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Benzothiazole and Pyrrolone Flavivirus Inhibitors Targeting the Viral Helicase

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Abstract

The flavivirus nonstructural protein 3 (NS3) is a protease and helicase, and on the basis of its similarity to its homologue encoded by the hepatitis C virus (HCV), the flavivirus NS3 might be a promising drug target. Few flavivirus helicase inhibitors have been reported, in part, because few specific inhibitors have been identified when nucleic acid unwinding assays have been used to screen for helicase inhibitors. To explore the possibility that compounds inhibiting NS3-catalyzed ATP hydrolysis might function as antivirals even if they do not inhibit RNA unwinding in vitro, we designed a robust dengue virus (DENV) NS3 ATPase assay suitable for high-throughput screening. Members of two classes of inhibitory compounds were further tested in DENV helicase-catalyzed RNA unwinding assays, assays monitoring HCV helicase action, subgenomic DENV replicon assays, and cell viability assays and for their ability to inhibit West Nile virus (Kunjin subtype) replication in cells. The first class contained analogues of NIH molecular probe ML283, a benzothiazole oligomer derived from the dye primuline, and they also inhibited HCV helicase and DENV NS3-catalyzed RNA unwinding. The most intriguing ML283 analogue inhibited DENV NS3 with an IC_{50} value of 500 nM and was active against the DENV replicon. The second class contained specific DENV ATPase inhibitors that did not inhibit DENV RNA unwinding or reactions catalyzed by HCV helicase. Members of this class contained a 4-hydroxy-3-(5-methylfuran-2-carbonyl)-2*H*-pyrrol-5-one scaffold, and about 20 μM of the most potent pyrrolone inhibited both DENV replicons and West Nile virus replication in cells by 50%.

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Supporting Information

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Experimental and characterization details for the synthesis of compounds **25**–**30** (PDF)

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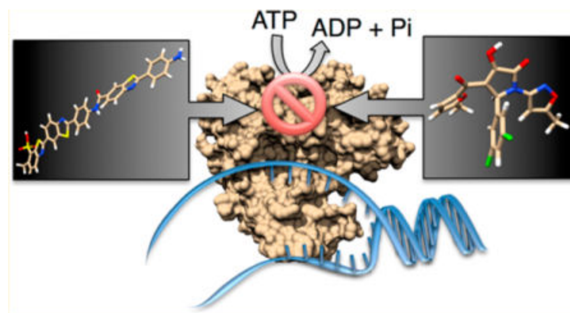

Keywords

nonstructural protein 3; ATPase; motor protein; direct acting antiviral; Dengue fever; West Nile virus; yellow fever virus; positive sense single-stranded RNA ((+)ssRNA) virus

Flaviviruses comprise a genus of positive-sense single-stranded RNA ((+)ssRNA) viruses. They include the important human pathogens yellow fever virus (YFV), Japanese encephalitis virus (JEV), Dengue virus (DENV), and West Nile virus (WNV). Many of these mosquito-borne viruses are endemic in tropical regions. Vaccines exist for only YFV and JEV. DENV is probably the most noteworthy human health threat because it infects an estimated 390 million people each year, causing “break-bone” fever, an extraordinarily painful disease with symptoms ranging from mild fever to a fatal hemorrhagic syndrome.[1]

The recent development of direct-acting antiviral (DAA) drugs to treat the hepatitis C virus (HCV), which is in the same Flaviviridae family as the flaviviruses, suggests that similar compounds might be useful to treat flavivirus infections or hemorrhagic fevers.[2] DAA targets encoded by flaviviruses include the viral structural proteins (capsid protein C, membrane protein M, envelope protein E) and nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). DAAs have been reported that inhibit the flavivirus capsid protein,[3] the NS5 polymerase,[4] the NS2B-NS3 protease/helicase,[5,6] the NS4B protein,[7] the NS5 guanylyltransferase,[8] and the NS5 polymerase.[4]

NS3 is one of the most thoroughly studied antiviral drug targets, and this work has led to approval of several new antiviral drugs to treat HCV.[9] NS3 proteins are unlike any others found in nature because they combine an *N*-terminal protease needed for viral polyprotein processing with a C-terminal domain functioning as an ATP-fueled RNA helicase. The new HCV drugs inhibit the NS3 protease function, but compounds that inhibit the helicase[10–13] and compounds that bind between the helicase and protease domains[14] also function as antivirals in cell culture.

The viruses in Flaviviridae encode the only known proteins that are both helicases and proteases. The helicase portion of these NS3 proteins is composed of two motor domains that are similar in all superfamily 1 and 2 helicases and a third domain very different from other helicases. ATP binds between the motor domains, and RNA binds between the motor domains and domain 3.[15] Detailed mechanistic studies have revealed how the NS3 helicase moves like an inchworm in a 3′ to 5′ direction along one strand of RNA while actively

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displacing the complementary strand in an ATP-fueled reaction.[10,16] Similar structural[15] and enzymatic analyses of the DENV helicase suggest that the hepacivirus and flavivirus proteins share a similar mechanism of action.

