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Development of a luminescence-based method for measuring West Nile Virus MTase activity and its application to screen for antivirals

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ABSTRACT

West Nile virus (WNV) is a flavivirus responsible for causing febrile illness and severe neurological diseases, with an increasing impact on human health around the world. However, there is still no adequate therapeutic treatment available to struggle WNV infections. Therefore, there is an urgent need to develop new techniques to accelerate the discovery of drugs against this pathogen. The main protein implicated in the replication of WNV is the non-structural protein 5 (NS5). This multifunctional protein contains methyltransferase (MTase) activity involved in the capping formation at the 5′-end of RNA and the methylation of internal viral RNA residues, both functions being essential for viral processes, such as RNA translation and escape from the innate immune response.

We have developed a straightforward luminescence-based assay to monitor the MTase activity of the WNV NS5 protein with potential for high-throughput screening. We have validated this method as a sensitive and suitable assay for the identification of WNV MTase inhibitors assessing the inhibitory effect of the broad MTase inhibitor sinefungin, a natural nucleoside analog of the universal methyl donor S-adenosyl methionine (SAM). The screening of a small series of purine derivatives identified an adenosine derivative as a dose-dependent inhibitor of the MTase activity. The antiviral efficacy of this compound was further confirmed in WNV infections, displaying a measurable antiviral effect. This result supports the utility of this novel method for the screening of inhibitors against WNV MTase activity, which can be of special relevance to the discovery and development of therapeutics against WNV.

Introduction

West Nile virus (WNV) is an arbovirus belonging to the family *Flaviviridae* (genus *Orthoflavivirus*), responsible for a zoonosis causing febrile illness and severe neurological diseases, including meningitis, encephalitis and acute flaccid paralysis in humans (David & Abraham, 2016). Despite the increasing exposure of the population to mosquito-borne flavivirus due to globalization and the expanding geographical distribution of these vectors under climate change, the resources assigned to the prevention and treatment of WNV infections remain limited (Cendejas & Goodman, 2024; Garcia, Padilla, & Castano, 2017). As a result, current treatments for diseases caused by this virus are primarily supportive, with no antiviral approved drug available. Hence, there is a critical need for new approaches in the control and treatment of WNV infections.

The WNV virion is approximately 50 nm in diameter and contains a positive-sense single-stranded RNA genome of about 11 kb. This genome encodes a single polyprotein, which is processed into three structural proteins (capsid, membrane, and envelope) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Barrows et al., 2018).

The NS5 protein is essential in the replication and transcription

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processes of WNV. On one side, the N-terminal domain of NS5 (amino acids 1–272) contains the methyltransferase (MTase) activity, responsible for generating the cap structure at the 5′ end of newly synthesized positive RNA strands, which promotes their translation (Fajardo et al., 2020; Zhou et al., 2007) and helps the virus to evade the hosts immune response (Fleith et al., 2018; Guo, Zhang, & Jia 2018). Specifically, NS5 uses S-adenosyl-L-methionine (SAM) as a methyl donor to produce N-7-methylguanine and 2′-O-methylribose in the viral RNA cap structure (Dong et al., 2014; Ray et al., 2006), as well as in internal viral RNA residues (Dong et al., 2012). On the other side, the C-terminal domain (amino acids 273–905) contains the RNA-dependent RNA polymerase (RdRp) activity, crucial for viral genome transcription and replication (Barrows et al., 2018; Selisko et al., 2006).

The crystal structure of the WNV MTase domain (Zhou et al., 2007) revealed conserved motifs observed in the three-dimensional architectures of other flavivirus MTases (Coloma, Jain, Rajashankar, Garcia-Sastre, & Aggarwal, 2016), including the conservation and spatial positioning of K61, D146, K182 and E218, which are the characteristic catalytic residues of the 2′-O-MTase activity in other viral methyltransferases (Egloff, Benarroch, Selisko, Romette, & Canard, 2002). All these residues are essential for this type of methylation, while only the integrity of D146 is crucial for N-7 methylation (Ray et al., 2006). This finding, suggesting dual methylation activities of NS5 methyltransferase, was later confirmed as a sequential methylation mechanism in which the GpppA-capped RNA is first methylated at position N-7 of G, and subsequently undergoes repositioning to allow 2′-O methylation (Ray et al., 2006) at the first nucleotide A generating the cap-1 structure. This mechanism was proven to be common to flaviviruses, facilitating efficient viral RNA cap synthesis (Dong et al., 2008; Zhou et al., 2007).

