The flavivirus methyltransferase (MTase) sequentially methylates the N7 and 2'-O positions of the viral RNA cap (GpppA-RNA→m7GpppA-RNA→m7GpppAm-RNA), using S-adenosyl-L-methionine (SAM) as a methyl donor. We report here that sinefungin (SIN), a SAM analog, inhibits several flaviviruses through suppression of viral MTase. The crystal structure of West Nile virus MTase in complex with SIN inhibitor at 2.0 Å resolution revealed a flavivirus-conserved hydrophobic pocket located next to the SAM-binding site. The pocket is functionally critical in the viral replication and cap methylations. In addition, the N7 methylation efficiency was found to correlate with the viral replication ability. Thus, SIN analogs with modifications that interact with the hydrophobic pocket are potential specific inhibitors of flavivirus MTase.

The genus Flavivirus in the family Flaviviridae is composed of more than 70 viruses (1). Many flaviviruses are arthropod-borne and cause significant human disease. The four serotypes of Dengue virus (DENV), yellow fever virus (YFV), West Nile virus (WNV), Japanese encephalitis virus (JEV), and tick-borne encephalitis virus (TBEV) are categorized as global emerging pathogens and are also NIAID Priority Pathogens (2). The World Health Organization has estimated annual human cases of more than 50 million, 200,000, and 50,000 for DENV (3), YFV (4), and JEV (5), respectively. Approximately 2.5 billion people are at risk of DENV infection (3). Since 1999, WNV has spread rapidly throughout the Western Hemisphere (6). Vaccines for humans are currently available only for YFV, JEV, and TBEV (2). No clinically approved antiviral therapy is available for treatment of flavivirus infections. Therefore, the development of vaccines and antiviral agents for prevention and treatment of flavivirus infections is a clear public health priority (6).
The flavivirus genomic RNA is single-stranded and of positive (i.e., mRNA) polarity. A type I cap (see details below) is present at the 5' end, followed by the conserved dinucleotide sequence 5'-AG-3' (m^7GpppAmG) (7). The 3' end of the genome terminates with 5'-CUOH-3' rather than with a poly(A) tract (8). The viral genome is approximately 11 kb in length, consisting of a 5' UTR, a single long open reading frame (ORF), and a 3' UTR (9). The single ORF encodes a polyprotein that is co- and post-translationally processed by viral and cellular proteases into three structural proteins (capsid [C], premembrane [prM] or membrane [M], and envelope [E]), and seven nonstructural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5) (10). The nonstructural proteins are assumed to be involved primarily in the replication of viral RNA, as components of a replicase complex. Among the NS proteins, NS3 is a multi-functional protein with activities of a serine protease, an RNA triphosphatase (RTPase), a nucleoside triphosphatase (NTPase), and a helicase (11-13); NS5 has the functions of an RNA-dependent RNA polymerase (RdRp) (14-16) and a MTase involved in methylation of the 5' RNA cap structure (17-20).

Most eukaryotic and viral mRNAs possess a 5'-cap that is important for mRNA stability and efficient translation (21). Based on what is known about cap formation for cellular mRNA (22,23), four enzymatic modifications are involved in the formation of the type 1 cap structure. (i) The 5' triphosphate of nascent pre-mRNA is hydrolyzed to a 5' diphosphate by an RNA triphosphatase; (ii) the diphosphate RNA end is capped with GMP by an RNA guanylyltransferase; (iii) the GpppN cap is methylated at the N7 position of guanine by an RNA guanine methyltransferase (N7 MTase), resulting in type 0 cap (m^7GpppN) (24); and (iv) the first and second nucleotides of many cellular and viral mRNAs are further methylated at the ribose 2'-OH position by a nucleoside 2'-O MTase, to form type 1 cap (m^7GpppNm) and type 2 cap (m^7GpppNmNm) structures, respectively (21). Both N7 and 2'-O MTases use S-adenosyl-L-methionine (SAM) as a methyl donor and generate S-adenosyl-L-homocysteine (SAH) as a by-product.

Since flaviviruses replicate in the host cytoplasm, they are assumed to encode their own capping enzymes, rather than using the host’s capping apparatus that is located in the nucleus. Among the four enzymes required for flavivirus m^7GpppAm-cap formation, the RNA triphosphatase has been mapped to NS3 (12,13). Recent studies suggest that the N-terminal methyltransferase (MTase) domain of NS5 has a guanylyltransferase activity, transferring the GMP moiety of GTP to the 5' end of ppA-RNA, resulting in GpppA-RNA (25,26). We, and others, have shown that recombinant NS5 proteins from various flaviviruses possess both N7 and 2'-O MTase activities (18-20,27-29). Compared with cellular and many other viral MTases, flavivirus MTase is unique, in that a single MTase domain catalyzes two methylation events, in the order of GpppA→m^7GpppA→m^7GpppAm (19,20). Additionally, both cap methylations are dependent on viral RNA sequence (27). Despite these two distinct methylation activities, the crystal structure of flavivirus methyltransferase exhibits only a single SAM-binding site as methyl donor (18,30-34).