Although they share key similarities, NS3 proteins from the hepacivirus and flavivirus genera are also remarkably different. First, NS3 proteases are most active only when combined with another viral peptide, but the HCV NS3 protease is activated by HCV NS4A,[17,18] and flavivirus NS3 is activated by NS2B.[19,20] Unlike the analogous NS4A protein in HCV, NS2B wraps like a belt around NS3 such that part of the cofactor contributes residues to the active site of the protease.[21,22] Second, the NS3 helicase and protease domains are oriented differently relative to each other in the two genera. In all X-ray crystal structures reported to date, the HCV NS3 protein folds in a compact conformation where the protease domain does not contact the known RNA- or ATP-binding sites on the helicase.[23] However, the DENV NS3 has been observed twice in more extended conformations. In the first observed conformation,[19] the DENV protease partially blocks the ATP-binding site. In the second conformation, the protease is rotated by about 180° so that both ATP and RNA can access the helicase active site.[24] These conformations appear to be biologically relevant because mutations that affect the flexibility of the helicase–protease linker also affect the ability of DENV to replicate in cells.[24] Third, the HCV helicase is capable of separating both DNA[25] and RNA[26] duplexes in vitro, but the flavivirus NS3 clearly prefers RNA in most assays. Finally, unlike the HCV proteins, the flavivirus helicase ATP-binding site can also accommodate RNA so that the protein can cleave the terminal 5′ phosphate to prepare genomic RNA for capping.[25]

A few flavivirus NS3 helicase inhibitors have been reported previously.[27] Recent examples include ivermectin, which was discovered to inhibit the helicase using molecular modeling,[28] ST-610, which was discovered using assays monitoring DENV cytopathic effects,[29] and suramin, which was discovered to inhibit DENV by screening compound libraries with a molecular beacon-based helicase assay.[30] Ndjomou et al.[12] also recently showed that some compounds resembling NIH molecular probe ML283[31] are potent inhibitors of DENV NS3-catalyzed ATP hydrolysis. We have therefore designed a new DENV helicase assay, optimized the new assay for high-throughput screening, and used the assay to analyze a small library of additional ML283 analogues and other compounds. Here, we report that some of the more specific ML283 analogues exert an antiviral effect against DENV and that another class of helicase inhibitors, which share a pyrrolone scaffold, specifically inhibit the ability of DENV NS3 to cleave ATP and DENV and WNV replication in cells.

RESULTS

After comparing several different ATPase assay protocols with several different flavivirus helicases, we found that the most cost-effective (at ~2.5¢/assay) was a colorimetric inorganic phosphate assay using DENV helicase. The recombinant flavivirus helicase we found to be most stable in vitro was a truncated enzyme from dengue virus serotype 2 (TSV01 strain). In this NS3 protein (called NS3h), the protease domain is replaced by

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thioredoxin to enhance solubility. NS3 residues 171–618 are present, and thioredoxin can be removed with enterokinase so the protein can be examined using X-ray crystallography.[15,32]

The colorimetric assay included poly(U) to mimic the stimulatory effect of RNA binding to the helicase (Figure 1A). In this way, compounds that restrict ATP hydrolysis, ATP binding, or RNA binding could be discovered. Like all helicases, NS3h hydrolyzes ATP more rapidly in the presence of nucleic acids than in their absence. ATP hydrolysis fuels helicase movements on RNA. In the procedure for automated HTS, assays were assembled, compounds added, and reactions initiated with 3 μL of an $MgCl_2$ solution and terminated after 30 min with 50 μL of the Biomol Green reagent (Enzo Life Sciences). Phosphate was then determined from absorbance at 620 nm (Figure 1B). The sensitivity of the Biomol reagent allowed the assays to be conducted at subsaturating ATP concentrations near the K_m observed for the enzyme (i.e., 100 μM ATP). At this ATP concentration, compounds that either affect the turnover number of the enzyme (k_{cat}) or the affinity of the enzyme for ATP (K_m) could, in theory, be detected in an HTS.

Under these conditions, pilot screens comparing DMSO and the nonspecific helicase inhibitor aurintricarboxylic acid (ATA)[11,34] led to excellent Z′ factors (Figure 1C).[33] Concentration–response assays with ATA and other nonspecific helicase inhibitors such as suramin[11] and titan yellow[35] yielded half-maximal inhibitory concentrations similar to those reported with other assays with the related NS3h protein from HCV (Figure 1D). The assay was also insensitive to DMSO concentrations up to 10% (v/v) (data not shown).

The assay was used to screen a library of 253 compounds that were reported to inhibit other helicases. Most of the compounds in this focused helicase inhibitor library were either previously reported to inhibit the homologous HCV helicase or the SV40 Tag helicase, or they were nucleic acid binding compounds reported to inhibit one or more helicases. When tested at 7 μM, only 41 of the compounds inhibited DENV NS3h-catalyzed ATP hydrolysis >50% in two separate assays (Figure 2A).

Two sets of hit compounds were selected for further analysis. The first set (Tables 1 and 2) were analogues of NIH molecular probe ML283 (compound **13**), which was developed to target the HCV helicase.[31] ML283 and most of its analogues were synthesized from a benzothiazole dimer purified from the yellow dye primuline.[36] The second set contained pyrrolones (Table 3, see the Supporting Information for synthesis). The most potent compound in the benzothiazole class (compound 5, Table 1) inhibited DENV NS3h-catalyzed ATP hydrolysis with a half-maximal inhibitory concentration (IC_{50}) that was about 100 times lower than the IC_{50} observed with the most potent compound in the pyrrolone class (compound 26, Table 3).

Representatives of each class of inhibitory compounds were next tested in helicase assays in which the ability of the enzyme to separate duplex RNA was monitored (Figure 3A). All of the compounds tested from the benzothiazole class also inhibited the ability of DENV NS3h to separate an RNA duplex in a concentration-dependent manner, but none of the pyrrolones inhibited RNA unwinding (Figure 3C; Tables 1–3). Most of the IC_{50} values obtained in the unwinding assays were similar to the IC_{50} values obtained in ATPase assays with a few

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exceptions. About 20 times more of compound **5** was needed to inhibit RNA unwinding to the same degree as was needed to inhibit ATP hydrolysis (Table 1), and about 8 times less of compound **20** was needed to inhibit RNA unwinding than ATP hydrolysis.