The essential role of this protein in the biology of the virus and the conserved motifs and similar structure observed in the three-dimensional architectures of WNV and other flavivirus NS5 proteins makes NS5 a prime target for WNV antiviral drug development (Fernandes, Chagas, Rocha, & Moraes 2021). However, no clinical candidates have emerged, highlighting the need to identify new inhibitors targeting NS5.

Here, we have developed a straightforward and cost-effective luminescence-based assay to monitor the methylation activity of WNV NS5 protein. The suitability of this method as a sensitive assay for identifying WNV MTase inhibitors has been validated using the well-characterized MTase inhibitor sinefungin as reference. The evaluation of a small series of purine-based compounds in this luminescence assay identified an adenosine derivative as a dose-dependent inhibitor. Moreover, this compound, when tested for antiviral efficacy in WNV infections in cell culture, afforded a measurable antiviral effect. This result supports the utility of this novel method to measure the WNV MTase activity that can be of special relevance to the discovery and development of therapeutics against WNV, which are currently unavailable.

Materials and methods

Reagents

LB medium powder, kanamycin (KAN), $MnCl_2$, $MgCl_2$, NaCl, $ZnSO_4$, glycerol, imidazole, $NH_4CH_3CO_2$, isopropyl-β-D-1-thiogalactopyranoside (IPTG), 1,4-dithiothreitol (DTT), phenylmethanesulfonyl fluoride (PMSF), and Tris base were acquired from AppliChem. KAN stock solutions were resuspended in PBS and stored at -80°C (at 40 mg/mL). The S-adenosylmethionine (SAM) was obtained from New England Biolabs. Electro-competent cells *Escherichia coli* BL21(DE3)-pRIL were produced following standardized protocols (Green & Sambrook 2012). ATP, SYTO-9 fluorescent dye, and HisPurTM Ni-NTA resin were obtained from Invitrogen. The MTase-Glo™ Methyltransferase Assay kit and the CellTiter-Glo™ Luminescent Cell Viability Assay kit were purchased from Promega. Cell Meter™ Colorimetric MTT Cell Proliferation Kit was from AAT Bioquest. Polyuridylic acid ssRNA (Poly-U), polycytidylic acid ssRNA (Poly-C) and polyadenylic acid ssRNA (Poly-A) were obtained from Amersham Biosciences. DNA primers were purchased from Eurofins Genomics. Tween-20, EDTA, trifluoroacetic Acid (TFA), dimethylsulfoxide (DMSO) and KOD DNA Polymerase were purchased from Sigma-Aldrich. The NucleoSpin™ Plasmid Miniprep kit and NucleoSpin™ Gel and PCR Clean-up kit for DNA extraction and purification, respectively, were acquired from Macherey-Nagel. Eagle's Modified Minimum Essential Medium (EMEM) and Penicillin/streptomycin was provided by ThermoFisher. L-glutamine was purchased from Corning. Fetal bovine serum was acquired from GE Healthcare.

Chemical samples

Sinefungin was purchased from MedChemExpress. The synthesis of 5′-(2,2,2-triphenylacetamide)-5′-deoxyadenosine (**1**) is described in the Supporting information. 5′-Amino-5′-deoxy-2′,3′-O-isopropylidinead enosine (**2**) was purchased from BLDPharma. The purine derivatives tested have been synthesized by us (Orduna, Del Rio, & Perez-Perez, 2023) and their chemical structures are included in the supporting information (Figure S2).

Construction of expression plasmids

The construction of expression vectors utilizing the pET28a backbone encoding either the WNV NS5 protein from isolate Serbia Novi Sad/12 (KC407673.1), as well as the expression vector encoding the catalytically inactive WNV NS5 RdRp domain mutant, termed pET28-WNV and pET28-GAA, respectively, has been previously described (Garcia-Zarandieta et al., 2023).

