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Two RNA Tunnel Inhibitors Bind in Highly Conserved Sites in Dengue Virus NS5 Polymerase: Structural and Functional Studies

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ABSTRACT Dengue virus (DENV) NS5 RNA-dependent RNA polymerase (RdRp), an important drug target, synthesizes viral RNA and is essential for viral replication. While a number of allosteric inhibitors have been reported for hepatitis C virus RdRp, few have been described for DENV RdRp. Following a diverse compound screening campaign and a rigorous hit-to-lead flowchart combining biochemical and biophysical approaches, two DENV RdRp nonnucleoside inhibitors were identified and characterized. These inhibitors show low- to high-micromolar inhibition in DENV RNA polymerization and cell-based assays. X-ray crystallography reveals that they bind in the enzyme RNA template tunnel. One compound (NITD-434) induced an allosteric pocket at the junction of the fingers and palm subdomains by displacing residue V603 in motif B. Binding of another compound (NITD-640) ordered the fingers loop preceding the F motif, close to the RNA template entrance. Most of the amino acid residues that interacted with these compounds are highly conserved in flaviviruses. Both sites are important for polymerase *de novo* initiation and elongation activities and essential for viral replication. This work provides evidence that the RNA tunnel in DENV RdRp offers interesting target sites for inhibition.

IMPORTANCE Dengue virus (DENV), an important arthropod-transmitted human pathogen that causes a spectrum of diseases, has spread dramatically worldwide in recent years. Despite extensive efforts, the only commercial vaccine does not provide adequate protection to naive individuals. DENV NS5 polymerase is a promising drug target, as exemplified by the development of successful commercial drugs against hepatitis C virus (HCV) polymerase and HIV-1 reverse transcriptase. High-throughput screening of compound libraries against this enzyme enabled the discovery of inhibitors that induced binding sites in the RNA template channel. Characterizations by biochemical, biophysical, and reverse genetics approaches provide a better understanding of the biological relevance of these allosteric sites and the way forward to design more-potent inhibitors.

KEYWORDS flaviviruses, dengue virus, nonstructural protein, RNA-dependent RNA polymerase, high-throughput screening, inhibitor, binding site, SPR, X-ray crystallography

Dengue virus (DENV) is a member of the *Flaviviridae* family, which includes other medically important human pathogens, such as Zika virus (ZIKV) and yellow fever virus (1–3). DENV annually infects more than 390 million people worldwide, leading to

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a broad spectrum of outcomes from a mild febrile illness to fatal hemorrhage and shock syndrome (3, 4). Approximately 500,000 cases develop into life-threatening severe disease each year. Currently, no clinically approved therapeutic measures are available, and the only available vaccine does not protect seronegative individuals (5, 6). Thus, there is an urgent need to develop effective antiviral agents to control dengue disease.

DENV encodes a single-stranded positive-sense (+) RNA genome of approximately 11 kb in length with a conserved type I 5' cap (m^7GpppA_mG) structure that is essential for RNA stability (7). The four serotypes of DENV have been found to possess 67% to 73% similarity at the nucleotide level and 69% to 78% similarity at the amino acid level (8, 9). The viral genome contains an open reading frame (ORF) that is flanked by highly structured and conserved 5' and 3' untranslated regions (UTRs). It encodes a single polyprotein of approximately 3,400 amino acids that undergoes co- and posttranslational cleavage by both host cellular proteases and viral encoded protease (NS2B/3) to produce three structural proteins (capsid, membrane, and envelope proteins) which are involved in viral particle formation and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) which assemble to form the viral replicative complex (RC) (7).

DENV NS5 is the largest nonstructural protein and possesses two enzymatic properties that are essential for viral RNA replication. Its N terminus encompasses N7 and 2'-O methyltransferase (MTase) activities (10), while the C terminus comprises RNA-dependent RNA polymerase (RdRp) activity (11). These two domains are linked by a short 10-amino-acid sequence that is poorly conserved across various flaviviruses (12–15). DENV MTase sequentially methylates the N-7 atom of guanine-0 and 2'-O atom of the ribose on the first adenosine at the 5' end of the viral genome, along with other internal adenosines (16–19). DENV RdRp initiates RNA synthesis via a *de novo* initiation (*dnl*) mechanism followed by processive RNA elongation (20, 21). Similar to those of other viral RdRps, the polymerase domain of DENV NS5 has a right-hand shape that includes a fingers, thumb, and palm subdomain (22–24). Within these subdomains, seven conserved amino acid sequence motifs play key roles for binding RNA, nucleoside triphosphates (NTPs), and metal ions and for catalysis (22–24). Structures of the apo-DENV RdRp were found to adopt a “closed” preinitiation state conformation, with a well-ordered priming loop projecting into a narrow RNA binding tunnel. Disordered peptide segments adjacent to motifs F and G (amino acids [aa] 455 to 469 and aa 406 to 417, respectively) as well as in the C-terminal end (aa 884 to 900) were observed.

NS5 is also the most conserved protein in flaviviruses with more than 70% sequence identity across the four DENV serotypes and with no equivalent in human host (11). The central role that NS5 plays in the flavivirus RC makes it an attractive drug target for antiviral intervention. Two broad categories of polymerase inhibitors may be developed to suppress DENV: (i) nucleoside/nucleotide inhibitors (NIs) that mimic the natural substrates and function as RNA or DNA chain terminators and (ii) nonnucleoside inhibitors (NNIs) that are noncompetitive and bind in allosteric pockets in the RdRp domain (reviewed in references 25–29). In our efforts to identify novel DENV NS5 polymerase NNIs, we previously carried out high-throughput screening (HTS) campaigns, using *in vitro* fluorescence-based alkaline phosphatase-coupled polymerase assays (FAPAs) to identify inhibitors of DENV NS5 polymerase *de novo* initiation (*dnl*) and/or elongation activities (30–32).

We subsequently reported two chemical scaffolds that bound in the RdRp RNA template binding groove at the interface between the fingers-thumb subdomains. The first compound, NITD-107, bound as dimers at this site and ordered loop 3 (residues 410 to 419, part of the G motif [33]) in the fingers subdomain. Lead optimization efforts with this class of compound were not successful. The second compound, NITD-29 (from *N*-sulfonyl-anthranilic acid class) interacts with M343 near the top of the thumb domain (34, 35). The most active compounds from this class had submicromolar activities in the RdRp elongation assay but were inactive in DENV cell-based assays.

We describe herein two additional compounds from our HTS campaigns that bind in the DENV RdRp RNA template tunnel. These compounds likely inhibit DENV RdRp

FIG 1 Flowchart of hit follow-up activities following diverse compound library screening. The primary HTS assay used a coupled enzyme fluorescence detection format that measured DENV4 *de novo* initiation activity (30, 31). Counterscreens to remove nonspecific inhibitors were described previously (30). After IC₅₀ reconfirmation, the hits were tested for binding to DENV-3 and -4 by SPR, followed by co-crystallization with DENV3 RdRp (36, 37).

activity by blocking protein conformational changes and/or RNA binding. One compound, NITD-434, induced the formation of a new pocket by displacing residue V603 in motif B, at the interface between the fingers and palm subdomains. Another compound, NITD-640, ordered the fingers loop preceding the F motif (aa 468 to 473) near the RNA tunnel entrance. We characterized these compounds and their binding sites using a combination of biophysical, biochemical, and cell-based assays. We found that the majority of the amino acid residues in both sites are important for *in vitro* polymerase activities and are essential for viral replication.

RESULTS

***In vitro* profiling of two hits from DENV NS5 polymerase HTS.** We previously described a high-throughput screening campaign and hit follow-up flowchart to identify inhibitors for DENV NS5 polymerase *de novo* initiation (*dnl*) activity (30). Briefly, 6,270 primary inhibitors were identified from an in-house library of 257,000 compounds, after elimination of nonspecific inhibitors by counterscreens and reconfirmation by an orthogonal liquid chromatography mass spectrometry (LC-MS)-based assay (30). Hits with attractive biochemical profiles (based on 50% inhibitory concentration [IC₅₀] value and hill slope) were further validated using surface plasmon resonance (SPR) (Fig. 1). In total, 1,360 compounds with *dnl* FAPA IC₅₀ of <20 μM and hill slopes between 0.8 and 1.5 were assessed by SPR using DENV3 and -4 RdRp domains (36, 37). Following single-point screening at 40 μM, 301 compounds were evaluated at 8-point dose-response concentrations. A total of 56 validated binders were followed up by co-crystallization with DENV3 RdRp domain to determine their binding modes. Co-crystal structures were attained for 6 compounds, and partial binding was observed for another six compounds (data not shown).