The specificity of each DENV helicase inhibitor was then evaluated by performing the same ATPase assay in the presence of HCV helicase instead of DENV helicase. The ability of each DENV helicase inhibitor to inhibit HCV helicase-catalyzed DNA and RNA unwinding was also evaluated using assays similar to those used for DENV, except that a different buffer system was used with HCV helicase because HCV helicase has poor unwinding activity at pH 7.5.[38,39]

None of the pyrrolone compounds inhibited any of the reactions catalyzed by HCV helicase, and more of each benzothiazole was needed to inhibit HCV helicase-catalyzed ATP hydrolysis than DENV-catalyzed ATP hydrolysis to the same extent (Table 1). When IC_{50} values obtained in DENV ATPase assays were compared with IC_{50} values obtained in various HCV helicase assays, the best correlations were between IC_{50} values obtained in HCV DNA unwinding assays (Table 1, column 6) and IC_{50} values obtained in DENV ATPase assays (Table 1, column 3). When these numbers were compared, most of the ML283 analogues displayed similar inhibitory potentials for HCV and DENV helicases with the noteworthy exception that compounds **5** and **24** were both more active against DENV than the HCV helicase (Tables 1 and 2).

For flavivirus helicase inhibitors to be used as molecular probes in cells, they must be relatively nontoxic and inhibit viral replication. Compounds in the focused helicase inhibitor library were therefore also tested in cell-based assays to determine the effects on cell viability and flavivirus replication (Figure 4).

The first set of assays used a subgenomic DENV-2 replicon designed by Whitby et al.[41] In the DENV-2 replicon, DENV-2 nucleotides 180–2342 are replaced by fusing the first 28 amino acids of the capsid protein with the *Renilla* luciferase, ubiquitin, and puromycin *N*-acetyltransferase (Pur^r) genes. An encephalomyocarditis virus internal ribosome entry site is also present between the reporter and the NS1 region (Figure 4A). These replicon assays measured the ability of 20 μM of each compound in the collection to inhibit *Renilla* luciferase after cells harboring replicons were exposed to compounds for 72 h (Figure 4B, *y*-axis). In the second set of assays, the effect of the same compound concentration on BHK cell viability was monitored (Figure 4B, *x*-axis).

At 20 μM, most of the compounds in the collection had some impact on replicon levels. About half of the compounds with the highest activity against replicons (>65% inhibition) were toxic, meaning that they reduced cell viability and replicon content to a similar degree (upper left quadrant, Figure 4B). Many of the compounds with a modest (20–50%) impact were in the benzothiazole class (ML283 analogues), and most of these were previously reported to have a similarly modest impact on subgenomic HCV replicon levels in Huh7.5 cells.[31,36] Only 22 compounds (9%) inhibited the replicon more than 65% and cell viability less than 20% (upper right quadrant, Figure 4B).

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The relatively nontoxic replicon inhibitors included compounds in both the benzothiazole and pyrrolone classes. The ability of members of each class to inhibit DENV replicons in a concentration-dependent manner was therefore monitored by applying each active compound to replicon cells in an eight-point two-fold dilution series starting at 100 μM. Compound efficacy was compared with ribavirin and the NS5 inhibitor BG323.[8] In these concentration–response assays, only one compound in the benzothiazole class inhibited replicon content more than 90% (compound **24**, Table 2). Like the compounds in Table 1, compound **24**, also inhibited reactions catalyzed by HCV helicase. For example, **24** inhibited HCV helicase-catalyzed RNA unwinding with an IC_{50} value of 2.0 ± 0.7 μM. None of the benzothiazoles were tested against authentic virus because of the limited solubility above 50 μM.

All of the pyrrolones tested inhibited the DENV replicon in a concentration-dependent manner (Table 3). The most potent in cells, compound **25**, inhibited both DENV helicase-catalyzed ATP hydrolysis and the DENV replicon with half-maximal inhibitory concentrations of 78 ± 23 and 36 ± 6 μM, respectively (Figure 4C). Each pyrrolone tested inhibited cell viability, also, but only at concentrations >50 μM (Figure 4D).

Each of the hits in the pyrrolone series was also tested for its ability to inhibit the replication of West Nile virus (Kunjin strain) in BHK cells by examining plaque titers after incubation with 50 μM of each compound (Figure 4E). Results paralleled those seen with the DENV replicon, with compound **25** lowering plaque-forming units (pfu) to the greatest extent (Figure 4E; Table 3). Effects on WNV were concentration-dependent with 22 μM of compound 25 exerting a half-maximal inhibitory effect (Figure 4F). In similar experiments, exposure of the same WNV-infected cells to 50 μM of BG323 also yielded about 10^6 pfu of West Nile virus at 72 h,[8] suggesting that BG323 and compound **25** yield similar antiviral effects.

DISCUSSION

Our inspiration for developing helicase inhibitors as drugs comes from the fact that helicase inhibitors are now potent antiviral agents that, in some clinical trials, rival many of the drugs traditionally used to treat virus infections.[42,43] Boehringer Ingelheim[44] and Bayer[45] discovered the first antiviral drugs that target the herpes simplex virus helicase. Boehringer Ingelheim identified their inhibitors by screening for compounds that inhibit helicase-catalyzed DNA strand separation. The Bayer compounds were discovered using cytoprotection assays.[45] The Bayer thiazolylamide compound BAY 57-1293 inhibits purified helicase-catalyzed ATP hydrolysis (IC_{50} = 30 nM), can be used to treat HSV in animal models,[46,47] and is being tested in humans as the drug pritelivir.[48] Astellas Pharma Inc. also developed a HSV helicase inhibitor called amenamevir (ASP2151), which inhibits helicase-catalyzed ATP hydrolysis (IC_{50} = 78 nM) and shortens the median time for lesion healing.[49,50]

Helicases are nevertheless challenging drug targets because helicases are difficult to inhibit with small molecules and potent inhibitors are often not specific. Many potent helicase inhibitors interact with the nucleic acid substrate or highly conserved regions of the helicase

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motor domains.[51] Prior high-throughput screens with DENV helicase have yielded inhibitors that were either nucleic acid binding agents[2] or nonspecific nucleic acid mimics such as suramin[30] and aurintricarboxylic acid (ATA).[34] Because previous screens for flavivirus helicase inhibitors used unwinding assays,[2,30] we set out to perform screens using an ATPase assay to test the hypothesis that compounds that inhibit helicase-catalyzed ATP hydrolysis might function as antivirals even if they do not inhibit the ability of the protein to unwind RNA.