To generate a catalytically inactive mutant of the WNV NS5 MTase domain, catalytic residues K61, D146, K182 and E218 were replaced with alanine using site-directed mutagenesis in two steps. Initially, a PCR reaction was conducted with KOD polymerase, employing MTase_D146A_Fw and MTase_E218A_rv primers (Supplementary Table S1) and utilizing the pET28-WNV plasmid as a template. The reaction conditions included an initial denaturation at 95°C for 3 min, followed by 30 cycles of denaturation at 98°C for 15 s, annealing at 55°C for 15 s, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. The PCR product was purified using the NucleoSpin™ Gel and PCR Clean-up kit and subsequently employed as a megaprimer in a second PCR reaction, performed with KOD polymerase and utilizing the pET28-WNV plasmid as a template. Reaction conditions consisted of an initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 98°C for 15 s and extension at 72°C for 4 min, with a final extension at 72°C for 10 min. The resulting product was digested with 20 U *Dpn*I at 37°C for 2 hours, followed by desalting through dialysis in distilled water prior to transformation into *E. coli* BL21(DE3)-pRIL. Plasmids from transformed bacteria were isolated using the NucleoSpin™ Plasmid Miniprep kit, and the presence of desired mutations was confirmed by sequencing. Subsequently, in a second step, this plasmid underwent a similar mutagenic PCR protocol as described above but employing MTase_K61A_Fw and MTase_K182A_rv primers (Supplementary Table S1). The resultant expression plasmid obtained after these sequential PCR was termed pET28-WNV-4A. This plasmid encodes for a mutant recombinant protein termed WNV NS5–4A.

E. coli BL21(DE3)-pRIL cells harboring the expression plasmids were preserved in 20% glycerol at -80°C for subsequent use.

Expression and purification of viral polymerases.

Overexpression of WNV NS5 WT, WNV NS5-GAA (a catalytically inactive mutant for the RdRp activity of NS5 obtained previously (Garcia-Zarandieta et al., 2023) and WNV NS5–4A recombinant proteins were carried out as described (Garcia-Zarandieta et al., 2023).

Luminescence based activity assay and optimization of the method.

Methyltransferase activity was assessed according to the MTase-Glo™ Methyltransferase protocol (Promega). This assay detects ATP, generated through the conversion of S-Adenosyl-L-homocysteine, which is the universal product of all methyltransferases, upon addition of MTase-Glo™ Methyltransferase reagents (Hsiao, Zegzouti, & Goueli 2016).

Briefly, the "standard MTase activity assay" was conducted in Eppendorf tubes with 9.5 μL reaction mixture containing 7.5 mM NaCl, 20 μM SAM, 50 mM Tris–HCl (pH 9), and 0.25 μg of poly-A (average size of 300 nucleotides approximately 0.25 μM). The reaction was initiated by adding 0.5 μL of recombinant WNV NS5 (1 μM), and the samples were then incubated for 60 minutes at 30°C. Following this, the samples were centrifuged at maximum speed for 1 minute, and 2 μL of 0.5% TFA were added to stop the reaction, followed by the addition of 2 μL of 5X MTase-Glo™ Reagent. After incubating the samples at 25°C for 30 minutes, 10 μL of MTase-Glo™ Detection solution was added. The reaction mixtures were then incubated for 30 additional minutes at 25°C, centrifuged at maximum speed for 1 minute, and transferred to individual wells of a white 96-well flat-bottom plate (Thermo Scientific). Finally, the luminescence emitted from each sample was quantified at 37°C using a Fluostar Optima fluorometer (BMG Labtech) until the signal stabilization.

Several experiments varying different experimental parameters were carried out to optimize this method and determine the combination of optimal conditions for the methylation. Variations regarding the "standard MTase activity assay" described above, such as different concentrations of enzyme, pH, reaction times, temperature, and/or the presence of reagents, are specifically indicated in each corresponding figure legend.

Fluorescence based polymerase activity assay

The polymerase activity of WNV NS5 WT recombinant protein was assessed, in the presence or absence of inhibitors, by real time fluorescence following the stablish protocol in our laboratory (Garcia-Zarandieta et al., 2023).

Screening of chemical samples as WNV MTase inhibitors.

Luminescence-based MTase activity assays were conducted to assess the influence of inhibitory compounds. The assays followed the above-described "standard MTase activity assay" protocol with the following variations: the MTase assay was performed on white 96-well flat-bottom plate from the beginning and incubated during 25 min at 30°C, with the addition of 0.5 μL of each compound (in DMSO) to 9 μL of the reaction mixture before initiating the reaction by addition of the recombinant WNV NS5 enzyme.