Viruses represent an attractive system for the study of RNA capping. The capping mechanisms of some viral mRNAs are different from those seen for the cellular mRNA (21). The differences between host and viral cap formation could potentially be used for development of antiviral therapy. Flavivirus MTase has been shown to be
essential for WNV (20,27), KUNV (35), YFV (36), and DENV replication (37). Furthermore, sinefungin (SIN), a SAM analog, was shown to inhibit both N-7 and 2’-O methylations of WNV MTase, as well as to inhibit WNV replication in cell culture (28). Those results suggest that SIN analogs have the potential to be developed for flavivirus therapy. However, since SAM is also a methyl donor for host RNA and protein methylations, its analogs would nonspecifically suppress host MTases, resulting in toxicity. Therefore, identification of features unique to the flaviviral MTase is critical for the design of specific inhibitors of viral MTase.

Here we extend our previous studies to show that SIN can not only inhibit WNV replication, but it can also inhibit the replications of several other flaviviruses, i.e., DENV and YFV. We have also determined the co-crystal structure of the WNV MTase-SIN complex at 2.0Å resolution. The crystal structure of the WNV MTase -SIN complex reveals the presence of a hydrophobic pocket extension of the SAM-binding pocket; this feature is conserved among various flaviviruses. Point mutations of conserved residues within the pocket impair, to various extents, either one or both of the N7 and 2’-O methylation activities. We further show that residues within the pocket are important in viral replication, and that the N7 methylation efficiency correlates with the viral replication ability. Thus, SIN analogs with modifications that interact with the hydrophobic pocket are potential specific inhibitors of flavivirus methyltransferase.

**EXPERIMENTAL PROCEDURES**

**Cloning, expression, and purification of the WNV, WNV mutant, YFV, and DENV-2 MTase**—The WNV MTase domain containing the N-terminal 300 amino acids of NS5 was prepared for co-crystallization and enzyme assays as described previously (19,20). A QuickChange II XL site-directed mutagenesis kit (Stratagene) was used to engineer the MTase mutants. A standard overlapping PCR-based and the WNV replicon that we developed previously (38).

DNA fragments representing the N-terminal 272 amino acids of DENV-2 were RT-PCR-amplified from the DENV-2 genomic RNA (NGC strain) and cloned into plasmid pET26b(+) (Novagen) at Nde I and Xho I sites. The DENV-2 MTase containing a C-terminal (His)6-tag was expressed and purified through a Ni-NTA column followed by a gel-filtration 16/60 Superdex column (Amersham).

**Crystallization, X-ray data collection, structure determination and refinement**—Co-crystals of the MTase-SIN complex were grown by methods described previously (20), except in the presence of 5 mM SIN. The co-crystals belong to space group $P1$, and are isomorphous to the native crystals that we grew previously (20) (Table 1). Prior to data collection, all crystals were transferred to a reservoir solution containing 25% glycerol, and then flashed-cooled under a nitrogen stream at 100K, and stored in liquid nitrogen. Diffraction data were collected to 2.0Å resolution at 100K at beamline X4A of the National Synchrotron Light Source (NSLS) (Brookhaven National Laboratory). All of the data were processed and scaled using HKL2000 (39) (Table 1).

The structure of the MTase-SIN complex was determined by the difference Fourier method, with the native structure of the WNV MTase (PDB code: 2OY0) used as a starting model (20). Structural refinement was carried out using CNS (40) (Table 1). At 2.8Å resolution, the final $R_{cryst}$ is 23%, with an $R_{free}$ of 26.9%. The final refinement statistics are summarized in Table 1.
In vitro MTase inhibition assay—The N7 and 2'-O methylation inhibition assays were performed as described previously for WNV (28,41), except that the 5'-end-labeled substrates G*pppA-RNA and m7G*pppA-RNA, representing the first 211 nucleotides of the DENV-2 genome (the asterisk indicates that the following phosphate is 32P labeled), were used. The N7 methylation was measured by conversion of G*pppA-RNA→m7G*pppA-RNA. The 2'-O methylation was monitored by conversion of m7G*pppA-RNA→m7G*pppAm-RNA. Both methylation assays were performed with 0.25 µg DENV-2 MTase, 25 µM SAM, and various concentrations of SIN. The methylation reactions were digested with nuclease P1 to release cap moieties (m7G*pppAm, m7G*pppA, and G*pppA). The cap molecules were separated on a thin-layer chromatograph (TLC), and quantified by a PhosphorImager (28,41).

Inhibition of virus replication by SIN—The assay for inhibition of DENV-2 and YFV by SIN was performed as described previously for WNV (28). BHK cells were infected with DENV-2 (NGC strain) or YFV (17D strain) at an MOI of 0.3 for 1 h at 37°C. The viral inocula were washed and replaced with DMEM plus 10% FBS containing various concentrations of SIN. The viral titers were determined by plaque assay (42). For the cytotoxicity assay, BHK cells were incubated with various concentrations of SIN and assayed for cell viability at 48 h post-infection using an MTT assay kit (American Type Culture Collection).