The DENV ATPase assay that we have optimized was designed so that it can, in theory, detect compounds that inhibit ATP binding or ATP hydrolysis or that prevent RNA from stimulating helicase-catalyzed ATP hydrolysis. Because RNA is present in the assay at a concentration needed to stimulate ATP hydrolysis to ~85% of its maximal rate, then compounds that either inhibit ATP hydrolysis or RNA binding will be identified as “hits”. The assay is simpler than other colorimetric phosphate assays based on the Fiske–SubbaRow method[52] or ammonium molybdate reagents that incorporate the dye malachite green[53, 54] because ATP need not be removed and multiple reagents need not be added in a precisely timed procedure.

When the new DENV ATPase HTS assay was used to screen a focused helicase inhibitor library composed of compounds reported to inhibit related helicases such as the HCV NS3 protein,[11,35] and more distantly related helicases such as the SV40 Tag protein,[55] results showed that the assay was robust (high Z′ factor, Figure 1C) and reproducible (Figure 2A). The ATPase assay was also more sensitive than unwinding assays in detecting helicase inhibitors. As evidence, higher concentrations of compounds were needed to inhibit DENV helicase in RNA unwinding assays than were needed to inhibit ATPase assays to the same extent. Some compounds that inhibited ATP hydrolysis failed to inhibit RNA unwinding even at much higher concentrations (Table 1; Figure 3C).

The most noteworthy of the new DENV helicase inhibitors reported here are a set of pyrrolones, which also inhibited the DENV replicon and WNV replication in cell culture (Figure 4; Table 3). Unlike other helicase inhibitors detected with the assay, the pyrrolones do not inhibit the related HCV helicase under any conditions tested. The pyrrolones were all built around a 4-hydroxy-3-(5-methylfuran-2-carbonyl)-2*H*-pyrrol-5-one scaffold (see the Supporting Information for synthesis and characterization). Three of the most potent compounds contained a 3,5-dichlorophenyl R_1 group with one of three R_2 groups. Replacement of the chlorines led to a marked decrease in biological activity. In the R_2 position, isoxazole was most active in cells (compound **25**), and compounds in which the isoxazole was replaced with either a benzyl (compound **26**) or a furanylmethyl (compound **29**) were about 3 times more potent inhibitors of DENV helicase-catalyzed ATP hydrolysis in vitro.

The other series of DENV helicase inhibitors that was analyzed in this study was composed of analogues of NIH molecular probe ML283 (compound **13**), which was designed to inhibit HCV helicase.[31] ML283 is a benzothiazole oligomer that acts by displacing nucleic acids from the HCV helicase.[35] Our comparison of the effects of these compounds on the DENV and HCV helicase yielded several noteworthy results. First, lower concentrations of these

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compounds inhibit HCV helicase-catalyzed unwinding than are needed to inhibit HCV helicase-catalyzed ATP hydrolysis (to the same extent). The reverse is true with DENV helicase. Higher concentrations of the compounds are needed to inhibit DENV helicase-catalyzed unwinding than ATP hydrolysis. Second, some ML283 analogues were better inhibitors of DENV helicase than the HCV helicase. The two most potent, compounds **5** (Table 1) and **24** (Table 2), were >10 times more active against the DENV protein. Although most ML283 analogues had some activity against the DENV replicon when tested at 20 μM, only compound **24** reduced replication of a subgenomic DENV replicon in cells in a concentration-dependent manner (Table 2).

If properly optimized, the two series of compounds reported here could be valuable probes to study the role of the helicase in viral replication, and they might lead to new direct-acting antivirals. Few direct-acting antiflavivirus agents have been reported, and some that were suspected to act via the helicase, such as ivermectin,[28] also affect host proteins.[56] NS3 is one of the most widely studied antiviral drug targets mainly because of the intense emphasis on HCV drug discovery. However, most HCV drug discovery has focused on the NS3 protease function. NS3 proteins are multifunctional, with the *N*-terminal domain acting as a protease needed for viral polyprotein processing and the C-terminal domain acting as a helicase. Three NS3 protease inhibitors have recently been approved to treat chronic infection with HCV, which is in the same family (Flaviviridae) as the flaviviruses. Flaviviridae members encode the only proteins known that are both proteases and helicases. The compounds reported here might also prevent flaviviruses from capping their RNA because the same site that cleaves ATP also prepares RNA for capping by removing the 5′ terminal phosphate from the (+) sense RNA genome.[25] Regardless of whether or not the new inhibitors here are studied further, the new assays reported here should be valuable tools for discovering additional compounds that inhibit the DENV helicase.