The half-maximal inhibitory concentrations (IC_{50}) of each compound were determined by conducting MTase activity assays with increasing compound concentrations. A control experiment, involving the addition of 0.5 μL of DMSO, demonstrated no significant effect on the MTase activity of recombinant WNV NS5 WT protein.

Antiviral activity in cultured cells.

Infectious virus manipulations were conducted in biosafety level 3 facilities. Vero CCL81 (ATCC) cells were cultured in EMEM supplemented with penicillin/streptomycin, 2 mM L-glutamine and 5% fetal bovine serum. Cells were infected with WNV strain NY99 (Genbank KC407666.1) at a multiplicity of infection (MOI) of 1 plaque forming units (PFU)/mL diluted in EMEM. After 1 h of incubation at 37°C, viral inoculum was removed and fresh medium supplemented with 1% fetal bovine serum and containing 10 or 50 μM of compound **1** or the same amount of drug vehicle (DMSO) was added. Infections proceeded 24 h at 37°C. The viral progeny released to the culture supernatant was titrated by standard plaque assay in semisolid agarose medium as previously described (Martin-Acebes & Saiz, 2011). To evaluate the potential cytotoxicity of the compounds, uninfected cells were treated in parallel, and the amount of cellular ATP was determined using CellTiter-Glo Luminescent Cell Viability Assay following the instructions provided by the manufacturer. To quantify the number of live cells, Cell Meter™ Colorimetric MTT Cell Proliferation Kit which MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was used as described by the manufacturer. The water-soluble MTT produces water-insoluble purple formazan in metabolically active cells, and the amount of formazan produced is directly proportional to the number of living cells.

Statistical analysis.

Data were expressed as mean ± SD. The number of experimental or biological replicates shown in each figure is indicated in its corresponding legend. Dose-response plots were obtained through nonlinear regression curve fitting. Nonlinear regression, one-way analysis of the variance (ANOVA) and Dunnett's multiple comparisons test to evaluate statistically significant differences on virus yield and cell viability were performed with GraphPad Prism 9. To evaluate the potential of luminescence-based MTase activity assays for high-throughput applications, the statistical estimator Z′ factor (Zhang, Chung, & Oldenburg, 1999) was calculated using the formula $1 - [(3SD_{c+} + 3SD_{c-})/(mean_{c+} - mean_{c-})]$; where "c+" represents the activity measured in a "standard MTase activity assay", and "c-" represents the activity measured in "standard MTase activity assay" in the absence of protein, used as negative control.

Results

Detection of WNV NS5 MTase activity by luminescence-based assay.

We have previously demonstrated that the polymerase activity of WNV NS5 can be efficiently measured using a straightforward fluorescence-based method with minimal components. This method has proven to be highly effective as a high-throughput platform for identifying compounds with antiviral activity against WNV (Garcia-Zarandieta et al., 2023). Its simplicity eliminates the need for complex setups or the use of radioactive compounds, facilitating its implementation in any molecular biology laboratory.

Encouraged by such results, in this work, we have new explored whether the full WNV NS5 protein could be employed to develop an easy-to-use platform to measure MTase activity using a luminescence-detection method. For this purpose, we have used the "MTase-Glo™ Methyltransferase assay" protocol as ground, a method capable of measuring the activity of any MTase irrespective of the acceptor substrate in the presence of the methyl donor substrate SAM (Hsiao et al., 2016). In this assay, the MTase catalyzes the transfer of the methyl group from SAM, leading to its conversion into S-adenosyl homocysteine (SAH). Subsequently, SAH is converted to ADP via MTase-Glo™ Reagent, and finally to ATP by MTase-Glo™ Detection Solution. The ATP generated is detected through a luciferase/luciferin luminescent reaction, with the recorded luminescence being directly proportional to the SAH produced during the MTase reaction (Fig. 1A).