Two scaffolds that bound in the RNA tunnel of the enzyme were further profiled. Compound NITD-434, a bis-chloro-diphenylamine, 2-aminoindan-2-carboxyl derivative (Fig. 2A), binds with moderate affinity to DENV4 RdRp (SPR dissociation constant [*K_d*] = 18 μM) (Fig. 2D and E) with IC₅₀ values ranging from 6 to 17 μM in DENV1 to -4 polymerase *dnl* assays (Table 1; Fig. 2F). X-ray crystallography showed that the com-

FIG 2 *In vitro* biophysical and biochemical profiles of DENV3 RdRp RNA tunnel inhibitors. Structures of compound NITD-434 (A), NITD-640 (B), and valsartan (C) (38) are shown. SPR measurements of the interaction of NITD-434 (D) and NITD-640 (G) with biotinylated DENV3 RdRp domain and 7-point, 2-fold serially diluted compounds, starting from 2.5 μ M, as described in Materials and Methods. The plots of steady-state response (RU) against NITD-434 (E) and NITD-640 (H) concentration are provided. Inhibition curves for NITD-434 (F) and NITD-640 (I) obtained from DENV4 RdRp *dnl* assay using 2-fold serially diluted compound concentrations (30).

pound binds deep in the DENV3 RdRp RNA tunnel near the junction between the fingers and palm subdomains (Fig. 3, resolution 2.01 Å; Table 2). The second compound, NITD-640 (Fig. 2B), comprising a central 1,4-*N*-bis-substituted-piperazine-3-carboxyl group fused to a diphenylethane group and a biphenyl-tetrazole moiety (sartan-like, resembling angiotensin II receptor blockers) (Fig. 2C) (38), binds also with moderate affinity to DENV4 RdRp (SPR K_d = 41 μ M) (Fig. 2G and H) but possessed better inhibitory

TABLE 1 Compound binding and inhibitory profiles

Parameter	Viral protein	Value	
		NITD-434	NITD-640
SPR K_d (μ M) (% R_{max})	DENV4 RdRp	18.0 (178)	41.5
<i>dnl</i> IC ₅₀ (μ M) (hill slope)	DENV1 FL ^a	6.6 (1.3)	4.9 (2.2)
	DENV2 FL	16.5 (1.8)	12.8 (1.1)
	DENV3FL	12.8 (1.3)	7.8 (1.5)
	DENV4 FL	6.1 (1.0)	0.9 (1.0)
	DENV4 RdRp	11.8 (1.0)	3.0 (0.9)
Elongation IC ₅₀ (μ M) (hill slope)	DENV4 FL	40.1 (1.7)	8.2 (1.4)
DENV cell-based assay EC ₅₀ (CC ₅₀) ^b (μ M)	DENV2	31.2 (>50)	10.7 (>50)

^aFL, finger loop.

^bCC₅₀, 50% cytotoxic concentration.

FIG 3 DENV3 NS5 RdRp with the two RNA tunnel inhibitors. Alternate views (180° difference) showing the locations of the inhibitors binding sites in the DENV3 RdRp domain. Compounds NITD-434 and NITD-640 are displayed as sticks colored in magenta and cyan, respectively. The RdRp regions corresponding to NLS, fingers, thumb, and palm subdomains are indicated in yellow, pink, purple, and green, respectively. Residues are color coded based on domain boundaries indicated by Yap et al. (22). The polymerase active site (GDD at the tip of the palm subdomain) and the priming loop that form a retractile platform for the initiation of RNA polymerization are labeled. Two zinc ions (white spheres) in the fingers and thumb region, and PEG monomethyl ether 550 (PEG) and glycerol (GOL) from the crystallization medium are displayed.

properties, with IC_{50} values ranging from 0.9 to 13 μ M in DENV1 to -4 polymerase *dnl* assays (Table 1; Fig. 2I). X-ray crystallography showed that this compound interacted with the RdRp fingers loops near the entrance of the RNA template tunnel (Fig. 3, resolution 1.95 Å; Table 2). Compared to that in the DENV4 polymerase *dnl* assay, both compounds demonstrated 7- to 9-fold poorer activities in the DENV4 polymerase elongation assay (Table 1). In addition, they possessed moderate inhibitory activities in the DENV2 cell-based assay (50% effective concentration [EC_{50}] = 31 and 11 μ M for NITD-434 and NITD-640, respectively) (Table 1).

Binding sites of NITD-434 and NITD-640. Binding of NITD-434 (comprising bis-chloro-diphenylamine, 2-aminoindan-2-carboxyl groups) in DENV3 RdRp displaced residue V603 projecting from the palm subdomain (upon inhibitor binding, its sidechain is rotated by 42°) and induced the formation of a new binding pocket close to the active site (Fig. 4). As a result, its 2,6-dichlorodiphenylamine moiety sits in a hydrophobic pocket surrounded by residues F398, F485, and V603. Residues K401 and N492 make key hydrogen bonds to the amide and carboxyl groups, respectively, of its indane ring. The latter points toward residues W795, S796, and I797, whereby their backbone carbonyl groups form several hydrogen bonds with water molecules present in the inhibitor binding pocket.

Residues F398 and K401 lie within the α/β region of the DENV RdRp nuclear localization sequence (NLS; residues aa 369 to 415) that was proposed to play a role in DENV NS5 nuclear localization (39, 40). Residues F485 and N492 are located after the conserved motif F (aa 468 to 473; interacts with incoming NTPs [41–43]), in helix α 10 in the fingers subdomain. Residue V603 resides in the conserved motif B (aa 597 to 613; responsible for nucleotide selection [44, 45]), adjacent to helix α 16 (aa 604 to 703) in the palm subdomain (22) and is in close proximity to the catalytic triad (G662-D663-D664 or motif C) (Fig. 4d). Residues W795, S796, and I797 belong to the priming loop (aa 782 to 809, thumb domain) that provides the structural platform for *dnl* complex formation (41, 46, 47).

NITD-640 binds near the RNA template entrance, to the “fingers-tip” region that encompasses motif F (aa 468 to 473) and which is disordered in the free polymerase structure. Upon NITD-640 binding, M454-K469 become well ordered (Fig. 5). The compound wraps around R457, making several side and main chain interactions. Key interactions involve the carboxyl group (from the central piperazine ring) which hydrogen bonds with R457 and E458 and the tetrazole group which hydrogen bonds to

TABLE 2 Data collection and refinement statistics of DENV3 RdRp co-crystals

Parameter	Value ^a	
	NITD-434	NITD-640
Wavelength (Å)	1.0	1.0
Resolution range (Å)	29.37–2.01 (2.08–2.01)	19.33–1.95 (2.02–1.95)
Space group	C222 ₁	C222 ₁
Unit cell (Å)	161.66, 176.24, 57.76; 90, 90, 90	161.53, 175.20, 57.75; 90, 90, 90
Total reflections (n)	364,018 (35,393)	395,201 (40,524)
Unique reflections (n)	54,495 (5,350)	59,691 (5,880)
Multiplicity	6.7 (6.6)	6.6 (6.9)
Completeness (%)	98.55 (97.66)	99.75 (99.85)
Mean I/sigma (I)	20.06 (2.77)	24.97 (3.37)
Wilson B-factor (Å ²)	39.41	33.98
R _{merge} ^b	0.051 (0.66)	0.045 (0.59)
R _{meas}	0.056 (0.72)	0.049 (0.64)
R _{pim}	0.022 (0.28)	0.019 (0.24)
CC _{1/2}	0.99 (0.88)	1 (0.90)
CC ^c	1 (0.97)	1 (0.97)
Reflections used in refinement (n)	54,487	60,023
Reflections used for R _{free} (n)	2,764 (1,090)	3,036 (1,201)
R _{work} ^c	0.17 (0.21)	0.17 (0.20)
R _{free} ^d	0.19 (0.29)	0.19 (0.25)
CC (work)	0.95	0.96
CC (free)	0.95	0.95
No. of nonhydrogen atoms	5,285	5,493
Macromolecules	4,803	4,922
Ligands	68	52
Solvent	414	519
Protein residues	584	599
RMS		
Bond length (Å)	0.007	0.009
Angle (°)	0.86	0.92
Ramachandran plot (%)		
Favored	97.06	96.39
Allowed	2.77	3.44
Outliers	0.17	0.17
Rotamer outliers (%)	2.7	1.14
Clash score	5.07	1.73
Avg B-factor (Å ²)	49.04	40.72
Macromolecules	48.31	39.67
Ligands	63.46	43.69
Solvent	56.50	50.44
PDB code	6XD0	6XD1

^aStatistics for the highest-resolution shell are shown in parentheses.