METHODS

Protein Expression and Purification

All oligonucleotides were purchased from Integrated DNA Technologies (Coral-ville, IA, USA). The helicase domain of dengue virus NS3 (NS3 amino acids 171–618) fused at its *N*-terminus to thioredoxin and a $(His)_6$ tag followed by an enterokinase cleavage site was expressed and purified in *Escherichia coli* Rosetta (DE3). A plasmid expressing this protein was obtained from Julien Lescar (Singapore),[32] and the protein was purified as previously described.[57]

ATP Hydrolysis (ATPase) Assay

Reactions were performed in 30 μL in clear 384-well microplates (Thermonunc), and they each contained 25 mM Tris, pH 7.5, 1.25 mM $MgCl_2$, 100 μM ATP, 33 μg/mL BSA, 0.07% (v/v) Tween 20, 0.3 mM DTT, 0.6% DMSO, 15 μM poly(U) RNA (expressed as nucleotide concentration), and 3 nM DENV NS3h (or 4 nM HCV NS3h). For automated screening, reactions containing all components except test compounds and $MgCl_2$ were assembled at 1.11 times their final concentration. After 27 μL of this reaction mixture was dispensed in each well, 200 nL of each test compound (6.67 μM final concentration) or DMSO was added

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(0.3% DMSO v/v, final). Reactions were initiated by adding 3 μL of 12.5 mM $MgCl_2$. After 30 min at 23 °C, 50 μL of Biomol Green reagent (Enzo Life Sciences) was added, and after a final 30 min of incubation at 23 °C, absorbance at 620 nm was read. Percent inhibition was calculated by normalizing the data to assays containing 6.7 μM ATA (100% inhibition) and reactions with DMSO only (0% inhibition).

RNA/DNA Unwinding Assay

Molecular beacon-based DNA and RNA unwinding assays were performed as described previously with HCV helicase.[37] For reactions with DENV helicase, the buffer described for ATPase assays (above) and 100 nM DENV was included in each reaction.

DENV Subgenomic Replicon Assay

A total of 258 compounds were screened, 86 in duplicate and 173 in singlicate. Stable baby hamster kidney (BHK-21) DENV-Rluc cells[41] were maintained in Dulbecco's modified Eagle's medium (DMEM, LifeTech) containing 4.5 g/L D-glucose, L-glutamine, and 110 mg/L sodium pyruvate and supplemented with 10% FBS, 100 U/mL each of penicillin and streptomycin, and 3 μg/mL puromycin to maintain replicon stability. Cells were grown at 37 °C and in atmosphere supplemented with 5% CO_2. DENV-*R*luc cells were seeded in clear 96-well culture plates (Corning 3599) without puromycin at a concentration of 1000 cells per well. Cells were allowed to adhere to the plate for a minimum of 4 h, but not for more than 24 h. Compounds dissolved in DMSO were first diluted in DMEM and then added to cells such that the final volume in the well was 100 μL, the final DMSO concentration was 1%, and the final compound concentration was 20 μM. Quadruplicate control wells containing DMSO only or 40 μM ribavirin were included on each assay plate. Cells were incubated for 72 h at 37 °C and 5% CO_2 atmosphere.

The effect of compounds on DENV replication was assessed by measuring *Renilla* luciferase reporter gene activity using a *Renilla* luciferase assay kit (Promega, Madison, WI, USA). Medium was aspirated and cells were washed with PBS before being lysed with 70 μL of *Renilla* luciferase lysis buffer. Cells were rocked for 1 h at room temperature, and then 50 μL of lysate was transferred to a black 96-well luminescence plate (Nunc 9502867). Luciferase activity was measured in a FluoSTAR Omega (BMG Labtech, Germany) after the injection of 25 μL of luciferase substrate and reading for 10 s. Percent inhibition was normalized to DMSO-only controls.

Compounds that inhibited at least 50% and reduced cell viability less than 20% were tested again in confirmatory "cherry pick" assays, and those that inhibited confirmatory assays at least 50% were assayed in eight-point two-fold concentration–response (from 200 μM to 1.6 μM final concentrations) to obtain IC_{50} values.

Cell Viability Assays

To determine compound toxicity, BHK DenV-Rluc cells were plated and treated as above, and cell viability was assessed using the CellTiter-Glo luminescent cell viability kit (Promega). At the end of 72 h, the medium was aspirated and an equal volume of CellTiter-Glo reagent and medium was added to each well. After incubation for 30 min at room

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temperature, luminescence was read for 5 s using a FLUOstar Omega (BMG Labtech, Germany) and converted to percentage viability by normalizing readings to those obtained from cells treated with DMSO only (i.e., negative controls).

West Nile Virus (Kunjin Subtype) Assay

Effects of compounds on West Nile virus were measured as previously described.[8]

Data Analysis

Z′ factors were calculated as described by Zhang et al.[33] Half-maximal inhibitory concentrations were calculated from concentration–response curves using nonlinear regression to fit data to a log(inhibitor) versus normalized response equation with variable slope. Therapeutic index (TI) was defined as CC_{50}/EC_{50}.

Instant JChem 6.0 (2013) was used for structure database management, search, and prediction (ChemAxon (http://www.chemaxon.com).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ATA	aurintricarboxylic acid
BHK	baby hamster kidney
HCV	hepatitis C virus
HTS	high-throughput screen
DENV	Dengue virus
NS3h	nonstructural protein 2 lacking the protease domain
WNV	West Nile virus

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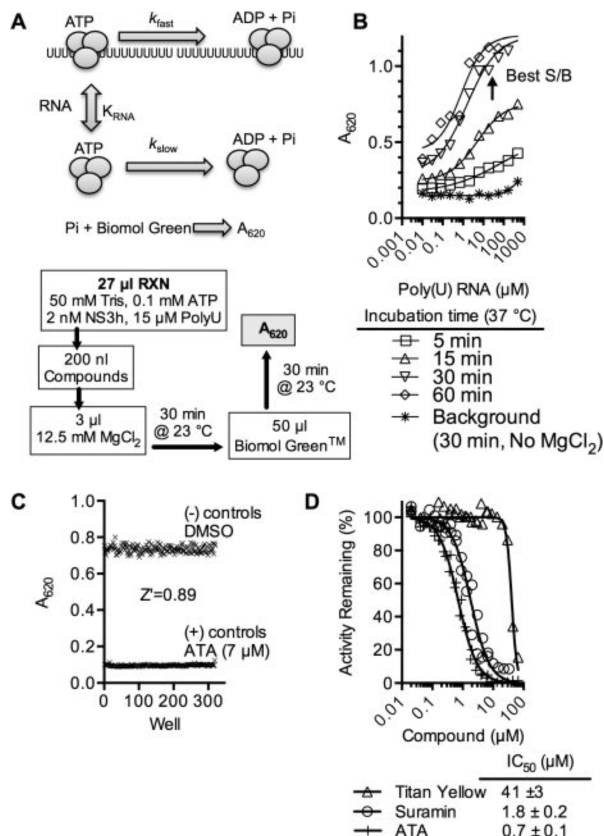

Figure 1.