The WNV NS5 WT, along with inactive catalytic mutants of both the RdRp (NS5-GAA) and a MTase (NS5–4A) domains of the WNV NS5 were overexpressed and purified as described previously (Garcia-Zarandieta et al., 2023) (Fig. 1B). These recombinant proteins were then used to develop the luminescence-based MTase activity assay. MTase activity was correlated with luminescence emission dependent on the presence of both an active WNV NS5 WT, SAM and RNA (Fig. 1C, compare column 1 with columns 2–4). However, control experiments performed in the presence of SAM showed a background noise (Fig. 1C, compare columns

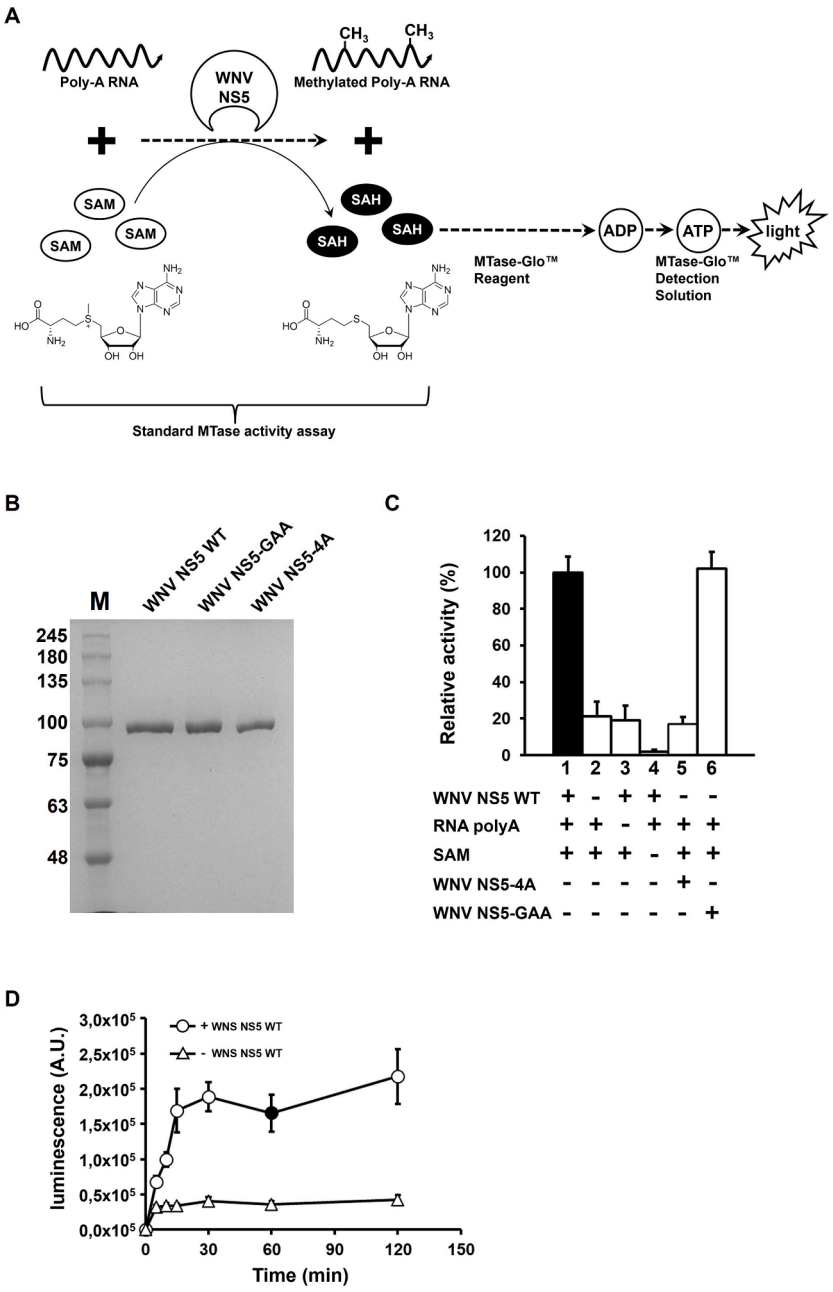

Fig. 1. Luminescence-based MTase assay using WNV NS5. A) Schematic of the steps involved in the luminescence-based methyltransferase assay developed in this work using the The MTase-Glo™ kit. The standard MTase activity assay stage is indicated. B) SDS-PAGE analysis of WNV NS5 WT, WNV NS5-GAA, and WNV NS5–4A recombinant proteins expressed in *E. coli* BL21(DE3)-pRIL and purified by affinity chromatography. "M", molecular marker. The molecular weight (in kDa) of each band is indicated. C) Relative activity of different recombinant WNV NS5 after 60 min of reaction in the presence (+) or absence (−) of the indicated reagents. The concentration of each reagent is the same as used in a standard MTase activity assay. 100 % activity (in black) corresponds to the standard MTase activity assay performed with WNV NS5 WT as established in Materials and Methods. D) Time course MTase activity of WNV NS5 in a standard MTase activity assay in the presence (circles) or in the absence (triangles) of WNV NS5 WT as described in Materials and Methods. Black circle corresponds to the activity displayed in a "standard MTase activity assay". Averages and ±SD of three independent experiments are represented.

2 and 3 with column 4), probably due to a contamination of SAH in the SAM sample, as previously documented (Hsiao et al., 2016). The disruption of the catalytic K-D-K-E motif led to background levels of MTase activity, while the RdRp catalytic domain integrity of the WNV NS5 protein did not affect this activity. (Fig. 1C, compare columns 5 and 6 with column 1). The maximum activity was recorded after 60 min of assay (Fig. 1D, circles), while the luminescence background remained constant throughout the entire reaction time (Fig. 1D, triangles). Therefore, for every subsequent experiment set, the luminescence signal of a control experiment lacking the WNV NS5 WT but in the presence of SAM was conducted in parallel and used as a negative control.

Several experiments were carried out to optimize the luminescence-based method. Different pH values, NaCl concentrations and temperatures were assessed, achieving optimal MTase activity at pH 9, 30°C and no additional NaCl, respectively (Figs. 2A to C), while the recording of luminescence showed linearity within the concentration range of WNV NS5 from 0.1 to 5 μM (Fig. 2D). These findings are in total agreement with the 2′-O methylation activity of WNV NS5 MTase domain observed previously using a different experimental approach (Zhou et al., 2007). Furthermore, poly-A, but not poly-C or poly-U, was efficiently