Replicon assay—Transient replication of wild-type (WT) and mutant WNV was performed using a replicon assay as described previously (43). Briefly, mutant replicons (G148A, R160A, R163A, V164A, and L184A) were prepared using a standard PCR-mediated mutagenesis method. Equal amounts of RNA (2 µg) for each replicon were electroporated into BHK cells. The transfected cells were resuspended in 25 ml of DMEM with 10% FBS; 1 ml of cell suspension was seeded into each well of a 12-well plate. Luciferase activity was measured at various time points. The protocols for in vitro RNA transcription, RNA transfection, and luciferase assays were reported previously (43).

RESULTS

Inhibition of flavivirus replication by SIN—SIN differs from SAM in that the S-CH3 sulfonium moiety is replaced with a C-NH2 amine (Fig. 1A). To examine whether SIN inhibits flaviviruses other than WNV, we performed viral titer reduction assays using DENV-2 and YFV. We found that SIN inhibits DENV-2 and YFV in a dose-responsive manner, with values for the EC50 (the concentration at which 50% of virus is inhibited) of 77 µM and 250 µM, respectively (Fig. 1B). Interestingly, DENV-2 was more sensitive to SIN inhibition than was YFV; at 250 µM, SIN reduced DENV-2 and YFV titers by about 6-fold and 50%, respectively (Fig. 1B). Interestingly, DENV-2 was more sensitive to SIN inhibition than was YFV; at 250 µM, SIN reduced DENV-2 and YFV titers by about 6-fold and 50%, respectively (Fig. 1B). A cell proliferation-based MTT assay (42) showed no cytotoxicity when uninfected cells were treated with SIN at concentrations up to 300 µM, indicating that the observed antiviral activity was not due to compound-mediated cytotoxicity (Fig. 1C).

SIN directly inhibits flavivirus MTase activities—To demonstrate that the observed antiviral activity of SIN was due to direct inhibition of the viral MTase, we expressed and purified the DENV-2 MTase domain (N-terminal 272 amino acids of NS5; Fig. 1D, left panel). The recombinant MTase was assayed for methylations in the presence or absence of SIN. The conversion of G*pppA-RNA→m7G*pppA-RNA [the asterisk indicates that the following phosphate is 32P labeled; the RNA represents the first 211
nucleotides of the DENV-2 genomic RNA] was measured for N7 methylation. The conversion of m7G*pppA-RNA → m7G*pppAm-RNA was measured for 2'-O methylation. As shown in Fig. 1D (middle and right panels), SIN inhibited both N7 and 2'-O methylations of DENV-2 MTase in a dose-responsive manner, with values for IC50 (the concentration at which 50% methylation activity is inhibited) of 0.7 µM and 19 µM, respectively. The difference in IC50 values suggests that, during the two methylation reactions, SAM is bound to MTase in two distinct conformations. Alternatively, it may suggest that the inhibitor affects how the distinct moieties (the base and the ribose) of the 5' end of the RNA substrates interact with the active site during the two methylation reactions. Nevertheless, the above results, together with our previous WNV data (28), demonstrate that SIN inhibits multiple members of flaviviruses through suppression of the viral MTase.

Crystal structure of WNV MTase in complex with SIN—Since SIN inhibits flavivirus replication, structural information about the MTase-SIN complex will be valuable to guide rational design of new inhibitors of flavivirus MTase. We determined the crystal structure of the WNV NS5 MTase domain in complex with SIN at 2.0 Å resolution (Table 1 and Fig. 2), with a final Rcryst of 23% and an Rfree of 26.9%.

In the final model, each chain of the MTase homodimer contains 262 amino acids and each molecule binds one SIN molecule (Fig. 2A). Although the completeness of diffraction data is less than ideal, the high resolution, 2.0 Å, conferred a good accuracy to the structural model of the WNV MTase, allowing the correct tracing of a fragment (amino acids 172-177) that had been poorly defined in the MTase-SAH complex structure at 2.8 Å resolution (Fig. 2B) (20). It should be noted that this loop is spatially distant from the active site of the MTase, as discussed previously (20). Therefore, the conformational discrepancy of this loop does not affect our original interpretation of the MTase function (20,28,29).

Structural comparison between the MTase-SIN and MTase-SAH complexes—The overall structure of the WNV MTase-SIN complex (Fig. 2B, C) is nearly identical to that of the MTase-SAH complex that was determined previously (20). The comparison yields an overall Cα root-mean-square deviation (rmsd) of 0.34 Å. No significant conformational change occurs upon replacement of SAH with SIN. In the SAH/SAM/SIN binding pocket, all of the residues superimpose very well (data not shown). The only significant conformational difference occurs for the side-chain of a nearby residue R163; both conformations are confirmed by clear electron densities in their respective structures. R163 is one of the important residues forming the extended flavivirus-conserved pocket (see below); mutation of R163 resulted in 50% reduction of the N7 methylation activity (see below). Nevertheless, the conformational differences seen for this residue may not be related to the SAH/SIN occupancy, since R163 is over 9 Å from the adenine base of SAH/SIN (Fig. 2D). Instead, given that R163 is surface-exposed, the difference may simply reflect a degree of conformational flexibility of surface residues.

