have been validated for mode-of-action analysis (11). The first assay used BHK-21 cells infected with VLPs containing an Rluc-expressing replicon RNA (Fig. 4A). VLPs were prepared by *trans* supplying WN structural proteins in a stable cell line containing Rluc-expressing replicons (WN Rluc-Neo-Rep) (Fig. 4B) (39). Infection of naive BHK-21 cells with such Rluc-VLPs (MOI of 1) could be used to examine whether an inhibitor blocks viral entry and replication. Treatment of the VLP-infected cells with triaryl pyrazoline suppressed Rluc signals, indicating that the compound could inhibit viral entry and/or replication. The EC_{50} value was estimated to be 14 μ M (Fig. 4A).

The second assay was based on a BHK-21 cell line harboring WN Rluc-Neo-Rep (Fig. 4B). The reporting cell line allows testing of inhibitors of viral replication (including viral translation and RNA replication) but not viral entry. Analyses using the WN virus Rluc-Neo-Rep cell line showed that the compound inhibited viral replication, with an EC_{50} of 19 μ M.

The third assay involved WN Rluc-Rep, in which an Rluc reporter was fused in frame with the ORF of the genome in which the structural genes were deleted (Fig. 4C). Transfection of BHK-21 cells with this replicon reveals two Rluc peaks, one at 1 to 10 h p.t. and another at >24 h p.t., which represent viral translation and RNA replication, respectively (29). To distinguish compound-mediated inhibition of viral translation from inhibition of RNA synthesis, we transfected BHK-21 cells with WN virus Rluc-Rep RNA, immediately incubated the cells with compound, and assayed them for Rluc activity at 2, 4, and 72 h p.t. (Fig. 4C). At 2 and 4 h p.t., Rluc signals from the cells treated with compound (at 33 and 100 μ M) were around 96 to 117% of those from the mock-treated cells. In contrast, at 72 h p.t., the compound suppressed Rluc activity by over 95%. The

A WN Rluc-VLP/BHK cell infection assay:

B Reporting replicon BHK cell line assay:

C Transient reporting replicon assay:

FIG. 4. Mechanism of triaryl pyrazoline-mediated inhibition of WN virus. (A) WN VLPs containing a luciferase-expressing replicon (Rluc-VLP) (39) were used to infect BHK-21 cells in the presence of indicated concentrations of the compound. (B) A BHK-21 cell line containing a WN virus replicon (Rluc-Neo-Rep) was incubated with the compound for 48 h and measured for luciferase activity. The average results from two experiments are shown in panels A and B. (C) A WN virus reporting replicon containing a luciferase reporter (WN Rluc-Rep) (fused in frame with the ORF) was used to measure the effects of the compound on viral translation and RNA replication. Values above each data point indicate percentages of luciferase signals from the compound-treated transfections compared with that from the mock-treated transfection. Error bars indicate standard deviations from three independent experiments.

data suggest that triaryl pyrazoline inhibits WN virus through suppression of viral RNA replication.

Time of addition of triaryl pyrazoline in WN viral infection. A time-of-addition experiment was performed to further de-

FIG. 5. Time-of-addition analyses of triaryl pyrazoline in WN virus infection. Vero cells were synchronously infected with WN virus, treated with compounds (100 μ M) at indicated time points after infection, and quantified for viral yields in culture medium at 24 h p.i. Because all compound treatments contained 1% DMSO, this concentration of DMSO was added to infected cells at 0 and 20 h p.i. to estimate its effect on viral yield production.

fine the mode of action of the compound. Vero cells were synchronously infected with an epidemic strain of WN virus. The compound (100 μ M) was added to infected cells at various time points postinfection. Viral titers in the culture medium were determined at 24 h p.i. For mock treatment, 1% DMSO was added at 0 and 20 h p.i. to estimate its effect on viral yield (because triaryl pyrazoline was tested at 1% DMSO). As shown in Fig. 5, a significant and steady level of suppression in viral titer (a reduction of 5×10^8 PFU/ml, equivalent to 11- to 12-fold reduction) was observed when the compound was added during the initial 10 h of infection. Inhibitory effects gradually diminished when the compound was added between 10 to 20 h p.i. We previously showed that during BHK-21 cell infection with an Rluc-expressing WN virus, initial translation occurred during the first 7.5 h p.i., while nascent genomic RNA began to accumulate after 10 h p.i. (39). Our time-of-addition data showed that the compound begins to lose its activity if added at 10 h p.i. or later, coinciding with the initiation of viral RNA replication. These results support the hypothesis that the inhibitor blocks WN virus at the stage of viral RNA synthesis.