^b $R_{\text{merge}} = \sum |I_j - \langle I \rangle| / \sum I_j$, where I_j is the intensity of an individual reflection, and $\langle I \rangle$ is the average intensity of that reflection.

^c $R_{\text{work}} = \sum |F_o| - |F_c| / \sum |F_c|$, where F_o denotes the observed structure factor amplitude and F_c is the structure factor amplitude calculated from the model.

^d R_{free} is as for R_{work} but calculated with 5% (3,044) of randomly chosen reflections omitted from the refinement.

R457 and K578 (located in strand $\beta 2$ in the fingers subdomain). Removal of this carboxyl group leads to elimination of binding altogether (data not shown). Two water-mediated hydrogen bonds are seen between the tetrazole ring and N452 as well as between the amide group and E458. The main hydrophobic interaction is attributed to the biphenyl tetrazole moiety and *n*-butyl group which are surrounded by residues F464, I473, and W474.

The majority of NS5 residues lining the binding pockets of NITD-434 and NITD-640 are conserved in all four DENV serotypes and in other flaviviruses, such as Japanese encephalitis virus (JEV), yellow fever virus (YFV), West Nile virus (WNV), and Zika virus (ZIKV) (Fig. 6). This suggests that these residues may play an important role in viral RNA replication, and the binding sites are attractive targets for the discovery of pan-

FIG 4 Interactions between DENV3 RdRp and NITD-434. (a) Close-up views (related by a rotation of 180° along a vertical axis) of the NITD-434 compound binding site with surrounding water molecules and NS5 residues that make direct contact with the inhibitor. NITD-434 is represented as ball and sticks with carbon atoms in magenta, chlorine in green, oxygen in red, and nitrogen atoms in blue, and surrounding residues forming the binding pocket are labeled. (b) Schematic flat representation of the protein-ligand interactions. Hydrogen bonds are represented by dashes with the donor-acceptor distances given in angstroms. The same color code is used throughout to indicate the subdomains to which residues belong (palm, green; fingers, orange; NLS, light yellow). An electron difference density map with Fourier coefficients $F_o - F_c$ with phases from the refined model (and where the inhibitor was omitted from the phase calculation) is displayed at a level of 3 sigmas over the mean (green mesh). (c) Close-up view indicating the induced fit in the NITD-434 pocket with the flipping of the aliphatic side chain residue V603 (green) by 42° (arrow), compared to PDB access code 4HHJ in white (33), to accommodate the dichlorophenyl ring of the NITD-434 inhibitor in the cavity. (d) Location of residues surrounding the compound binding site that were subjected to site-directed mutagenesis. Mutated residues are highlighted with thicker bonds and labeled (a and d). The RdRp subdomains and NLS are colored coded as per Fig. 3.

flavivirus inhibitors. The only exceptions are residue 604 (DENV3 numbering), which corresponded to either G (DENV-1, -3, and -4) or V (DENV2, JEV, YFV, WNV, and ZIKV) in the NITD-434 binding site, and residue 578, which comprised either K (DENV-3 and -4, JEV, YFV, WNV, and ZIKV) or the conservative substitution R (DENV-1 and -2) in the NITD-640 binding site.

Mutagenesis of residues in compound binding pockets. To guide the design of more-potent analogs of NITD-434 and -640, we characterized the roles of specific residues in the compound binding sites. We mutated the amino acid residues that made contacts with NITD-434 and -640 to alanine and evaluated the effects on both *in vitro* NS5 polymerase activities and virus fitness. We selected DENV serotype 4 for the *in vitro* polymerase activity studies due to the better protein stability (31, 32).

Compared to wild-type (WT) RdRp, alanine mutations of NLS (F398 and K401) and fingers (N492) residues that encircled NITD-434 decreased *de novo* initiation (*dnl*) and

FIG 5 Interactions between DENV3 RdRp and NITD-640. (a) Close-up view of the NITD-640 compound binding site with NITD-640 (cyan sticks) and the surrounding residues that line the pocket. An electron difference density map with Fourier coefficients $F_o - F_c$ with phases from the refined model (and where the inhibitor was omitted from the phase calculation) is displayed at a level of 3 sigmas over the mean (green mesh). (b) Flat representation of the ligand-protein interactions. Residues M453 to G469 preceding motif F (main-chain α -carbon atoms are displayed as a black “tube”) (c) become ordered upon NITD-640 binding (d). Location of residues targeted by mutagenesis are highlighted with thicker bonds and labeled (a and d). The RdRp subdomains and NLS are colored coded as per Fig. 3.

elongation activities by 60% to 93% (Fig. 7A and B; Table 3). These mutants were also lethal in the DENV4 reporter replicon. No renilla luciferase activity or viral protein expression was detected during the 4-day study period (Fig. 8A and C; Table 3). On the other hand, fingers residue mutant F485A exhibited marginal reduction in polymerase activities (20% to 35%) compared to that of the WT.

Mutation of the priming loop residue W795, which is near the indane group of NITD-434, increased polymerase activities by approximately 25% (Fig. 7A; Table 3). This finding is unlike that in our previous study, where alanine mutations of priming loop residues (S796, H798, H800, Q802, and W803) decreased RdRp activities by 50% to

NLS (α7)				F-motif and α10				β2			
DENV1	395	EEFTRK	VRSN 404	451	NMMGKREKK	LGEFGKAKGS	RAIWYMWLGA	RFLEFEALGF	MNEDHW	495	574 KVVVRVQ 579
DENV2	396	EEFTRK	VRSN 405	452	NMMGKREKK	LGEFGKAKGS	RAIWYMWLGA	RFLEFEALGF	LNEDHW	496	575 KVVVRVQ 580
DENV3	396	EEFTKK	VRTN 405	452	NMMGKREKK	LGEFGKAKGS	RAIWYMWLGA	RYLEFEALGF	LNEDHW	496	575 KVVVRVQ 580
DENV4	397	EEFISK	VRSN 406	453	NMMGKREKK	LGEFGKAKGS	RAIWYMWLGA	RFLEFEALGF	LNEDHW	497	576 KVVVRVQ 581
WNV	401	EEFIRK	VNSN 410	457	NMMGKREKK	PGEFGKAKGS	RAIWFWWLGA	RFLEFEALGF	LNEDHW	501	580 KVVKVM 585
YFV	400	EEFIAK	VRSH 409	456	NMMGKREKK	LSEFGKAKGS	RAIWYMWLGA	RYLEFEALGF	LNEDHW	500	579 KVVKVL 584
JEV	401	EEFIK	VNSN 410	457	NMMGKREKK	PGEFGKAKGS	RAIWFWWLGA	RYLEFEALGF	LNEDHW	501	580 KVVKVM 585
ZIKV	398	EEFINK	VRSN 407	454	NMMGKREKK	QGEFGKAKGS	RAIWYMWLGA	RFLEFEALGF	LNEDHW	498	577 KVVKVL 582

B-motif and α16				C-motif				priming loop			
DENV1	596	QRGSG	QVGTYGLNTF	TNMEVQLIRQ	MESE	624	658	ISGDD	662	785	DWVPTS RTTWSIHAAH QWMTTEDM 808
DENV2	597	QRGSG	QVVTYGLNTF	TNMEAQLIRQ	MEGE	625	659	ISGDD	663	786	HWVPTS RTTWSIHATH EWMTTEDM 809
DENV3	597	QRGSG	QVGTYGLNTF	TNMEAQLVRQ	MEGE	625	659	ISGDD	663	786	HWVPTS RTTWSIHAAH QWMTTEDM 809
DENV4	598	QRGSG	QVGTYGLNTF	TNMEVQLIRQ	MEAE	626	660	ISGDD	664	787	EWFTS RTTWSIHAAH QWMTTEDM 810
WNV	602	QRGSG	QVVTYALNTF	TNLAVQLVRM	MEGE	630	664	VSGDD	668	791	NWVPTG RTTWSIHAGG EWMTTEDM 814
YFV	601	QRGSG	QVVTYALNTI	TNLKVQLIRM	AEAE	629	663	VSGDD	667	790	SWVPQG RTTWSIHGKG EWMTTEDM 813
JEV	602	QRGSG	QVVTYALNTF	TNLAVQLVRL	MEAE	630	664	ISGDD	668	791	DWVPTG RTTWSIHSGK EWMTTEDM 814
ZIKV	599	QRGSG	QVVTYALNTF	TNLVVQLIRN	MEAE	627	661	VSGDD	665	788	DWVPTG RTTWSIHGKG EWMTTEDM 811

FIG 6 Alignment of amino acid residues forming the compound binding pockets across different members of the flavivirus family. The RdRp sequences are numbered and labeled. Conserved motifs B, C, and F are boxed (42–44). Amino acids that contact NITD-434 and NITD-640 within their respective binding sites are colored in red and blue, respectively.