SIN binds to the SAM pocket of the MTase in a conformation similar to that of SAH in the MTase-SAH complex. The overall rmsd for all SIN and SAH atoms is about 0.47 Å, which is slightly higher than the overall Cα rmsd of the MTase molecules in the two structures. In general, SIN binds more deeply in the pocket than SAH does (Fig. 2B). In the MTase-SIN complex, the free amine Nε of the C-NH2 group, i.e., the
group that replaces the S-CH₃ group of SAM, points outward from the pocket. Interestingly, the free amine NE of SIN is only 3.7 Å-3.9 Å away from the carboxyl side-chain atom OD₁ of D146 (Fig. 2D). D146 was previously shown to be essential for both N7 and 2'-O methylation activities of the WNV MTase (20). It is reasonable to postulate that in a MTase-SAM complex, the CH₃ donor group of SAM will be at a position similar to that of SIN NE. The proximity of the donor methyl group of SAM and the D146 side chain provides structural evidence for a potential catalytic role of D146 in the N-7 MTase function. Nevertheless, the conformational similarity between the MTase/SAH and MTase/SIN complexes is consistent with the fact that SIN and SAM/SAH do not have significant structural differences.

An additional pocket near the adenosine base of SIN—Upon examination of the crystal structures of the WNV MTase-SIN and MTase-SA H complexes (20), we observed an additional pocket which is an extension of the cavity holding the adenine base of SAM or the analogs (SAH or SIN) (Fig. 2C). The extended pocket is nearly square-shaped with approximate dimensions of 8 Å × 9 Å, and ~ 5 Å deep. The additional pocket and the SAM-binding pocket together form a large continuous pocket.

The nature of the extended pocket is mainly hydrophobic, with a deep hole in the bottom along the wall away from the adenine base (Fig. 2E, left panel). That side of the pocket is occupied with six ordered water molecules (only five are visible, the sixth is deeply trapped inside the hole) that form hydrogen bonds with the main-chain and side-chain atoms of residues forming the pocket. Four of the water molecules are located above the pocket (i.e., out in the solvent), forming hydrogen bonds with R163 side-chain atom NH₁, and with E149 N and R160 O. Interestingly, these four water molecules, which form hydrogen bonds with one another, are organized in such a way that they could be connected to form a ring structure as shown by a continuous electron density in the map (map not shown). The other two water molecules are trapped inside the deep hole (one visible, one deeply buried), forming hydrogen bonds with main-chain atoms (I147 O, I147 N, K182 O, and L184 N) (data not shown).

Further analysis indicated that a similar pocket exists in all known structures of flavivirus MTases (18,20,30-34,44). Fig. 2E shows the surface of the additional pocket of MTases of representative flaviviruses DENV-2 (18) and YFV (33) (middle and right panels), although the deep hole is not seen in the latter two structures. These results indicate that the additional pocket is a general feature for flavivirus MTases. In addition, upon examination of known structures of SAM-utilizing proteins (over 100 structures available), we found that the extended pocket is specific to the flaviviruses, as it has not been seen in structures of other SAM-utilizing proteins. Figure 2F shows the surface representation of the SAM-binding region of several representative human SAM-utilizing proteins, including SET-like histone lysine MTase (PDB: 3FPD) (45), RNA MTase domain of human TARBP1 (2HA8) (46), small molecule catechol-O-MTase domain containing 1 (2AVD) (47), and dimethyladenosine transferase (1ZQ9) (48). Clearly, none of these SAM-utilizing proteins present the extended pocket as seen for the flavivirus MTases.

The extended pocket is formed by residues F133, I147, G148, E149, R160, R163, V164, and L184 of the WNV MTase (Fig. 2D). The long side chain of R163 and part of F133 form the steep wall of one side of the pocket, whereas the opposite side is lined up by the long side chains of R160 and
Residues I147 and V164 together with part of F133 form the floor of the pocket. L184 is located inside the deep hole of the pocket. G148 stabilizes the conformation of E149, a residue critical for the N7 methylation (19,28).

It should be noted that the SAM-binding pocket and the additional pocket together form one continuous pocket. Several residues such as F133, I147 and E149 are shared by the two portions of the pocket and they participate in SAM-binding, whereas others are associated only with either the extension portion or the SAM-binding portion. Among these residues, K105, H110, and D131 form the steep wall side of the SAM-binding pocket to hold the adenine base of the SAM/SAH/SIN cofactor/by-product/inhibitor; these three residues are not part of the additional pocket. Within this additional pocket, residues G148, R160, R163, V164, and L184 do not interact directly with the inhibitor SIN or co-factor/by-product SAM/SAH (Fig. 2D).

**Residues that form the additional pocket are conserved**—Sequence alignment of flavivirus MTases indicated that residues at positions 133, 147, 148, 149, 160, 163, and 164, and 184 of the WNV MTase are conserved among flaviviruses (Table 2, supplemental Figs. 1 and 2) (20,34). Among these residues, G148 and E149 are completely conserved. Of 61 flaviviruses with known sequences, residues I147, R160, and L184 are nearly completely conserved, with only one or two conservative (one exception) variations for each residue (Meaban virus (V147), Rio Bravo virus (K160), Tamana bat virus (G160, M184), and Kedougou virus (F184)). V164 is conserved in more than 92% of flavivirus MTases, with remaining ones (five out of 61) showing a conserved substitution of Ile at this position. R/K163 is present in over 95% of flavivirus MTases, with three viruses (Rio Bravo virus (RBV), Modoc virus (MODV), Apoi virus (APOIV)) showing a Glu, Thr, or Gln at this position, respectively. The majority (37 out of 56, 61%) of flavivirus MTase sequences show a Phe at position 133; other bulky residues such as His and Tyr are seen for a few flaviviruses (about 23%) at this position.