DENV helicase high-throughput ATPase assay. (A) Assay setup. DENV helicase (gray) cleaves ATP at a slow basal rate (k_{slow}) and more rapidly in the presence of RNA (k_{fast}). The Biomol Green reagent reacts with inorganic phosphate to produce a product that absorbs light at 620 nm. In automated screening, compounds are added to a reaction mix, and ATP hydrolysis is initiated with Mg^{2+}. In addition to key reagents noted, all assays were performed at pH 7.5 and contained 5 μg/mL BSA, 0.01% (v/v) Tween 20, 5 mM DTT, and 0.3% DMSO (final concentration). (B) Effect of poly(U) RNA on inorganic phosphate released from ATP in the presence of 2 nM DENV NS3h after indicated times. Conditions with the best signal compared to control assays where reactions were not initiated with $MgCl_2$ were chosen for HTS. (C) To assess assay quality, a Z′ factor[33] was calculated by performing 192 positive control reactions (7 μM aurintricarboxylic acid (ATA)) and 192 negative control reactions (DMSO only) arranged in a checkerboard fashion in a 384-well plate. (D) Concentration–response assays using nonspecific helicase inhibitors.[11, 34]

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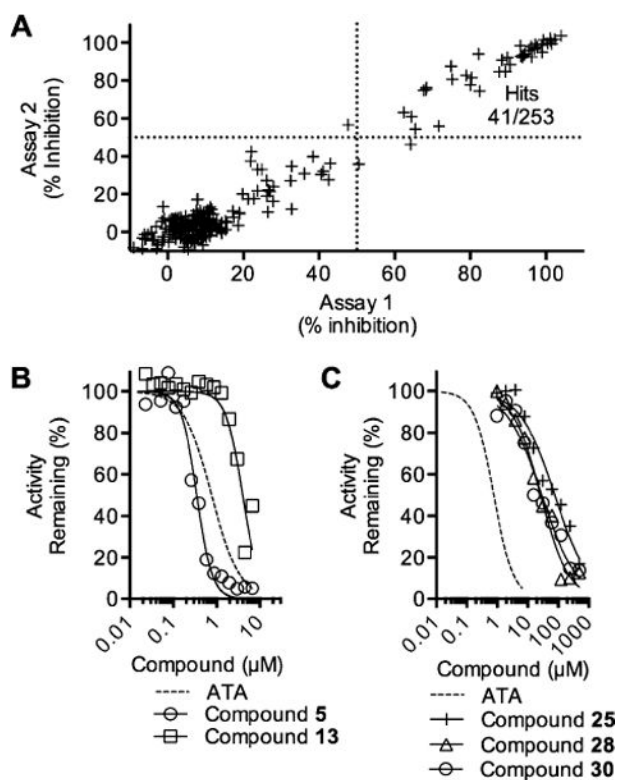

Figure 2.

Screen of a focused helicase inhibitor library (A) Each compound was tested twice, and the result from the first assay (*x*-axis) was compared to the result from the second (*y*-axis) to assess assay reproducibility. (B) Concentration–response assays with selected inhibitors in the benzothiazole class. (C) Concentration–response assays with representatives of the pyrrolone class of compounds. In panels B and C results are compared with data obtained with the potent nonspecific helicase inhibitor ATA (dotted lines).

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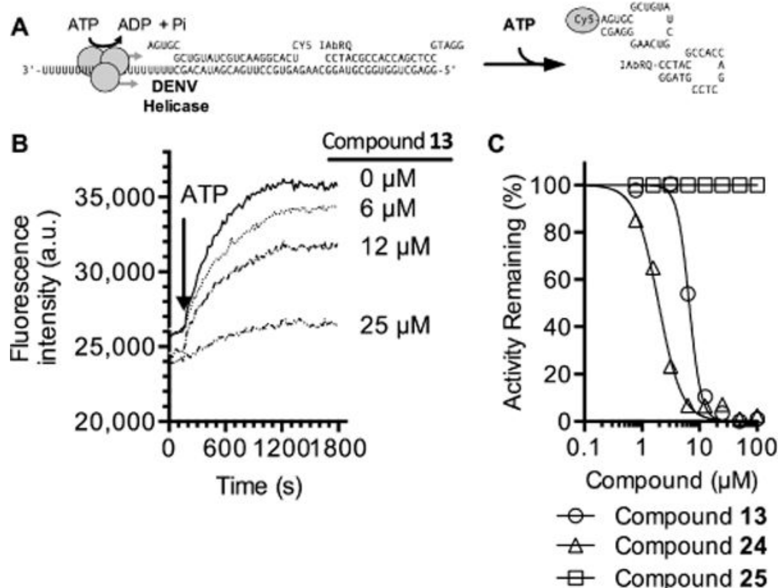

Figure 3.