An HTS assay for identifying inhibitors of DEN virus replication. To test whether the mechanism of triaryl pyrazoline-mediated inhibition of DEN virus is similar to that of WN virus, we developed a replicon of DEN-1 virus in which an Rluc-Ubi-Neo-EMCV IRES fragment was substituted for the viral structural genes (DEN-1 Rluc-Neo-Rep) (Fig. 6A). The DEN-1 Rluc-Neo-Rep fragment retained the 37 N-terminal amino acids of the capsid protein and the 31 C-terminal amino acids of the envelope protein. Upon transfection of Vero cells with DEN-1 Rluc-Neo-Rep RNA, the Rluc-Ubi-Neo polypeptide driven by the DEN-1 5' UTR was processed through Ubi-mediated cleavage, resulting in individual Rluc and Neo proteins; the NS proteins were translated through the EMCV IRES. G418 selection of the transfected cells allowed establishment of cell lines containing persistently replicating replicons. Analyses of the established cell lines showed that 100% of the cells expressed viral proteins (Fig. 6B) and exhibited a high level of Rluc activity (Fig. 6C). Cells passaged for more than 4 months retained robust expression of viral proteins and the Rluc reporter (data not shown).

FIG. 6. Inhibition of a cell line containing a luciferase-expressing replicon of DEN-1 virus. (A) A replicon of DEN-1 virus (Rluc-Neo-Rep) was constructed by replacing the viral structural genes with an Rluc-Ubi-Neo-EMCV IRES fragment. G418 selection of the Rluc-Neo-Rep-transfected Vero cells allowed establishment of cell lines containing persistently replicating replicons. (B) Rluc-Neo-Rep-containing cell lines were analyzed by IFA using DEN-1 virus immune mouse ascites fluid and goat anti-mouse IgG conjugated with Texas Red as primary and secondary antibodies, respectively. The same views of an IFA stained with Texas Red (left panel) and a differential interference contrast (right panel) are presented. (C) The reporting Vero cells containing DEN-1 Rluc-Neo-Rep were used in an antiviral assay in a 96-well format. The reporting cells were incubated with MPA, glycyrrhizin, and triaryl pyrazoline at the indicated concentrations for 48 h and assayed for luciferase activity.

We converted the DEN-1 virus replicon-containing cell line into an HTS assay. Seeding of 2×10^4 cells per well of a 96-well plate and incubation of the cells for 48 h consistently showed an assay window of 1×10^6 to 2×10^6 (Rluc signal from replicon-bearing cells divided by the background signal from naive Vero cells). To validate the assay for HTS, we incubated the DEN-1 Rluc-Neo-Rep cell lines with various concentrations of MPA, a known inhibitor of DEN virus (12). MPA suppressed the Rluc signal in a dose-responsive manner (Fig. 6C). Since the CC_{50} of MPA in Vero cells was previously reported to be $>300 \mu$ M (34), the reduction of Rluc in the DEN-1 virus replicon cells was not due to its cytotoxicity. In contrast, no suppression of Rluc activity was detected when cells were incubated with glycyrrhizin, a compound previously reported to exhibit weak inhibition of WN virus (9). The EC_{50} values were estimated to be 0.3 and $>100 \mu$ M for MPA and glycyrrhizin, respectively. Next, we tested triaryl pyrazoline in the DEN-1 Rluc-Neo-Rep-containing cells in a 96-well plate. Incubation of the cells with the compound inhibited Rluc activity, yielding an EC_{50} of 17 μ M (Fig. 6C). The results demonstrate that (i) the reporting cell line can be used as an HTS

assay for identifying inhibitors of DEN-1 virus and that (ii) triaryl pyrazoline inhibits DEN-1 virus replication.