100% (37). Nevertheless, similar to that for these latter mutations, growth of the W795A mutant replicon was markedly slower than WT (84% decline) (Fig. 8A and C; Table 3). Growth attenuation by W795A was also confirmed with an infectious DENV2 clone; W795A generated smaller and more-diffuse plaques than WT DENV2 (Fig. 8E). The

FIG 7 Alanine mutations of residues that contact RNA tunnel inhibitors and their effects on *in vitro* RdRp enzymatic activity. *In vitro* dnl (A and C) and elongation (B and D) enzymatic activities of alanine mutants of residues that interact with NITD-434 (A and B) or NITD-640 (C and D) were tested using biochemical assays as described in Materials and Methods. Each data point is the average from duplicates or triplicates, and error bars represent the standard deviations from at least two independent experiments. The amino acid residues indicated correspond to DENV3 numbering.

TABLE 3 *In vitro* polymerase activities and replicon fitness of alanine mutants of amino acid residues in NITD-434 and NITD-640 binding sites

Compound	Amino acid residue(s) mutated to alanine		Activity compared to that of WT after 2 h (%)		Thermodenaturation (protein T_m [°C])	Fitness compared to that of WT after 72 h (%) for DENV4 replicon
	DENV3	DENV4	<i>De novo</i> initiation	Elongation		
NITD-434	WT	WT	100	100	37	100
	F398	F399	32.8 ± 9.2	40.6 ± 7.1	38.7	0
	K401	K402	7.0 ± 3.3	38.5 ± 9.7	37.6	0
	F485	F486	64.9 ± 10.4	79.6 ± 1.9	36.6	ND ^a
	N492	N493	13.3 ± 4.1	29.6 ± 10.7	36.8	0
	G604	G605	106.8 ± 12.1	83.9 ± 14.8	38.6	ND
	T605	T606	1.62 ± 0.11	3.41 ± 0.18	37	ND
	Y606	Y607	21.7 ± 4.7	126.5 ± 23.1	37.6	0
	N609	N610	0.3 ± 0.4	5.7 ± 3.8	37.2	0
	W795	W796	125.2 ± 10.3	124.1 ± 18.3	38.6	16
NITD-640	WT	WT	100	100	37	100
	N452A	N453A	4.4 ± 1.1	23.8 ± 2.7	37	0.02
	R456A	K457A	44.0 ± 15.4	85.3 ± 4.2	37	0
	R457A	R458A	51.1 ± 2.3	110.8 ± 1.0	37	0.05
	R456A/R457A	K457A/R458A	33.9 ± 10.7	62.1 ± 10.9	38	0
	E458A	E459A	18.4 ± 4.7	72.3 ± 5.7	37	0.05
	F464A	F465A	106.2 ± 27.6	124.3 ± 4.6	37	1.09
	W474A	W475A	42.7 ± 7.7	76.9 ± 1.8	37	0.09
	K578A	K579A	53.0 ± 12.7	86.8 ± 8.6	37	114
	D663A	D664A	0	0	37	0

^aND, not determined.

reason for the observed disparity between the *in vitro* polymerase activities and replicon fitness of mutant W795A is uncertain.

Due to the proximity of the NITD-434 to motif B of the RdRp, we further mutated G604, T605, Y606, and N609 to alanine and assessed the impact on polymerase activities (Fig. 7A and B; Table 3). Mutant G604A was not significantly changed from WT protein. Mutant Y606A displayed approximately 78% less *dnl* activity but 27% higher elongation activity. T605A and N609A mutants were completely inactive in the polymerase assays. Both Y606A and N609A also abolished replicon growth (Fig. 8A and C). Overall, these results are in agreement with the importance of motif B residues in selection of ribonucleoside triphosphates over deoxynucleoside triphosphates (dNTPs) for viral RNA synthesis (43, 44).

We next explored the functional importance of residues that contacted NITD-640. In general, the effects of alanine mutations on the fingers-tip residues (aa 456 to 578) were more pronounced in the *dnl* assay than in the elongation assay (Fig. 7C and D; Table 3B). Mutants R457A, W474A, and K578A (interact with the carboxyl and tetrazole moieties of NITD-640) retained 43% to 53% and 77% to 111% activities in *dnl* and elongation assays, respectively, compared to that for the WT protein. Mutant E458A (interacts with the carboxyl and amide groups of NITD-640) retained 18% and 72% *dnl* and elongation activities, respectively. Mutant N452A (makes a water-mediated interaction with the tetrazole ring) has markedly reduced polymerase activities (4.4% and 23.8%), while mutant F464A (interacts with biphenyl tetrazole moiety of NITD-640) was equally active as WT protein. Unlike a previous report (43), the double mutant R456A/R457A (K457A/R458A in DENV4) retained significant polymerase activities (34% and 62%).

All mutants proximal to the motif F sequence (aa 468 to 473), including F464A, strongly restricted DENV4 replicon growth (Fig. 8B and D; Table 3). K456A, R457A, and K456A/R457A replicons, like the catalytically dead RdRp mutant D663A, are inactive and displayed no luciferase activity over the 4-day study period. N452A, E458A, F464A, and W474A replicons maintained very low residual activities of 0.02% to 1.09% compared to that of the WT replicon. In contrast, growth of the K578A replicon was delayed in the first 24 h but replicated to comparable levels as the WT replicon (114%) by day 3.

FIG 8 Alanine mutations of residues that contact RNA tunnel inhibitors and their effects on growth fitness of DENV replicons and infectious virus. BHK-21 cells were electroporated with equal amounts of WT and mutant DENV4 replicon RNA (A to D) or WT and mutant DENV2 infectious virus (E) harboring alanine substitutions of residues in the binding sites of compound NITD-434 (A, C, and E) and NITD-640 (B and D). The amino acid residues indicated correspond to DENV3 numbering. At various time points, transfected cells were lysed for luciferase activity measurements (A and B) or assessed for viral NS3 protein and RNA expression (C to E). Growth of DENV4 replicons is denoted by $\log_{10}$ value of renilla luciferase signal (RLU) of the average from duplicates readings, and error bars represent the standard deviations from at least two independent experiments (A and B). Expression of viral NS3 protein (red) and viral dsRNA (green) in DENV4 WT and mutant replicons was detected by immunofluorescence assays (C and D) with nucleus staining (DAPI, blue) as described in Materials and Methods. Plaque assays were performed as described in Materials and Methods using a DENV2 infectious virus (E). ND, no plaques were detected. Mutant D663A (part of motif C, the catalytic motif) was included as a negative control (A and E).

DISCUSSION

Our lead finding strategy that utilizes SPR and X-ray crystallography with the DENV3 RdRp domain enabled us to identify several compounds that bound in the enzyme RNA template tunnel. Interestingly, binding of compound NITD-434 induced the formation of a pocket near the palm and fingers subdomains by displacing the side chain of residue V603 located in motif B (aa 597 to 613). Binding of a second scaffold NITD-640 to the fingers loop near the RNA template entry site led to the ordering of the region comprising residues M454 to K469 adjacent to motif F (aa 468 to 473). Thus, while the regions encompassing the G motif (aa 409 to 416) and C-terminal residues (aa 884 to 900) are disordered in DENV3 RdRp co-crystals with both NITD-434 and NITD-640, residues aa 454 to 469 are visible in the latter.

Both compounds show low- to high-micromolar inhibitory activities in *in vitro* DENV NS5 polymerase biochemical, biophysical, and DENV cell-based assays. In addition, they were 7- to 9-fold less active in the DENV elongation assay than in the *dnl* assay. This suggests that binding of these two inhibitors is impaired when the RdRp RNA tunnel is occupied by double-stranded RNA (dsRNA) during the elongation phase. It is also possible that their binding sites undergo conformational changes during the template elongation phase, leading to weaker binding.