Ability of hit compounds to inhibit DENV helicase-catalyzed RNA strand separation. (A) In the assay, the helicase loads on the 3′ single-stranded tail of the substrate, and translocates 3′ to 5′ upon addition of ATP to separate a Cy5-labeled annealed RNA strand from an annealed DNA strand labeled with the fluorescence quencher Iowa black RQ (IAbRQ, Integrated DNA Technologies).[12,37] (B) Concentration-dependent inhibition of DENV helicase-catalyzed RNA unwinding by ML283 (compound **13**). Fluorescence traces with only three ML283 concentrations are shown for clarity. (C) Concentration–response plots obtained for select inhibitors. Activity was normalized by first calculating initial rates of fluorescence increase and then normalizing these rates to those observed in negative control reactions containing DMSO alone. IC_{50} values obtained by fitting data with GraphPad PRISM are shown in Table 1.

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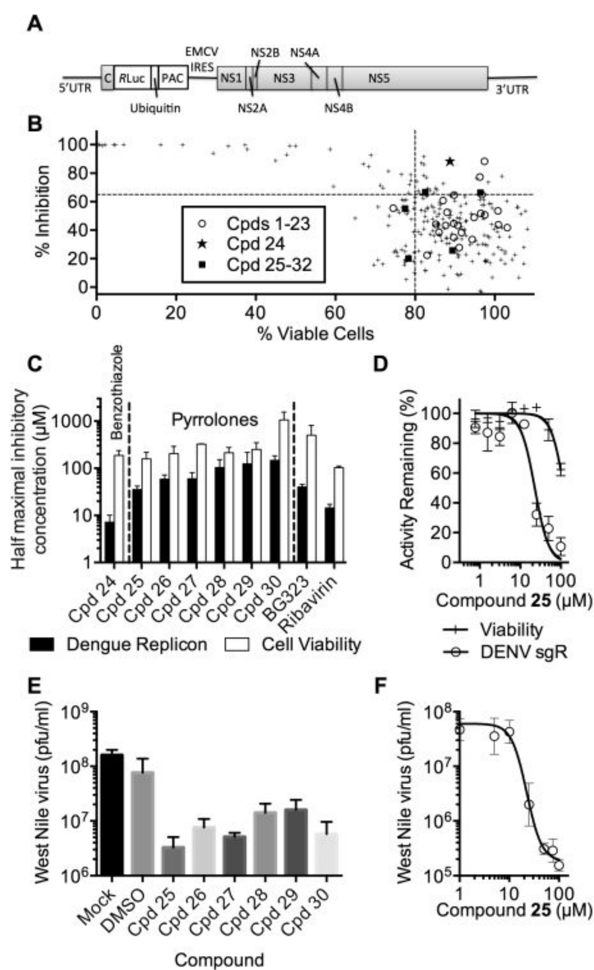

Figure 4.

Effects of helicase inhibitors on cellular flavivirus replication. (A) Subgenomic DENV replicon used in this study. (B) Percent of DENV replicon remaining in cells treated with 20 μM of each compound in the focused helicase inhibitor library plotted against the effect of 20 μM of each compound on cell viability. Dotted lines mark arbitrary cutoffs. (C) Effects of compounds in Tables 2 and 3 on DENV replicon content and cell viability (av ± SD, n = 3). (D) Effect of various concentrations of **25** on cell viability (+) and subgenomic replicon (sgR) content (○). (E) BHK cells were infected with West Nile virus in the presence of 50 μM of indicated compounds and titered 72 h later (av ± SD, n = 3). (F) Effect of various concentrations of compound 25 on West Nile virus titers (av ± SD, n = 3).

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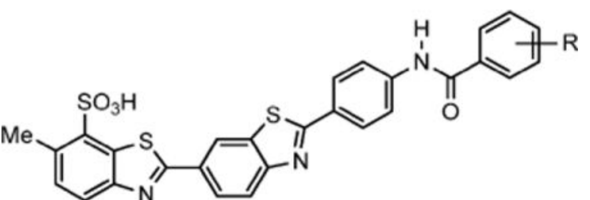
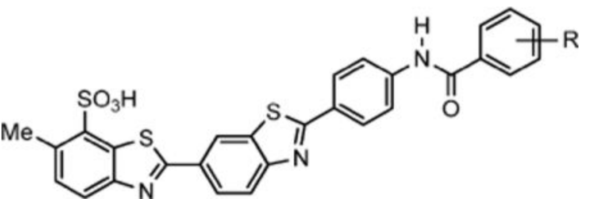
Table 1

Ability of Selected ML283 Analogues To Inhibit DENV Helicase-Catalyzed ATP Hydrolysis and RNA Unwinding