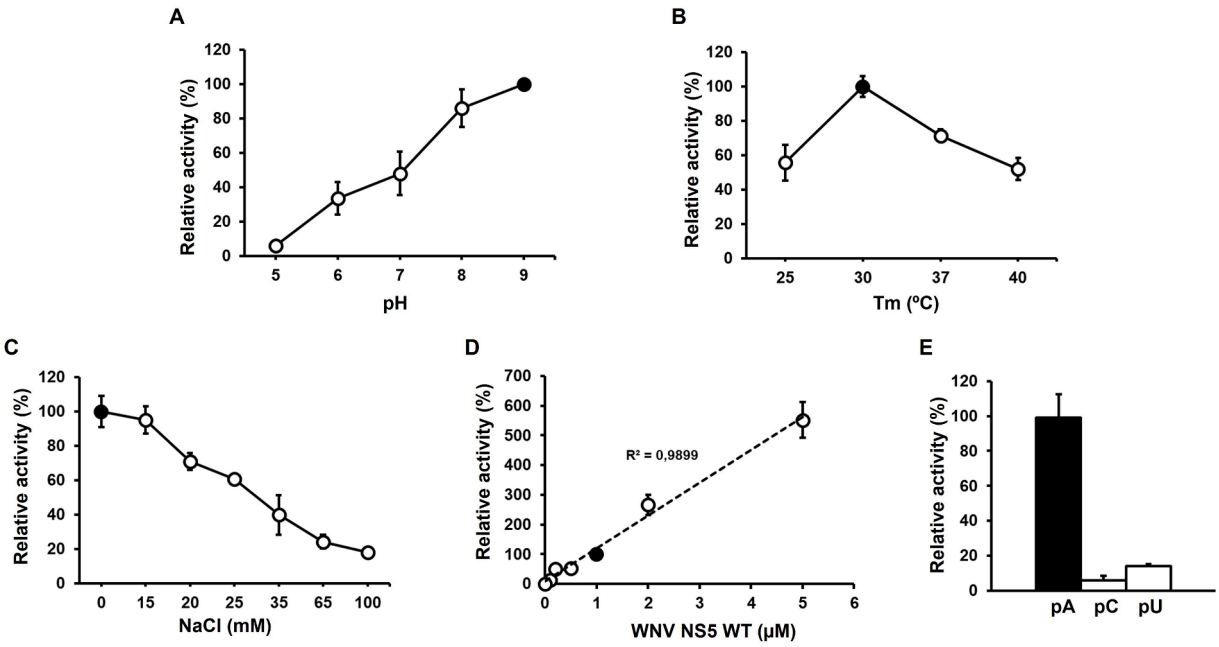

Fig. 2. Optimization of conditions for the WNV NS5 methylations. Relative activity of WNV NS5 varying the pH (A), temperature (B), concentrations of NaCl (C), concentration of WNV NS WT (D) and methyl acceptor RNA (E), while keeping the other three parameters constant at the standard MTase activity assay levels. For each parameter, activities are related to the optimal level, expressed as 100 % (black circles or bar). Averages and ±SD of three independent experiments are represented. The R squared value of the linear function that relates the amount of WNV NS WT to the activity is given in D.

methylated, as occurs with both Dengue (DENV) and Zika (ZIKV) NS5 MTases (Coutard et al., 2017; Dong et al., 2012) (Fig. 2E). Altogether, these data indicate that WNV NS5 displays MTase activity, employing preferentially adenosine as acceptor in the methylation reaction with no requirement of a specific viral RNA sequence.

Identification of a potential WNV NS5 inhibitor through luminescence-based screening.

To assess the reproducibility of the luminescence-based assay, the Z' factor was determined (Zhang, Chung, & Oldenburg, 1999), as a measure of the method's appropriateness for high-throughput screening, obtaining a factor value of 0.53 (Supplementary Figure S1). In parallel, to evaluate the capacity of our method for screening WNV MTase inhibitors, it was challenged by performing a dose-dependent inhibition experiments in the presence of sinefungin (Fig. 3A) (Fuller & Nagarajan, 1978), a well-characterized broad-spectrum inhibitor of MTases, including WNV NS5 (Dong et al., 2008). The IC_{50} of this inhibitor in the luminescence-based assay was 3.6 ± 1.8 µM (Fig. 3B), in the same range of previous results using a radiolabeled based assay (Dong et al., 2008).