FIG 8 (Continued)

Notably, the variation in inhibitory properties of NITD-434 across DENV1 to -4 NS5 (approximately 3-fold difference) is lower than for NITD-640 (about 14-fold difference). This is likely due to the more exposed and flexible nature of the NITD-640 binding site (at the RNA template entrance, proximal to F motif, aa 468 to 473), which in turn affects compound binding affinity. In particular, motif F and its flanking sequences have been observed in crystal structures of DENV2 and DENV3 NS5 proteins to adopt different conformations and to mediate interactions with the MTase domain during protein transition states when the interdomain linker is extended but not when it is a coiled form (13, 15, 48).

We characterized the compound binding sites using X-ray crystallography and studied the amino acid residues lining these pockets to facilitate the design of better analogs. Residues from the α/β NLS (aa 369 to 415), motif B (aa 597 to 613), and priming loop (aa 782 to 809) line the pocket that accommodates NITD-434, while NITD-640 interacts with motif F (aa 468 to 473). These regions are known to play important roles in enzyme function, such as nuclear translocation, ribonucleotide triphosphate (rNTP) entry, rNTP selectivity, and *de novo* initiation. Not surprisingly, they are highly conserved across flaviviruses, and mutation of these residues reduced RdRp *dnl* and elongation activities and abolished DENV replication. This suggests that compounds that interact with these sequences may be developed as pan-DENV or pan-flavivirus inhibitors.

Two other chemical scaffolds (NITD-107 and HeE1-2Tyr) have been reported to bind

FIG 9 Summary of RNA tunnel inhibitors of DENV3 NS5 RdRp. Overall view of the locations of the various DENV RdRp allosteric inhibitors that bind in the RNA tunnel (middle) and close-up views of their respective binding sites: (i) NITD-434 (cyan), (ii) NITD-107 dimers (white), (iii) HeE1-2Tyr (blue), (iv) fragment JF-31-MG46 (yellow), (v) compound 27 (cyan), and (vi) fragment RK-0404678 (orange, site II). The RdRp subdomains and NLS are colored coded as per Fig. 3.

near the NITD-434 site (Fig. 9). NITD-107 bound as dimers and made similar amino acid interactions as NITD-434, in the NLS, motif B, and priming loop sequences (33). One of its monomers (NITD107-2) ordered residues from motif G (aa 407 to 418) through contacts with V411, F412, and T413. Binding of the bulky compound, HeE1-2Tyr, resembled that of NITD-107 dimers, with its phenolic ring stacked over its pyridobenzothiazole moiety, but formed few amino acid contacts, primarily with W795 and N492 (49). Like in NITD-434, V603 was flipped by 180° to accommodate the cyclohexane moiety of HeE1-2Tyr.

Overall, NITD-434 bound deeper in the RNA tunnel, making more amino acid contacts than HeE1-2Tyr but fewer than the NITD-107 dimer. Binding modes of NITD-434 and HeE1-2Tyr do not overlap, with NITD-434 binding closer to the NLS than HeE1-2Tyr. The position of the terminal cyclohexane ring of HeE1-2Tyr is located in the vicinity of the phenyl ring of the indane moiety of NITD-434, which points toward residue I797 of the priming loop.

The binding site of NITD-434 is close to that of monomer 1 of NITD-107. When superimposed, the benzofuran ring of NITD107-1 lies almost in the same position as the indane group of NITD-434. Carboxyl groups in both moieties point toward the same direction but in somewhat different orientations. They make interactions with side chains of residues N492 (NITD-434 and NITD107-1), K401 (NITD107-1), and E493 (NITD107-1, via a bridging water molecule). We speculate that replacing the 2-amino-indan-2-carboxyl moiety in NITD-434 with other carboxyl-substituted fused heterocyclic moieties may enable interactions with K401 and E493 and increase its binding to the RdRp. Alternatively, reversing the orientation of the amide linker in NITD-434 or replacing it with a sulfonamide moiety may promote additional interactions with these charged residues.

Furthermore, we observed that the chloro-substituted phenol ring of monomer

NITD107-2 is in a similar position as the central phenyl ring of NITD-434 and points toward the side chain of F485. It would be interesting to explore various substitutions (such as chloro, fluoro, methyl, and amine) on this ring in NITD-434 to facilitate more amino acid interactions.

Recently, Shimizu et al. (50) identified a fragment, RK-0404678 (2-oxo-1,3-benzoxathiol-5-yl acetate) that bound in two sites (I and II) in DENV2 RdRp. Amino acid residue interactions in site II comprised E510, G511 (palm subdomain), S661 (motif C), and C709 (motif E). It induced movement of motif B (residue T607) and disordered part of it (α 16-helix and its flanking loop). When co-crystallized with DENV3 RdRp, RK-0404678 bound only in site I in the thumb subdomain. Pan-DENV nanomolar inhibitors, compounds 27 and 29, and its original fragment, JF-31-MG46, also interacted with the priming loop and motif E (aa 709 to 729) residues (36, 37). Overall, we believe that the regions of the RdRp encompassing motifs B, C, and E and the priming loop represent hot spots for inhibitor binding and that it is possible to design potent pan-DENV RdRp inhibitors targeting these regions, as exemplified by compounds 27 and 29 (36, 37).

Compound NITD-640 binds near the entrance of the RNA tunnel and ordered the sequence from residues M454 to K469 that precedes motif F (aa 468 to 473). Motif F participates in binding of the incoming NTP in other viral RdRps (41, 42). Recently, it was reported to interact with DENV viral RNA 5' UTR at stem-loop A during *de novo* RNA synthesis (43). Mutation of residues that contact NITD-640 impacted DENV *dnl* activity more than elongation activity and impeded DENV replication in cells. These findings corroborate other reports that motif F and the fingers-thumb loop region are critical for RdRp activity (41–43). Nevertheless, designing more-potent NITD-640 analogs is challenging. The compound does not bind to a well-defined cavity due to the flexible nature of motif F and the presence of surrounding fingers-thumb loops, including motif G (aa 407 to 418). Indeed, the segment comprising motif F has been shown to adopt different conformations in DENV (13, 15, 48), ZIKV (51), and JEV (52) full-length NS5 proteins.

Attempts to select dengue viruses that are resistant to compound NITD-434 and NITD-640 were unsuccessful. This may be attributed to the modest EC_{50} values of both compounds (10.7 and 31.2 μ M, respectively). This is not a surprising result, and, for instance, Shimizu et al. (50) were also not successful in raising resistant dengue viruses to the compound RK-0404678 (DENV2 EC_{50} = 7 μ M), which binds near NITD-434 (50).

In summary, we present an extensive characterization of two DENV NS5 RdRp RNA tunnel inhibitors. We propose that the binding site of NITD-434 is promising for rational design and offers the opportunity to generate pan-DENV or even pan-flavivirus inhibitors for treatment of additional medically important pathogens, such as ZIKV. Design of more-potent analogs of NITD-640 that bind in the fingers-thumb interconnecting loops may be challenging due to the intrinsic flexibility of this region and the plausible entropic cost incurred in binding.

MATERIALS AND METHODS

Protein production, co-crystallization with compounds, and X-ray structure determination.

Protein production, co-crystallization with compounds, and X-ray structure determination for DENV3 RdRp were previously described (34, 36, 37). Briefly, protein was expressed in BL21(DE3) RIPL cells (Agilent Technologies) and purified by Ni-affinity, thrombin cleavage, reverse Ni-affinity, and size exclusion chromatography. Purified protein at 10 mg/ml was used to set up sitting-drop vapor diffusion crystallization trials in 0.1 M Tris-HCl, pH 8.2 to 8.4, 26% to 28% polyethylene glycol (PEG) 550 monomethyl ether (MME) (1 μ l plus 1 μ l, protein plus reservoir). For compound soaking, 100 mM compound stock in 100% dimethyl sulfoxide (DMSO) was mixed with crystallization solution to obtain a final concentration of 10 mM with 10% DMSO, 0.1 M Tris-HCl, pH 8.2, and 26% PEG 550 MME. DENV3 RdRp crystals were transferred to the soaking solution and incubated overnight at room temperature with the same well solution. Crystals were harvested the next day by transferring them to a soaking solution supplemented with 10% glycerol for a few seconds for cryoprotection and quickly freezing them in liquid nitrogen. X-ray diffraction data for NITD-434 were collected at Advanced Photon Source beamline 17ID and, for NITD-640, at Swiss Light Source beamline X10SA (PXII). Data were processed by autoPROC (53), and the structure was refined using BUSTER (54) starting with coordinates for DENV3 RdRp obtained from PDB (access code [2J7U](https://www.rcsb.org/structure/2J7U)).