compd (CID)[a]	R	DENV ATPase IC_{50}[b] (μM)	DENV RNA- helicase IC_{50}[c] (μM)	HCV ATPase IC_{50}[d] (μM)	HCV DNA- helicase IC_{50}[e] (μM)	HCV RNA-helicase IC_{50}[f] (μM)
1 (49849280)	H	5.3 ± 1.2	nd[g]	69 ± 6	11 ± 1.5	8.5 ± 2
2 (49849300)	4-NH_2	2.8 ± 0.6	7.6 ± 1.6	58 ± 10	10 ± 2.4	7.0 ± 1*
3 (49849294)	4-F	3.0 ± 0.4	6.6 ± 0.9	32 ± 7	5.2 ± 0.6	4.2 ± 2*
4 (49849282)	4-OCH_3	3.0 ± 0.5	nd	115 ± 14	10 ± 2.6	nd
5 (49849290)	4-CO_2CH_3	0.4 ± 0.1	7.9 ± 2.5	>200	9.7 ± 4.6	nd
6 (49849302)	4-Cl	1.4 ± 0.2	5.6 ± 1.2	53 ± 4	3.4 ± 0.3	3.3 ± 1
7 (49849286)	4-CH_3	1.1 ± 0.2	4.3 ± 1.9	61 ± 1	3.3 ± 0.3	3.9 ± 1
8 (49849276)	4-CF_3	1.2 ± 0.2	5.2 ± 0.6	111 ± 60	1.8 ± 0.4	2.8 ± 2
9 (49849299)	4-t-Bu	4.0 ± 0.5	10 ± 3.4	>200	8.2 ± 1	8.8 ± 3*
10 (49849284)	4-$N(CH_3)_2$	3.4 ± 0.3	nd	>200	11 ± 6.7	8.6 ± 2
11 (50930740)	4-Br	1.1 ± 0.1	5.0 ± 1.2	21 ± 6	5.2 ± 4	3.8 ± 0.4*
12 (46839370)	4-NHFmoc	1.3 ± 0.3	3.5 ± 1.7	39 ± 31	5.4 ± 1	2.8 ± 1
13 (50930730)	3-Cl	4.0 ± 0.8	5.1 ± 0.9	24 ± 5	2.6 ± 1	5.9 ± 2
14 (50930737)	3,4-di-Cl	2.7 ± 0.2	3.6 ± 0.9	53 ± 3	3.7 ± 1	3.9 ± 2*
15 (50930748)	2-CF_3	5.4 ± 0.8	nd	71 ± 23	14 ± 1	22.8 ± 5
16 (50930755)	3-CF_3	9.7 ± 3.1	nd	>200	20 ± 12	22.4 ± 8
17 (50930745)	2-F,6-CF_3	5.3 ± 6.8	nd	93 ± 16	17 ± 6	32.5 ± 7
18 (50930751)	2-F,3-CF_3	4.3 ± 0.3	nd	42 ± 14	9.2 ± 3	11.9 ± 4*
19 (50930743)	3-F,4-CF_3	3.5 ± 1.1	nd	55 ± 42	17 ± 7	5.4 ± 2
20 (50930749)	3,5-di-CF_3	63 ± 20	7.7 ± 1.8	>200	22 ± 4	14.2 ± 2*



compd (CID)[a]	R	DENV ATPase IC_{50}[b] (μM)	DENV RNA- helicase IC_{50}[c] (μM)	HCV ATPase IC_{50}[d] (μM)	HCV DNA- helicase IC_{50}[e] (μM)	HCV RNA-helicase IC_{50}[f] (μM)
21 (50930741)	2-F,5-CF_3	3.8 ± 0.2	5.4 ± 1.3	16 ± 2	6.4 ± 2	7.5 ± 2
22 (50930733)	3-F,6-CF_3	7.2 ± 0.8	nd	65 ± 10	19 ± 15	nd
23 (50930732)	3-F,5-CF_3	11 ± 5.7	nd	>200	28 ± 7	9.8 ± 3

[a] PubChem[40] compound identification number (CID).

[b] Half-maximal inhibitory concentration observed in assays monitoring DENV NS3h-catalyzed ATP hydrolysis.

[c] Half-maximal inhibitory concentration observed in assays monitoring DENV NS3h RNA unwinding.

[d] Half-maximal inhibitory concentration observed in assays monitoring HCV NS3h-catalyzed ATP hydrolysis.

[e] Half-maximal inhibitory concentration in assays monitoring HCV NS3h DNA unwinding. Data from Li et al.[36]

[f] Half-maximal inhibitory concentration in assays monitoring HCV NS3h RNA unwinding. Values marked with asterisks (*) were published previously in Ndjomou et al.[12]

[g] nd, not determined.

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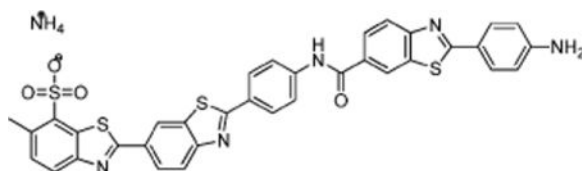
Table 2

ML283 Analogue with Optimal Antiviral Activity in Cells



compd (CID)	DENV ATPase IC_{50}[a] (μM)	DENV helicase IC_{50}[a] (μM)	replicon EC_{50}[b] (μM)	TI[c]
24 (49849289)	0.5 ± 0.1	1.5 ± 0.2	7.1±3	17

[a]As defined in Table 1.

[b]Half-maximal inhibitory concentration observed in assays monitoring BHK cell DENV replicon content.

[c]Half-maximal inhibitory concentration observed in BHK cell viability assays (CC_{50})/replicon EC_{50}.

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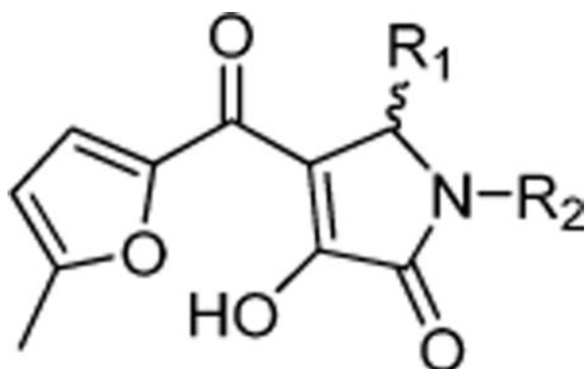
Table 3

DENV Helicase Inhibitors Active against DENV Replicons but Not the HCV Helicase



Cmpd (CID)	R_1, R_2	ATPase IC_{50} (μM)[a]	Replicon EC_{50} (μM)[b]	T.I.[b]
25 (45382104)		78±23	36±6	4.5
26 (45382103)		27±8	59±14	3.5
27 (4594423)		147±72	60±21	5.4
28 (45382102)		93±25	104±48	2.1
29 (4473963)		30±8	125±93	2.0
30 (45382099)		29±9	147±38	7.2

[a] As defined in Table 1.

[b] As defined in Table 2.