Triaryl pyrazoline-mediated inhibition of DEN-1 virus RNA replication. To further compare the mode of action of triaryl pyrazoline inhibition of DEN viruses to that of WN viruses, we established a DEN-1 virus reporting replicon which can differentiate between viral translation and RNA replication. Initially, we constructed a replicon in which an Rluc gene replaced viral structural genes (DEN-1 Rluc-Rep) (Fig. 7A). Upon transfection, the replicon was expected to express an Rluc fusion protein containing an extra 40 N-terminal amino acids (37 residues from the capsid protein and 3 residues from the NotI site engineered for cloning purposes) and an extra 33 C-terminal amino acids (2 residues from the BsiWI site engineered for cloning and 31 residues from the envelope protein). Transfection of BHK-21 cells with DEN-1 Rluc-Rep yielded only a single Rluc peak during the initial 10 h p.t. (Fig. 7B), and no further Rluc activity was detected up to 96 h p.t. (data not shown). Samples after 96 h p.t. were not analyzed for Rluc activity due to overgrowth of the transfected cells. In agreement with the Rluc profile, the IFA showed no viral protein expression in the transfected cells by 60 h p.t. (Fig. 7C, left panel). The results suggest that DEN-1 Rluc-Rep RNA was successfully transfected into cells (judging by the translation of the input RNA) but that no replication followed.

To rescue RNA replication, we inserted an FMDV 2A sequence into the original replicon to mediate a cleavage between Rluc and the C-terminal fragment of the envelope protein (DEN-1 Rluc-2A-Rep) (Fig. 7A). Since the FMDV 2A sequence cleaves at its C terminus, the Rluc expressed from the DEN-1 Rluc-2A-Rep would be C-terminally fused to FMDV 2A. Transfection of BHK-21 cells with DEN-1 Rluc-2A-Rep yielded two Rluc peaks: the first peak was observed during the initial 10 h p.t., and a second peak was observed after 10 h p.t. (Fig. 7B). An identical time frame for the first Rluc peak was observed for both DEN-1 Rluc-2A-Rep- and DEN-1 Rluc-Rep-transfected cells, except that the Rluc signal derived from the 2A-containing replicon was more robust (twofold greater) than that derived from the non-2A replicon. This was expected because the Rluc protein expressed from the non-2A replicon had a longer C-terminal fusion partner (33 amino acids) than that expressed from the 2A-containing replicon (19 amino acids). In addition, due to the transmembrane nature of the C-terminal fragment of the envelope protein, it was likely that the Rluc protein remained anchored onto the ER membrane through its fusion partner. Remarkably, the 2A-containing replicon replicated efficiently, as indicated by a robust second Rluc peak after 10 h p.t. (Fig. 7B) as well as IFA-positive cells at 60 h p.t. (Fig. 7C, right panel). Similar two-peak Rluc kinetics were observed upon transfection of DEN-1 Rluc-2A-Rep RNA into Vero cells (data not shown). These results suggest that (i) the DEN-1 Rluc-2A-Rep is replication competent and that (ii) the two Rluc peaks observed during the initial 10 h p.t. and after 10 h p.t. represent viral translation and RNA replication, respectively.

Using the above-described DEN-1 Rluc-2A-Rep, we determined the effects of triaryl pyrazoline on viral translation and RNA replication. BHK-21 cells were transfected with Rluc-2A-Rep RNA, incubated immediately with the compound (at

FIG. 7. Inhibition of viral translation and RNA replication of DEN-1 virus by triaryl pyrazoline. (A) Two reporting replicons were constructed for DEN-1 virus. One replicon contains a luciferase fused in frame with the ORF at a position where the structural genes were deleted (DEN-1 Rluc-Rep). The other replicon is identical to Rluc-Rep except that an FMDV 2A sequence was fused to the C terminus of the luciferase (DEN-1 Rluc-2A-Rep). (B) BHK-21 cells were electroporated with identical amounts of DEN-1 Rluc-Rep and DEN-1 Rluc-2A-Rep and assayed for luciferase activity at various time points posttransfection. The double lines on the vertical axis indicate that the scales of the top and bottom portions of the diagram are different. (C) BHK-21 cells transfected with equal amounts of DEN-1 Rluc-Rep (left) and DEN-1 Rluc-2A-Rep (right) were analyzed by IFA at 60 h p.t. (D) The transient replicon system was used to quantify the inhibitory effects of triaryl pyrazoline on viral translation (2 and 4 h p.t.) and RNA replication (48 h p.t.) in DEN-1 virus. Values above each bar indicate percentages of luciferase activity derived from the compound-treated transfections versus the luciferase signal derived from the mock-treated transfection. Error bars represent standard deviations from three independent experiments.