Crystal contact analysis of the WNV MTase structure indicated that as many as 20 residues are within 4Å distance to contact the bound SAH molecule (Supplemental Fig. 1). These residues are S56, G58, G81, C82, G83, G85, G86, W87, T104, K105, G106, H110, E111, V130, D131, V132, F133, D146, I147, and E149. With the exception of three residues, K105 (30% conserved), V130 (40%), and F133 (61%), all of the residues are well conserved (including eight invariant ones) among flavivirus MTases (Supplemental Fig. 1). Therefore, the SAM/SAH-binding pocket, together with the pocket extension described above, is highly conserved (Supplemental Figs. 1 and 2).

We next investigated whether the sequence conservation described above exists for other SAM-utilizing proteins. Structure-based sequence alignment indicated that the sequence conservation of these residues only occurs among flavivirus MTases, but not among other SAM-dependent MTases, nor, on a broader scale, among other SAM-utilizing proteins (data not shown, and (18)). Consistently, as we discussed above, the additional pocket adjacent to the SAM-binding pocket is not observed in the structures of other SAM-utilizing proteins. Therefore, the flavivirus-conserved additional pocket is a unique feature of flavivirus MTases.

**Distinct effects of the additional pocket on the WNV N7 and 2'-O methylations**—We performed structure-based mutagenesis to explore the function of the additional
A panel of five mutants, each with an Ala substitution for one of the conserved residues in the pocket, were prepared and assayed for cap methylations, as previously described (19). Fig. 3A depicts the locations of the mutated residues: G148, R160, R163, V164, and L184. SDS-PAGE analysis showed that the recombinant wild-type (WT) and mutant MTases (N-terminal 300 amino acids of NS5) had similar purities, of > 90% (Fig. 4B). Different mutant MTases exhibited distinct effects on the N7 (Fig. 3C) and 2’-O methylation activities (Fig. 3D). Based on the methylation results, the five mutants could be categorized into three groups: the Group I mutant (G148A) was defective in both N7 and 2’-O methylation; the Group II mutants (R160A and R163A) were defective in N7 methylation, but not in 2’-O methylation; and the Group III mutants (V164A and L184A) were selectively defective in 2’-O methylation, but not N7 methylation.

Previous studies have established functional significance of conserved residues within the SAM-binding pocket (19,20,28,37). Those studies showed that mutations of residues predicted to be involved in SAM-binding (such as S56, G81, G83, G85, W87, K105, H110, E111, D131, I147, D146, and E149) reduced, and in some cases abolished, either the N7 or the 2’-O methylation, or both. Except K105, all of these residues are well conserved among flavivirus MTases (Supplemental Fig. 1). Together with our results in this manuscript, these results indicated the functional significance of residues within the SAM-binding pocket and the pocket extension described above.

**Effects of the additional pocket on WNV replication**—To validate the biological relevance of the identified pocket, we performed mutagenesis using a luciferase-reporting replicon of WNV (38). The replicon contained a luciferase reporter in-frame fused with the viral ORF, in the position where structural genes were deleted (Fig. 4A) (38). Five mutant replicons were prepared, each with an Ala-substitution within the MTase hydrophobic pocket (Fig. 4B). For every mutant, two of the three nucleosides within the codon were changed to avoid potential reversion (Fig. 4B). The transfection experiments showed comparable luciferase activities at 2 h post-transfection (p.t.; indicative of input replicon translation) for all replicons, suggesting that transfection efficiencies were similar. In contrast, luciferase activities at ≥ 24 h p.t. (indicative of RNA synthesis) were dramatically different among the various replicons (Fig. 4B). Based on the luciferase profile, the replication kinetics of replicons could be ranked as WT ≥ V164A > L184A > R163A > G148A > R160A. The luciferase curve for replicon R160A was identical to that of a non-replicative replicon (containing mutations in the polymerase GDD active site; data not shown). However, at 96 h p.t., the luciferase levels from mutants V164A, L184A, and R163A were comparable to that from the WT replicon (Fig. 4B). To exclude the possibility that the late luciferase signals were due to reversion of the engineered mutations to the WT sequence, we sequenced the replicative RNAs recovered from the transfected cells at 96 h p.t. The sequencing results showed that the engineered mutations were indeed retained in the replicative RNAs (Fig. 4C), and that no second site mutation was present in the NS5 gene (data not shown). The replication delay of mutant replicons could be due to the low methylation activity of MTase, and an accumulated level of mutant NS5 protein is required to efficiently methylate the viral RNA cap to assume viral replication. Overall, the results have demonstrated that the hydrophobic pocket is important for WNV replication.