Cloning of pET28a-DENV4MY01-NS5-22713 NS5 mutants and activity measurements. Template plasmid pET28a-D4MY01-NS5-22713 bearing full-length DENV4 NS5 (32) was used to perform site-

directed mutagenesis with a Quik Change II XL kit (Agilent Technologies) as per the manufacturer's instructions and then transformed into BL21 RIL *Escherichia coli* cells (Agilent Technologies) for protein expression. *In vitro de novo* initiation (*dnl*) and elongation activities of purified DENV4 NS5 wild type (WT) and mutant enzymes were measured using a fluorescence-based alkaline phosphatase-coupled polymerase assay (FAPA) as described previously (30–32, 37). A thermo-fluorescence assay was used to determine the protein stability of expressed proteins (12, 37).

Surface plasmon resonance binding assay. Biotinylated DENV3 and DENV4 RdRp were captured on flow cells 2 and 4, respectively, in 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 2 mM dithiothreitol (DTT), 0.05% Tween 20, and 3% DMSO at 4°C. Flow cells 1 and 3 were left blank to serve as a reference (36, 37). Compounds were tested in a 7-point 2-fold serial dilution, starting from 60 μ M, and a zero-concentration sample run was subtracted from each run. Compounds were injected at a flow rate of 30 μ l/min, with 45- or 60-s contact time and 60-s dissociation, starting from the DMSO control and finishing with the highest concentration. The experiments were performed using a Biacore T200 instrument, and the data were analyzed using Biacore T200 Evaluation software, version 2.0.

Compound testing with DENV *de novo* initiation FAPA assay. IC₅₀ values for inhibitors were determined in DENV 4 *dnl* FAPA assay by dose-response testing of compounds (10-point, 3-fold serially diluted compounds from 0 to 2 μ M or 0 to 100 μ M) with WT and mutant enzymes as described previously (30, 37). Briefly, compounds were incubated for 20 min with enzyme alone in 384-well, black opaque plates (Corning), after which, reactions were started with addition of single-stranded RNA (ssRNA) and nucleotide substrates and allowed to proceed for 1 to 3 h. Reactions were stopped by addition of 2.5 $\times$ STOP buffer with 25 nM calf intestinal alkaline phosphatase (CIP), reincubated for 60 min, and read on a Tecan Safire II microplate reader (excitation [Ex_{max}] and emission [Em_{max}] wavelengths at 422 nm and 566 nm, respectively). All enzymatic reaction steps were performed at room temperature. IC₅₀ curves were plotted using GraphPad Prism software and fitted to a four-parameter logistic equation yielding average IC₅₀ values and hill slopes. All data points were measured either in duplicates or triplicates.

Generation of NS5 mutant DENV replicons. Mutations in the DENV4 NS5 (GenBank accession number AF326825) sequence were engineered into the subclone, pACYC-DENV4-F shuttle, using the QuikChange II XL site-directed mutagenesis kit according to the manufacturer's protocol (Agilent Technologies). Following sequence verification, the plasmids were digested with NotI and KpnI and inserted with a PCR product of the sequence comprising nucleotides 1 to 7563 downstream of the T7 promoter in which the region from nucleotides 217 to 2291 in this cDNA had been replaced by renilla luciferase and foot-and-mouth disease virus 2A protease cDNAs as described previously (37). WT and mutant DENV4 replicon plasmids were linearized with XhoI and *in vitro* transcribed (IVT) using a T7 mMESSAGE mMACHINE kit according to the manufacturer's protocol (Thermo Fisher Scientific). Following electroporation of IVT RNA into BHK-21 cells, growth of DENV4 replicons was monitored as previously described by renilla luciferase activity and immunofluorescence (37).

Immunofluorescence assays. Replicon RNA-transfected or DENV-infected cells were seeded onto an 8-well chamber slide and incubated in 5% CO₂ at 37°C. At designated time points, the medium was removed, and the cells were washed with phosphate-buffered saline (PBS) before fixing in cold methanol for 30 min at –20°C. The fixed cells were then blocked with PBST (composed of 1% fetal bovine serum [vol/vol], 1% bovine serum albumin [wt/vol], and 0.05% Tween 20 [vol/vol] in PBS) for 1 h at room temperature (RT) followed by incubation for 1 h at RT with anti-NS3 helicase protein rabbit antibody (NITD, Singapore) and anti-dsRNA mouse monoclonal antibody (SCICONS). Cells were then incubated with Alexa Fluor 568 goat anti-rabbit IgG or Alexa Fluor 488 goat anti-rabbit IgG (Thermo Fisher Scientific) and fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (Sigma) or Alexa Fluor 568 donkey anti-mouse IgG (Thermo Fisher Scientific), respectively, for 1 h at RT. After PBS washing, mounting agent containing 4',6-diamidino-2-phenylindole (DAPI) was added. The slide was visualized under a fluorescence microscope, and cell images were captured on camera at $\times 20$ magnification.

Construction of DENV2 W795A mutant virus. Template plasmid TSV01-F subclone containing DENV 2 TSV01 NS4B-NS5cDNA-3'UTR plus hepatitis D virus ribozyme (HDVr) (55) was used to perform site-directed mutagenesis using the QuikChange II XL site-directed mutagenesis kit according to the manufacturer's protocol (Agilent Technologies) to introduce the various alanine mutations, followed by digestion with NruI and ClaI to obtain the 3.1-kb fragment encompassing the mutated NS5 region. It was then cloned into the pACYC-FL TSV01 plasmid (bearing infectious cDNA clone of WT DENV-2 pACYC TSV FL, strain TSV01) using the same restriction enzyme sites. The subsequent recombinant plasmid was linearized with ClaI and *in vitro* transcribed using a T7 mMESSAGE mMACHINE kit (Thermo Fisher Scientific).

Plaque assay. RPMI 1640 medium (Sigma) supplemented with 2% fetal bovine serum (FBS) and 1% penicillin/streptomycin was used for viral serial dilution during plaque assay studies. Viral titers of culture medium collected from BHK-21 (ATCC) cells at day 4 posttransfection were quantified through a single layer plaque assay; 2×10^5 BHK-21 (NITD) cells were seeded per well in a 24-well plate. Cells were incubated for 24 h at 37°C with 5% CO₂ to reach confluence. A series of 1:10 dilutions was made by mixing 100 μ l of cell supernatant from viral RNA-electroporated cells with 900 μ l of RPMI medium from 10^{-1} to 10^{-9} dilutions. RPMI medium with 10% FBS was aspirated from the seeded cells, and aliquots containing 100 μ l of appropriate dilutions were inoculated onto respective wells containing cell monolayers. The virus was allowed to infect the cells at 30°C with 5% CO₂ for 1 h. The virus medium was then replaced with 600 μ l of 0.8% methyl-cellulose RPMI overlay medium (Calbiochem) containing 2% FBS. Plates were then incubated for 4 days at 37°C with 5% CO₂. After the incubation period, cells were fixed

in 3.7% formaldehyde (Sigma) and stained with 1% crystal violet solution. The plaque number was determined by visual inspection of plaque-forming units (PFU).

Data availability. The structural data for the DENV3 RdRp co-crystal structures with compounds NITD-434 and NITD-640 are available in the Protein Data Bank under accession numbers 6XDO and 6XD1, respectively.

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We declare a conflict of interest. T.M.S., T.E.B., R.A., F.Y., and Y.L.C. are current employees of Novartis. In addition, T.E.B. and T.M.S. hold stock in Novartis.