33 and 100 μ M), and assayed for Rluc activity at 2, 4, and 48 h p.t. (Fig. 7D). Triaryl pyrazoline reproducibly inhibited Rluc signals by 11 to 31% at 2 and 4 h p.t. By 48 h p.t., the compound inhibited Rluc activity by approximately 90%. The results indicate that triaryl pyrazoline potently inhibits RNA replication of DEN-1 virus.

TABLE 1. Comparison of efficacies and cytotoxicities of triaryl pyrazoline in four WN virus assays

Assay (infected cell line)	EC ₅₀ (μM)	CC ₅₀ (μM) ^a	TI ^b
WN Rluc-Neo-Rep (BHK-21)	19	≥300	≥16
WN Rluc-VLP (BHK-21)	14	≥300	≥21
WN Rluc (Vero)	23	≥300	≥13
Authentic WN virus (Vero)	28	≥300	≥11

^a CC₅₀ values were determined for both BHK-21 and Vero cells.

^b TI (therapeutic index) is the CC₅₀ value divided by the EC₅₀ value.

DISCUSSION

Using WN virus as a model, we recently established three cell-based HTS assays: a full-length reporting virus, packaged VLPs containing replicon RNA, and replicon-harboring cells, each of which contains an Rluc reporter (39). Here, we have employed these assays to identify and characterize triaryl pyrazoline as a novel inhibitor of flavivirus infection in cell culture. Since the infection assay with full-length reporting virus covers the complete viral life cycle, we chose this system as our primary screening assay (Fig. 1). Once a “hit” was identified, the inhibitor was subjected to two other HTS assays (Fig. 4A and B) as well as an authentic WN virus infection assay (Fig. 2). As summarized in Table 1, the three HTS assays yielded comparable EC₅₀ values for triaryl pyrazoline. Importantly, these EC₅₀ values are similar to that obtained with the authentic viral infection assay. These results demonstrate that the three HTS systems can be used reliably for anti-WN virus drug discovery.

We characterized the mode of WN virus inhibition by triaryl pyrazoline. Because the three reporting assays described above encompass multiple but discrete steps of the viral life cycle, they could be used to discriminate among inhibition of viral entry, replication, and virion assembly by any compound. The comparable EC₅₀ values derived from the three reporting assays (Table 1) indicate that the compound inhibits WN infection at a step that is shared by all three HTS assays: viral replication. Further analyses using a transient replicon system showed that the compound significantly blocks RNA replication (by ≥95%) without suppression of viral translation (Fig. 4C). In agreement with the above-described results, time-of-addition experiments showed that a significant suppression in viral titer requires treatment of infected cells with the compound during the first 10 h p.i., after which the inhibitory effects gradually diminished (Fig. 5). It should be noted that during BHK-21 cell infection with an Rluc-expressing WN virus or an Rluc-expressing VLP, initial translation occurred during the first 7.5 h p.i., while nascent genomic RNA began to accumulate after 10 h p.i. (39). In combination, the results strongly suggest that triaryl pyrazoline inhibits WN virus through suppression of viral RNA replication.

As with WN virus, the compound inhibited DEN-1 viral infection through suppression of viral replication, as suggested by its antiviral activity in a DEN-1 virus replicon-containing cell line (Fig. 6C). However, analyses using a transient replicon system of DEN-1 virus consistently showed that besides exerting a significant inhibition of viral RNA replication (by 90%), the compound weakly suppressed viral translation (by 11 to 31%) (Fig. 7D). These results have raised the question, in the case of DEN-1 virus, of whether the antiviral activity was due

to a dual effect of the compound on both viral translation and RNA replication. Alternatively, the observed antiviral activity was caused solely by the compound's effect on translation, which in turn resulted in suppression of viral RNA replication. However, at this point, we could not rule out the possibility that the weak suppression of viral translation of the DEN-1 virus replicon was due to compound-mediated inhibition of FMDV 2A activity. More experiments are required to discriminate among these possibilities.