**DISCUSSION**
Many flaviviruses cause significant human disease. However, currently no clinically approved antiviral therapy is available for treatment of flavivirus-associated diseases. Given that we previously found that the N-7 methylation activity is essential for the WNV life cycle, the viral methyltransferase represents a novel target for flavivirus therapy (19,20,28,29,49).

SIN was previously shown to inhibit WNV in cell culture with EC$_{50}$ and CC$_{50}$ values of 23 µM and 4.5 mM, respectively (28). In the present study, we have demonstrated that SIN has antiviral activity in two other flaviviruses (Fig. 1). Due to the high similarity between SAM and SIN, toxicity is a major concern for the development of SIN as a therapeutic agent, since SIN could inhibit many SAM-utilizing enzymes of the host. To explore the potential for modification of the inhibitor, we determined the crystal structure of the WNV MTase in complex with SIN. Analysis of the SIN-MTase structure revealed an additional pocket that is continuous with the SAM-binding pocket. Re-visit of the structure of the WNV MTase-SAH complex determined previously (20) and examinations of the structures of MTases from several other flaviviruses indicated that this additional pocket is conserved among flavivirus MTases with known structures. In addition, sequence alignment indicated that the residues forming the additional (extended) pocket are conserved among flavivirus MTases. Furthermore, the sequence and structure conservations of this additional pocket are not seen among other SAM-utilizing proteins. The recognition that the SAM-binding pocket has an extended and flavivirus-conserved pocket beyond the SAM adenosine base has provided us with a unique opportunity to use SIN as a lead compound for drug development. We noticed that within the extended pocket, four ordered water molecules form a ring-like structure (Fig. 2E). Therefore, it may be possible to modify the adenine base of SIN with a covalently bound ring structure. Indeed, a docking analysis indicated that the additional pocket can easily accommodate a cyclopentanediol ring next to the adenine base of SIN (data not shown). SIN analogs with added moieties that interact with the additional pocket are expected to show enhanced binding affinities, leading to more potent and specific inhibitors of flavivirus MTase.

Using both enzymatic and replicon-based approaches, we performed mutagenesis to examine the function of the additional pocket. Five residues within the pocket were individually mutated to Ala; the effects of the mutations on N7 and 2'-O methylations, as well as on viral replication, were examined. As summarized in Fig. 4D, mutations with dramatic negative effect on N7 methylation (e.g., G148A and R160A) significantly impaired viral replication. We further note that residues G148, E149, and R160, all of which are critical for N7 methylation, form a hydrogen-bonding network (Fig. 2D). The G148 O hydrogen bonds to S150 N, whereas R160 NH1 atom forms two hydrogen bonds with the carboxyl oxygen atoms of residues E149 and S150. Therefore, the maintenance of the local conformation around E149 is critical for the viral MTase function.

In contrast to the above-noted mutations which indicated essential role of N7 methylation activity in viral replication, mutations that have a dramatic effect on 2'-O methylation did not significantly affect replicon replication. The correlation between the N7 methylation activity and viral replication is supported by previous mutagenesis results, for other regions of the WNV MTase (Fig. 4D). The overall results strongly suggest that, when MTase

Figures and data not shown.
inhibitors are being developed, emphasis should be placed on compounds that can achieve inhibition of N7 methylation activity. However, we note that such a correlation between N7 methylation and viral replication has so far only been observed in WNV; experiments are needed to test whether this correlation can be extended to other flaviviruses.

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The atomic coordinates and structure factors (codes 3LKZ) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).
References


Figure legends

FIG. 1. Inhibition of flavivirus replication through suppression of viral MTase by SIN. (A) Chemical structures of SAM and SIN. (B) Inhibition of DENV-2 and YFV by SIN. Data represent mean values and standard deviations (n \( \geq 3 \)). (C) Cytotoxicity of SIN. (D) Inhibition of N7 and 2’-O methylations of DENV-2 MTase by SIN. Left panel: SDS-PAGE analysis of recombinant DENV-2 MTase. Molecular masses of protein markers are labeled. Right panel: SIN-mediated inhibitions of N7 and 2’-O methylations of DENV-2 MTase were analyzed on TLC plates. The N7 methylation was measured by conversion of G*pppA-RNA\( \rightarrow \)m\(^7\)G*pppA-RNA (the asterisk indicates that the following phosphate is \(^{32}\)P labeled; the RNA represents the first 211 nucleotides of the DENV-2 genome). The 2’-O methylation was monitored by conversion of m\(^7\)G*pppA-RNA\( \rightarrow \)m\(^7\)G*pppAm-RNA. The spots representing different cap structures on TLC plates were quantified by a PhosphorImager. The remaining enzymatic activities are presented below the TLC plates. The methylation activities without SIN were set at 100%. The position of the origin and the migration positions of the G*pppA, m\(^7\)G*pppA, and m\(^7\)G*pppAm molecules are labeled on the side of the TLC images.