REFERENCES

- Wikan N, Smith DR. 2016. Zika virus: history of a newly emerging arbovirus. *Lancet Infect Dis* 16:e119–e126. [https://doi.org/10.1016/S1473-3099\(16\)30010-X](https://doi.org/10.1016/S1473-3099(16)30010-X).
- World Health Organization. 2017. Yellow fever – Brazil. <http://www.who.int/csr/don/04-april-2017-yellow-fever-brazil/en/>. Accessed 14 August 2019.
- Shepard DS, Undurraga EA, Halasa YA, Stanaway JD. 2016. The global economic burden of dengue: a systematic analysis. *Lancet Infect Dis* 16:935–941. [https://doi.org/10.1016/S1473-3099\(16\)00146-8](https://doi.org/10.1016/S1473-3099(16)00146-8).
- Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, Drake JM, Brownstein JS, Hoen AG, Sankoh O, Myers MF, George DB, Jaenisch T, Wint GR, Simmons CP, Scott TW, Farrar JJ, Hay SI. 2013. The global distribution and burden of dengue. *Nature* 496:504–507. <https://doi.org/10.1038/nature12060>.
- Halstead SB. 2018. Safety issues from a phase 3 clinical trial of a live-attenuated chimeric yellow fever tetravalent dengue vaccine. *Hum Vaccin Immunother* 14:2158–2162. <https://doi.org/10.1080/21645515.2018.1445448>.
- WHO. 2018. Dengue vaccine: WHO position paper – September 2018. *Wkly Epidemiol Rec* 93:457–476.
- Chambers TJ, Hahn CS, Galler R, Rice CM. 1990. Flavivirus genome organization, expression, and replication. *Annu Rev Microbiol* 44: 649–688. <https://doi.org/10.1146/annurev.mi.44.100190.003245>.
- Rico-Hesse R. 1990. Molecular evolution and distribution of dengue viruses type 1 and 2 in nature. *Virology* 174:479–493. [https://doi.org/10.1016/0042-6822\(90\)90102-w](https://doi.org/10.1016/0042-6822(90)90102-w).
- Holmes EC, Twiddy SS. 2003. The origin, emergence and evolutionary genetics of dengue virus. *Infect Genet Evol* 3:19–28. [https://doi.org/10.1016/S1567-1348\(03\)00004-2](https://doi.org/10.1016/S1567-1348(03)00004-2).
- Koonin EV. 1993. Computer-assisted identification of a putative methyltransferase domain in NS5 protein of flaviviruses and lambda 2 protein of reovirus. *J Gen Virol* 74:733–740. <https://doi.org/10.1099/0022-1317-74-4-733>.
- Koonin EV. 1991. The phylogeny of RNA-dependent RNA polymerases of positive-strand RNA viruses. *J Gen Virol* 72:2197–2206. <https://doi.org/10.1099/0022-1317-72-9-2197>.
- Lim SP, Koh JHK, Seh CC, Liew CW, Davidson AD, Chua LS, Chandrasekaran R, Cornvik TC, Shi P-Y, Lescar J. 2013. A crystal structure of the dengue virus non-structural protein 5 (NS5) polymerase delineates interdomain amino acid residues that enhance its thermostability and *de novo* initiation activities. *J Biol Chem* 288:31105–31114. <https://doi.org/10.1074/jbc.M113.508606>.
- Zhao Y, Soh TS, Zheng J, Chan KWK, Phoo WW, Lee CC, Tay MYF, Swaminathan K, Cornvik TC, Lim SP, Shi P-Y, Lescar J, Vasudevan SG, Luo D. 2015. A crystal structure of the dengue virus NS5 protein reveals a novel inter-domain interface essential for protein flexibility and virus replication. *PLoS Pathog* 11:e1004682. <https://doi.org/10.1371/journal.ppat.1004682>.
- Zhao Y, Soh TS, Chan KW, Fung SS, Swaminathan K, Lim SP, Shi PY, Huber T, Lescar J, Luo D, Vasudevan SG. 2015. Flexibility of NS5 methyltransferase-polymerase linker region is essential for dengue virus replication. *J Virol* 89:10717–10721. <https://doi.org/10.1128/JVI.01239-15>.
- El Sahili A, Soh TS, Schiltz J, Gharbi-Ayachi A, Seh CC, Shi PY, Lim SP, Lescar J. 2019. NS5 from dengue virus serotype 2 can adopt a conformation analogous to that of its Zika virus and Japanese encephalitis virus homologues. *J Virol* 94:e01294-19. <https://doi.org/10.1128/JVI.01294-19>.
- Egloff MP, Benarroch D, Selisko B, Romette JL, Canard B. 2002. An RNA cap (nucleoside-2'-O)-methyltransferase in the flavivirus RNA polymerase NS5: crystal structure and functional characterization. *EMBO J* 21: 2757–2768. <https://doi.org/10.1093/emboj/21.11.2757>.
- Ray D, Shah A, Tilgner M, Guo Y, Zhao Y, Dong H, Deas T, Zhou Y, Li H, Shi PY. 2006. West Nile virus 5'-cap structure is formed by sequential guanine N-7 and ribose 2'-O methylations by nonstructural protein 5. *J Virol* 80:8362–8370. <https://doi.org/10.1128/JVI.00814-06>.
- Dong H, Ren S, Zhang B, Zhou Y, Puig-Basagoiti F, Li H, Shi RY. 2008. West Nile virus methyltransferase catalyzes two methylations of the viral RNA cap through a substrate-repositioning mechanism. *J Virol* 82: 4295–4307. <https://doi.org/10.1128/JVI.02202-07>.
- Dong H, Chang DC, Hua MHC, Lim SP, Chionh YH, Hia F, Lee YH, Kukkaro P, Lok SM, Dedon PC, Shi PY. 2012. 2'-O Methylation of internal adenosine by flavivirus NS5 methyltransferase. *PLoS Pathog* 8:e1002642. <https://doi.org/10.1371/journal.ppat.1002642>.
- Tan BH, Fu J, Sugrue RJ, Yap EH, Chan YC, Tan YH. 1996. Recombinant dengue type 1 virus NS5 protein expressed in *Escherichia coli* exhibits RNA-dependent RNA polymerase activity. *Virol* 216:317–325. <https://doi.org/10.1006/viro.1996.0067>.
- Ackermann M, Padmanabhan R. 2001. *De novo* synthesis of RNA by the dengue virus RNA-dependent RNA polymerase exhibits temperature dependence at the initiation but not elongation phase. *J Biol Chem* 276:39926–39937. <https://doi.org/10.1074/jbc.M104248200>.
- Yap TL, Xu T, Chen YL, Malet H, Egloff MP, Canard B, Vasudevan SG, Lescar J. 2007. Crystal structure of the dengue virus RNA-dependent RNA polymerase catalytic domain at 1.85-angstrom resolution. *J Virol* 81: 4753–4765. <https://doi.org/10.1128/JVI.02283-06>.
- Caillet-Saguy C, Lim SP, Shi PY, Lescar J, Bressanelli S. 2014. Polymerases of hepatitis C viruses and flaviviruses: structural and mechanistic insights and drug development. *Antiviral Res* 105:8–16. <https://doi.org/10.1016/j.antiviral.2014.02.006>.