Besides WN and DEN viruses, triaryl pyrazoline also inhibited other flaviviruses, specifically YF (17D) and SLE viruses. Furthermore, the compound suppressed other plus-strand and minus-strand RNA viruses, as represented by WEE virus, MHV, and VSV. In contrast, the compound did not inhibit HIV-1 and influenza virus (Fig. 3). Interestingly, both HIV-1 and influenza virus replicate in the nucleus, whereas the compound-sensitive viruses (flaviviruses, WEE virus, MHV, and VSV) replicate in the cytoplasm. In line with those results, the compound did not inhibit herpes simplex virus (a DNA virus that replicates in the nucleus) (data not shown). These results clearly indicate that the compound inhibits a broad spectrum of RNA viruses with specificity, probably through blocking a target required for replication of the compound-sensitive viruses. It is currently not known whether the compound exerts its functions through direct interaction with a host factor or a viral protein. A number of approaches to define the target of the compound are being explored. Testing the compound in biochemistry assays (RNA-dependent RNA polymerase, protease, helicase, and NTPase) may indicate whether the inhibitor interferes directly with these viral functions. Alternatively, if resistant viruses or replicons could be selected, then sequencing of resistant viruses or replicons may point to the targets of the compound.

It is confirmatory that besides the compound shown in Fig. 1B, one other triaryl pyrazoline analogue from the library was identified as having antiviral activity (data not shown). However, this analogue was less potent, suggesting that different functional groups within this analogue are important for antiviral efficacy. Efforts are being made to generate a triaryl pyrazoline core-based library for improvement of the antiviral potency. At this time, due to the small number of analogues synthesized, we could not conclude any specific structural and functional relationships.

The DEN-1 virus replicon-containing cell lines (Fig. 6) and the transient Rluc replicon (Fig. 7) described in this study will be useful for HTS of compound libraries for inhibitors of DEN virus and for studying DEN virus replication. The utility of the DEN-1 virus replicon-containing cell line for drug screening was validated in a 96-well format with a known inhibitor (MPA) and the newly identified triaryl pyrazoline. For MPA, the EC₅₀ value (0.3 μM) (Fig. 6C) derived from the DEN-1 virus replicon cell line was consistent with those derived from the authentic viral titer reduction assay (0.3 to 1.9 μM) (12). For triaryl pyrazoline, we performed the DEN-1 virus replicon cell line assay and the authentic viral infection assay in Vero cells. The experiments yielded comparable EC₅₀ values of 17 μM (Fig. 6D) and 23 μM (Fig. 3), respectively. The results clearly demonstrate that the DEN-1 virus replicon-containing cell line could be used for HTS of inhibitors of DEN virus. Compared to the traditional viral titer reduction assay, the new

system offers superior speed and sensitivity. Establishment of such HTS assays for DEN virus is important, considering the large scale of disease caused by this virus (see Introduction).

It was surprising that the DEN-1 virus replicon containing a direct fusion of the Rluc gene with the N-terminal capsid and the C-terminal envelope (DEN-1 Rluc-Rep) (Fig. 7) was replication defective. Replication of the DEN-1 virus replicon requires an FMDV 2A-mediated cleavage between Rluc and the residual envelope fragment (DEN-1 Rluc-2A-Rep) (Fig. 7). A similar observation was recently reported for a DEN-2 virus replicon (2). The requirement for 2A cleavage for DEN-1 virus (this study) and DEN-2 virus (2) replicons contrasts with our WN virus replicon, whose replication does not require the 2A cleavage between Rluc and its downstream envelope fragment (29). However, comparison of replication kinetics (as indicated by the Rluc profile) showed a dramatic difference between the WN virus and DEN-1 virus replicons. The first Rluc peak during the initial 10 h p.t. was observed for both WN virus and DEN-1 virus replicons. The second Rluc peak appeared after 24 h p.t. for the WN virus replicon (without 2A cleavage), whereas the second peak appeared immediately after 10 h p.t. for DEN-1 Rluc-2A-Rep (Fig. 7B). These data indicate that the delay in RNA replication of the WN virus replicon is likely due to the fusion of Rluc to the C-terminal transmembrane domain of the envelope (anchored into the ER membrane), thereby interfering with RNA replication. The data also suggest that replication of DEN-1 virus is more sensitive to such interference than replication of WN virus.

In summary, the identification and characterization of triaryl pyrazoline represent the first step toward the development of this compound for potential antilavivirus therapy. Its broad spectrum of antiviral activity without detectable cytotoxicity in cell culture warrants future *in vivo* study. The establishment of luciferase-expressing replicons for DEN-1 virus has made possible HTS of compound libraries for inhibitors of DEN virus.

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