FIG 2. Crystal structure of the WNV MTase-SIN complex. (A) A representative omit (\(F_o-F_c\)) electron density map (magenta) showing the bound SIN molecule and its interactions with the surrounding MTase residues. The MTase is shown in a ribbon representation (green). The bound SIN is shown in ball-and-stick representation, with atom colors as follows: carbon (yellow), oxygen (red), nitrogen (blue), and sulfur (green). Hydrogen bonds are shown as orange dashed lines. (B) Superposition of the crystal structures of the MTase-SIN complex (pink) and the MTase-SAH complex (cyan) (20). SAH and SIN are shown in stick representation. (C) Surface representation of the WNV MTase, showing the additional pocket adjacent to the adenine of SIN (stick representation with green and blue bond colors, partially buried and see below for the close-up view (Fig. 2E)). The surface of the pocket is colored according to atomic colors: carbon, yellow; oxygen, red; nitrogen, blue. The rest of the MTase surface is colored white. SIN is shown as stick representation with carbon colored green and nitrogen blue. (D) Amino acids involved in the formation of the additional pocket. Residues are labeled and shown in a ball-and-stick representation, with atom colors as follows: carbon, yellow; oxygen, red; and nitrogen, blue. Hydrogen bonds are shown as orange dashed lines. (E) Close-up view of the flavivirus-conserved pocket continuous with the SAM-binding pocket in the WNV MTase-SIN complex (this study), with surface and atom colors the same as used in panel C (left panel). Six water molecules (red spheres) binds to the additional pocket of the WNV MTase (left panel). Similar pockets exist in the DENV-2 MTase (PDB code: 1L9K (18); (middle panel)) and YFV MTase (PDB code: 3EVA (33); (right panel)); in each frame the SAH/SAM/SIN molecule is dipicted in stick representation. (F) Close-up view of the SAM-binding region of representative human SAM-utilizing proteins (PDB codes are labeled), including SET-like histone lysine MTase (PDB: 3FPD) (45), RNA MTase domain of human TARBP1 (2HA8) (46), catechol-O-MTase domain containing 1 (2AVD) (47), and dimethyladenosine transferase (1ZQ9) (48). The images were produced using PyMOL (50).

FIG 3. Structure-based mutagenesis of the additional pocket in WNV MTase. (A) Locations of five conserved amino acids within the additional pocket of the WNV MTase on the surface. To better view the locations of each mutated residues, the molecule is shown in an
orientation slightly different from that shown in Figs. 2C and 2E. Left panel: Surface representation of the WNV MTase in complex with SIN. Right panel: Close-up view of the additional pocket showing the five amino acids selected for mutagenesis analysis (G148, red; R160, green; R163, blue; V164, yellow; and L184, magenta). The MTase protein was rendered with partial transparency to permit the complete molecule of SIN to be viewed, with atom colors as following: carbon, green; oxygen, red; and nitrogen, blue. The images were produced with PyMOL (50). (B) SDS-PAGE analysis of recombinant WT and mutant MTases of WNV. Molecular masses of protein markers are labeled. (C) Effects of mutations of the additional pocket on WNV N7 cap methylation. The assay measured the G*pppA-RNA→m²G*pppA-RNA conversion. (D) Effects of mutations of residues of the additional pocket on WNV 2’-O cap methylation. The assay quantified the m²G*pppA-RNA→m²G*pppAm-RNA conversion. The methylation efficiencies of mutant MTases were compared against that of the WT MTase (set at 100%). The positions of the origin, G*pppA, m²G*pppA, and m²G*pppAm are indicated to the side of the TLC images. The assays were performed as reported previously (20).

FIG 4. Effects of mutations of residues of the additional pocket in WNV replication. (A) Schematic of a luciferase reporting replicon of WNV (38). (B) Transient replication of WT and mutant replicons of WNV. An average of triplicate results is shown. (C) Sequencing of the replicating mutant replicons. Total cellular RNA was extracted using RNeasy (Qiagen), and subjected to RT-PCR, using primers targeting the WNV NS5 gene. The RT-PCR products were directly sequenced. The sequencing results of mutated regions are presented. (D) Summary of N7 methylation, 2’-O methylation, and viral replication. Effects of a panel of mutations, engineered in various regions of the WNV MTase sequence, on cap methylations and viral replication. In the present study, viral replication was monitored by replicon, with wild-type replication level set as +++ (strong), mutants replication levels as ++ (medium), + (weak), and – (non-replicative). In previous studies (20,28), viral replication was monitored by plaque assay, with wild-type replication level set as +; symbols “+” and “-” indicate that genome-length RNAs containing the MTase mutations are replicative and non-replicative, respectively.
Table 1. Diffraction data collection and structure refinement statistics

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<td>Space group</td>
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<td>Cell parameters</td>
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<tr>
<td>a=39.36 Å, b=65.74Å, c=77.12Å</td>
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<tr>
<td>$\alpha=112.01^\circ$, $\beta=102.65^\circ$, $\gamma=90.19^\circ$</td>
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<td>Completeness (%)</td>
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<tr>
<td>Average $I/\sigma(I)$</td>
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<tr>
<td>$R_{sym}$ (%)</td>
<td>10.4 (33.3)</td>
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<table>
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<td>$R_{free}$ (%)</td>
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<td>Sinefungin</td>
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<td>Average B (Å$^2$)</td>
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<tr>
<td>rmsd bond angle (º)</td>
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$^1$Values in parentheses are those for the highest-resolution shell.
Table 2: Amino acid (AA) sequence alignment of residues that form the conserved additional pocket of flavivirus MTases.