24. El Sahili A, Lescar J. 2017. Dengue virus non-structural protein 5. *Viruses* 9:91. <https://doi.org/10.3390/v9040091>.
25. Lim SP. 2019. Dengue drug discovery: progress, challenges and outlook. *Antiviral Res* 163:156–178. <https://doi.org/10.1016/j.antiviral.2018.12.016>.
26. Lim SP, Noble CG, Shi PY. 2015. The dengue virus NS5 protein as a target for drug discovery. *Antiviral Res* 119:57–67. <https://doi.org/10.1016/j.antiviral.2015.04.010>.
27. Chen YL, Yokokawa F, Shi PY. 2015. The search for nucleoside/nucleotide analog inhibitors of dengue virus. *Antiviral Res* 122:12–19. <https://doi.org/10.1016/j.antiviral.2015.07.010>.
28. Lim SP, Wang QY, Noble CG, Chen YL, Dong H, Zou B, Yokokawa F, Nilar S, Smith P, Beer D, Lescar J, Shi PY. 2013. Ten years of dengue drug discovery: progress and prospects. *Antiviral Res* 100:500–519. <https://doi.org/10.1016/j.antiviral.2013.09.013>.
29. Noble CG, Chen YL, Dong H, Gu F, Lim SP, Schul W, Wang QY, Shi PY. 2010. Strategies for development of dengue virus inhibitors. *Antiviral Res* 85:450–462. <https://doi.org/10.1016/j.antiviral.2009.12.011>.
30. Smith TM, Lim SP, Yue K, Busby SA, Arora R, Seh CC, Wright SK, Nutiu R, Niyomrattanakit P, Wan KF, Beer D, Shi PY, Benson TE. 2015. Identifying initiation and elongation inhibitors of dengue virus RNA polymerase in a high-throughput lead-finding campaign. *J Biomol Screen* 20:153–163. <https://doi.org/10.1177/1087057114551141>.
31. Niyomrattanakit P, Wan KF, Chung KY, Abas SN, Seh CC, Dong H, Lim CC, Chao AT, Lee CB, Nilar S, Lescar J, Shi PY, Beer D, Lim SP. 2015. Stabilization of dengue virus polymerase in *de novo* initiation assay provides advantages for compound screening. *Antiviral Res* 119:36–46. <https://doi.org/10.1016/j.antiviral.2015.04.007>.
32. Niyomrattanakit P, Abas SN, Lim CC, Beer D, Shi PY, Chen YL. 2011. A fluorescence-based alkaline phosphatase-coupled polymerase assay for identification of inhibitors of dengue virus RNA-dependent RNA polymerase. *J Biomol Screen* 16:201–210. <https://doi.org/10.1177/1087057110389323>.
33. Noble CG, Lim SP, Chen YL, Liew CW, Yap L, Lescar J, Shi PY. 2013. Conformational flexibility of the dengue virus RNA-dependent RNA polymerase revealed by a complex with an inhibitor. *J Virol* 87: 5291–5295. <https://doi.org/10.1128/JVI.00045-13>.
34. Niyomrattanakit P, Chen YL, Dong H, Yin Z, Qing M, Glickman JF, Lin K, Mueller D, Voshol H, Lim JY, Nilar S, Keller TH, Shi PY. 2010. Inhibition of dengue virus polymerase by blocking of the RNA tunnel. *J Virol* 84: 5678–5686. <https://doi.org/10.1128/JVI.02451-09>.
35. Yin Z, Chen YL, Kondreddi RR, Chan WL, Wang G, Ng RH, Lim JY, Lee WY, Jeyaraj DA, Niyomrattanakit P, Wen D, Chao A, Glickman JF, Voshol H, Mueller D, Spanka C, Dressler S, Nilar S, Vasudevan SG, Shi PY, Keller TH. 2009. N-sulfonylanthranilic acid derivatives as allosteric inhibitors of dengue viral RNA-dependent RNA polymerase. *J Med Chem* 52: 7934–7937. <https://doi.org/10.1021/jm901044z>.
36. Noble CG, Lim SP, Arora R, Yokokawa F, Nilar S, Seh CC, Wright SK, Benson TE, Smith PW, Shi PY. 2016. A conserved pocket in the dengue virus polymerase identified through fragment-based screening. *J Biol Chem* 291:8541–8548. <https://doi.org/10.1074/jbc.M115.710731>.
37. Lim SP, Noble CG, Seh CC, Soh TS, El Sahili A, Chan GK, Lescar J, Arora R, Benson T, Nilar S, Manjunatha U, Wan KF, Dong H, Xie X, Shi PY, Yokokawa F. 2016. Potent allosteric dengue virus NS5 polymerase inhibitors: mechanism of action and resistance profiling. *PLoS Pathog* 12:e1005737. <https://doi.org/10.1371/journal.ppat.1005737>.
38. Siragy HM. 2002. Angiotensin receptor blockers: how important is selectivity? *Am J Hypertens* 15:1006–1014. [https://doi.org/10.1016/s0895-7061\(02\)02280-x](https://doi.org/10.1016/s0895-7061(02)02280-x).
39. Pryor MJ, Rawlinson SM, Butcher RE, Barton CL, Waterhouse TA, Vasudevan SG, Bardin PG, Wright PJ, Jans DA, Davidson AD. 2007. Nuclear localization of dengue virus nonstructural protein 5 through its importin alpha/beta-recognized nuclear localization sequences is integral to viral infection. *Traffic* 8:795–807. <https://doi.org/10.1111/j.1600-0854.2007.00579.x>.
40. Hannemann H, Sung PY, Chiu HC, Yousuf A, Bird J, Lim SP, Davidson AD. 2013. Serotype-specific differences in dengue virus non-structural protein 5 nuclear localization. *J Biol Chem* 288:22621–22635. <https://doi.org/10.1074/jbc.M113.481382>.
41. Butcher SJ, Grimes JM, Makeyev EV, Bamford DH, Stuart DI. 2001. A mechanism for initiating RNA-dependent RNA polymerization. *Nature* 410:235–240. <https://doi.org/10.1038/35065653>.
42. Lesburg CA, Cable MB, Ferrari E, Hong Z, Mannarino AF, Weber PC. 1999. Crystal structure of the RNA-dependent RNA polymerase from hepatitis C virus reveals a fully encircled active site. *Nat Struct Biol* 6:937–943. <https://doi.org/10.1038/13305>.
43. Iglesias NG, Filomatori CV, Gamarnik AV. 2011. The F1 motif of dengue virus polymerase NS5 is involved in promoter-dependent RNA synthesis. *J Virol* 85:5745–5756. <https://doi.org/10.1128/JVI.02343-10>.
44. Poch O, Sauvaget I, Delarue M, Tordo N. 1989. Identification of four conserved motifs among the RNA-dependent polymerase encoding elements. *EMBO J* 8:3867–3874. <https://doi.org/10.1002/j.1460-2075.1989.tb08565.x>.
45. Gohara DW, Crotty S, Arnold JJ, Yoder JD, Andino R, Cameron CE. 2000. Poliovirus RNA-dependent RNA polymerase (3Dpol): structural, biochemical, and biological analysis of conserved structural motifs A and B. *J Biol Chem* 275:25523–25532. <https://doi.org/10.1074/jbc.M002671200>.
46. Bressanelli S, Tomei L, Rey FA, De Francesco R. 2002. Structural analysis of the hepatitis C virus RNA polymerase in complex with ribonucleotides. *J Virol* 76:3482–3492. <https://doi.org/10.1128/jvi.76.7.3482-3492.2002>.
47. Selisko B, Potisopon S, Agred R, Priet S, Varlet I, Thillier Y, Sallamand C, Debart F, Vasseur JJ, Canard B. 2012. Molecular basis for nucleotide conservation at the ends of the dengue virus genome. *PLoS Pathog* 8:e1002912. <https://doi.org/10.1371/journal.ppat.1002912>.
48. Wu J, Ye H-Q, Zhang Q-Y, Lu G, Bo Zhang LB, Gong P. 2020. A conformation-based intra-molecular initiation factor identified in the flavivirus RNA-dependent RNA polymerase. *PLoS Pathog* 16:e1008484. <https://doi.org/10.1371/journal.ppat.1008484>.
49. Tarantino D, Cannalire R, Mastrangelo E, Croci R, Querat G, Barreca ML, Bolognesi M, Manfroni G, Cecchetti V, Milani M. 2016. Targeting flavivirus RNA dependent RNA polymerase through a pyridobenzothiazole inhibitor. *Antiviral Res* 134:226–235. <https://doi.org/10.1016/j.antiviral.2016.09.007>.
50. Shimizu H, Saito A, Mikuni J, Nakayama EE, Koyama H, Honma T, Shirouzu M, Sekine SI, Shioda T. 2019. Discovery of a small molecule inhibitor targeting dengue virus NS5 RNA-dependent RNA polymerase. *PLoS Negl Trop Dis* 13:e0007894. <https://doi.org/10.1371/journal.pntd.0007894>.
51. Ferrero DS, Ruiz-Arroyo VM, Soler N, Usón I, Guarné A, Verdaguer N. 2019. Supramolecular arrangement of the full-length Zika virus NS5. *PLoS Pathog* 15:e1007656. <https://doi.org/10.1371/journal.ppat.1007656>.
52. Lu G, Gong P. 2013. Crystal structure of the full-length Japanese encephalitis virus NS5 reveals a conserved methyltransferase-polymerase interface. *PLoS Pathog* 9:e1003549. <https://doi.org/10.1371/journal.ppat.1003549>.
53. Vonnrhein C, Flensburg C, Keller P, Sharff A, Smart O, Paciorek W, Womack T, Bricogne G. 2011. Data processing and analysis with the autoPROC toolbox. *Acta Crystallogr D Biol Crystallogr* 67:293–302. <https://doi.org/10.1107/S0907444911007773>.
54. Bricogne G, Blanc E, Brandl M, Flensburg C, Keller P, Paciorek W, Roversi P, Sharff A, Smart O, Vonnrhein C, Womack TO. 2013. BUSTER version 2.11.2. Global Phasing Ltd., Cambridge, United Kingdom.
55. Zou G, Chen YL, Dong H, Lim CC, Yap LJ, Yau YH, Shochat SG, Lescar J, Shi PY. 2011. Functional analysis of two cavities in flavivirus NS5 polymerase. *J Biol Chem* 286:14362–14372. <https://doi.org/10.1074/jbc.M110.214189>.