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<tr>
<th>AA positions</th>
<th>133</th>
<th>147</th>
<th>148</th>
<th>149</th>
<th>160</th>
<th>163</th>
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<th>184</th>
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<td>F</td>
<td>I</td>
<td>G</td>
<td>E</td>
<td>R</td>
<td>K</td>
<td>V</td>
<td>L</td>
</tr>
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</table>

### Mosquito-borne flaviviruses

- **WNV**
- **CYV**
- **DENV1**
- **DENV2**
- **DENV3**
- **DENV4**
- **ILHV**
- **JEV**
- **LAMV**
- **SLEV**
- **ZIKV**
- **AROV**
- **BAGV**
- **ERV**
- **KOKV**
- **SEPV**
- **WESSV**
- **BANV**
- **BKV**
- **JUGV**
- **YFV**
- **MVEV**
- **USUV**
- **BODV**
- **NOUV**
- **SABV**
- **UGSV**
- **KEDV**

### Tick-borne flaviviruses

- **WTBEV**
- **FETBEV**
- **LIV**
- **OHFV**
- **STBEV**
- **GGEV**
- **GGYV**
- **KSIV**
- **LGTV**
- **POWV**
- **RFV**
- **SREV**
- **SSEV**
- **TSEV**
- **TYUV**
- **AHFV**
- **KFDV**
- **KADV**
- **MEAV**

### Flaviviruses with no known arthropod vector

- **MNV**
- **AEFV**
- **ENTV**
- **MARN**
- **GARV**
- **TEFV**
- **CFAV**
- **RVY**
- **MODV**
- **APDV**
- **TABV**

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1. All virus abbreviations used in the alignment are those of the International Committee on Taxonomy of Viruses: (1) **mosquito-borne flaviviruses**: West Nile virus (WNV), Chaoyang virus (CYV), Dengue virus serotype 1-4 (DENV1-4), Ilheus virus (ILHV), Japanese encephalitis virus (JEV), Lammi virus (LAMV), St. Louis encephalitis virus (SLEV), Zika virus (ZIKV), Aroa virus (AROV), Bagaza virus (BAOV), Edge Hill virus (EHV), Kokobera virus (KOKV), Sepik virus (SEPV), Wesselsbron virus (WESSV), Banzi virus (BANV), Barkedji virus (BKV), Jugra virus (JUGV), Yellow fever virus (YFV), Murray Valley encephalitis virus (MVEV), Usutu virus (USUV), Boubou virus (BOUV), Nounané virus (NOUV), Potiskum virus (POTV), Saboya virus (SABV), Uganda virus (UGSV), Kedougou virus (KEDV); (2) **tick-borne flaviviruses**:...
Western, Siberian, and Far Eastern subtypes of the tick-borne encephalitis virus (WTBEV, STBEV, FETBEV), Louping ill virus (LIV), Omsk hemorrhagic fever virus (OHFV), Greek goat encephalitis virus (GGEV), Gadgets Gully virus (GGYV), Karshi virus (KSIV), Langat virus (LGTV), Powassan virus (POWV), Royal Farm virus (RFV), Saumarez Reef virus (SREV), Spanish sheep encephalitis virus (SSEV), Turkish sheep encephalitis virus (TSEV), Tyuleniy virus (TYUV), Alkhurma hemorrhagic fever virus (AHFV), Kyasanur Forest disease virus (KFDV), Kadam virus (KADV), Meaban virus (MEAV); (3) flaviviruses with no known arthropod vector: Montana myotis leukoencephalitis virus (MMLV), Aedes flavivirus (AEFV), Entebbe bat virus (ENTV), Nakiwogo virus (NAKV), Yokose virus (YOKV), Culex flavivirus (CFV), Kamiti River virus (KRV), Quang Binh virus (QBV), Cell fusing agent virus (CFAV), Rio Bravo virus (RBV), Modoc virus (MODV), Apoi virus (APOIV), Tamana bat virus (TABV).
Figure 4

A) WNV Replicon

B) Luciferase activity (Log light units)


D) Table of mutation sites, amino acid changes, N7 methylation, 2-O methylations, and viral replication.

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<thead>
<tr>
<th>Mutation site</th>
<th>Amino acid</th>
<th>N7 methylation</th>
<th>2'-O methylation</th>
<th>Viral replication</th>
<th>Reference</th>
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<tr>
<td>Hydrophobic pocket</td>
<td>Wild type</td>
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<td>100%</td>
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<td>This study</td>
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<tr>
<td></td>
<td>G148A</td>
<td>14%</td>
<td>40%</td>
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<td></td>
<td>R160A</td>
<td>22%</td>
<td>120%</td>
<td>-</td>
<td></td>
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<td></td>
<td>R163A</td>
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<td>5%</td>
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<td>(28)</td>
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<td>Wild type</td>
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Structural and functional analyses of a conserved hydrophobic pocket of flavivirus methyltransferase
Hongping Dong, Lihui Liu, Gang Zou, Yiwei Zhao, Zhong Li, Siew Pheng Lim, Pei-Yong Shi and Hongmin Li

J. Biol. Chem. published online August 4, 2010

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