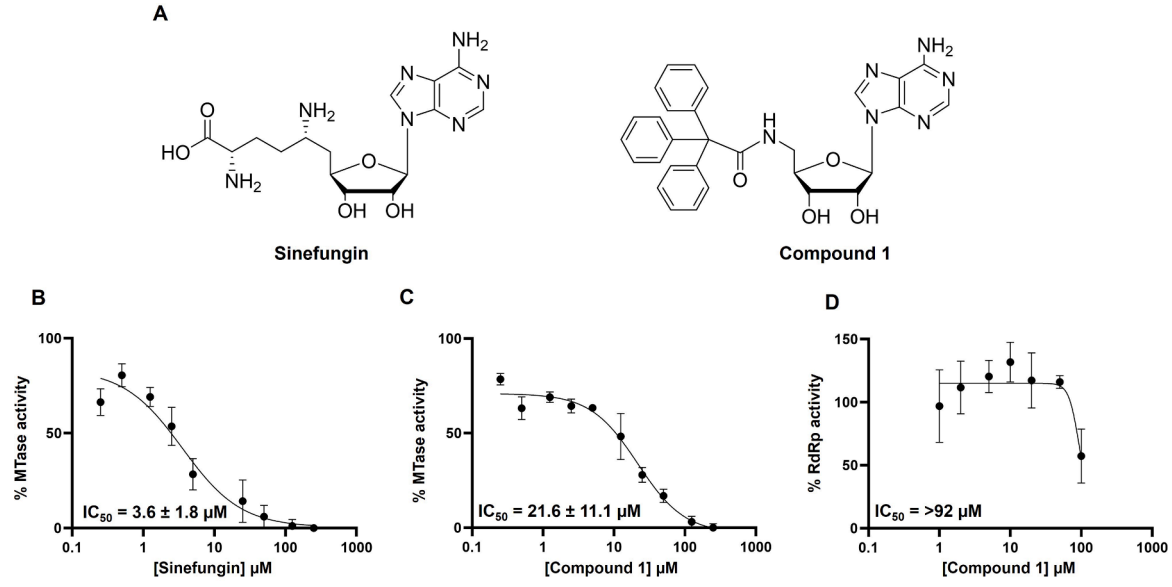

Fig. 3. Inhibitory effects of different inhibitors against the WNV NS5 WT Mtase or RdRp activities. A) Chemical structures of sinefungin and compound **1**. B) MTase activity dose-response inhibition curves and IC_{50} values of the WNV NS5 WT exerted by sinefungin. C) MTase activity dose-response inhibition curves and IC_{50} values of the WNV NS5 WT exerted by compound **1**. D) RdRp activity dose-response inhibition curve and IC_{50} value of the WNV NS5 WT exerted by compound **1**. IC_{50} calculation for each compound is indicated within the corresponding graph. Averages and ±SD of at least three independent experiments are represented.

Then, a small series of purine derivatives and two adenine nucleosides (Supplementary Figure S2) were tested as potential MTase inhibitors at a final concentration of 50 μM. From them, only compound **1** (Fig. 3A) demonstrated measurable inhibition of the luminescence signal. The other compounds (Supplementary Figure S2) did noy provide inhibition at this concentration. The inhibitory potency of compound **1** was measured yielding an IC_{50} value of 21.6 ±11.1 μM (Fig. 3C). Therefore, these results confirm the robustness of this method and its effectiveness as a screening tool for high-throughput prospection of inhibitors targeting the MTase activity of WNV NS5.

Since the protein used contains both the MTase and the RdRp domains, and we had previously described a fluorescence-based method to measure RdRp inhibition (Garcia-Zarandieta et al., 2023), the RNA polymerization assay was performed in the presence of compound **1**. As shown in Fig. 3D, no RdRp inhibition was observed in the concentration range used. Thus, these findings confirm that the luminescence method is specific for identifying compounds that impact MTase activity when employing the bifunctional WNV NS5 protein.

Antiviral effect of compound 1.

The antiviral activity of compound **1** against WNV was assessed in cultured Vero cells. To this end, cells were infected with WNV and treated with 10 or 50 μM of the compound or the same amount of the drug vehicle (DMSO) as a control. When the viral progeny was titrated at 24 h postinfection, a reduction was observed in samples treated with 50 μM of compound **1** (Fig. 4A). The analysis of the effect of the compound on cell viability, estimated by ATP measurement (Fig. 4B) and MTT assay (Fig. 4C), confirmed that the reduction in virus multiplication observed was not related to a reduction in cell viability, thus supporting the specificity of the antiviral activity exerted by the compound.

Discussion

West Nile virus (WNV) has experienced a significant increase in outbreaks around the world in the last decades due to the globalization of human activity (Wang, Liu, Gao, Wang, & Xiao, 2024), and influenced by the climate change that affects the proliferation of mosquito vectors carrying WNV (Kilpatrick 2011). Due to the lack of clinically approved therapeutic options, there is a great need for drugs that can specifically inhibit enzymes essential for the flavivirus life cycle. The methylation activity performed by the viral NS5 protein is crucial for the WNV life cycle, making the viral MTase an attractive target for flavivirus therapy. Despite substantial efforts invested and the discovery of several anti-WNV compounds displaying varying degrees of success targeting the NS5 MTase activity (Aouadi et al., 2017; Brecher et al., 2015; Chen et al., 2013; Lim et al., 2011), no drugs are available in the clinic. Therefore, the development of a robust method for screening compounds with targeting crucial WNV proteins is highly desirable in order to identify new anti-WNV drugs.

Various methods have been developed for screening compounds targeting flavivirus MTase, employing different experimental approaches. The ideal method should allow a reproducible, easy-to-use, and high-throughput analysis of the samples. Most established assays for evaluating potential inhibitory compounds rely on fluorometric methods (Aouadi et al., 2017; Geiss et al., 2011; Samrat et al., 2023) or the use of radioactive reagents (Chen et al., 2013; Fiorucci et al., 2024; Lim et al., 2008; Mensah et al., 2023). However, both approaches suffer from limitations that may reduce the success of the screening campaigns or limit their implementation. In the former, interference from screened compounds with autofluorescence can compromise the reliability of the results obtained (Gul & Gribbon 2010; Simeonov & Davis 2004). The latter requires high safety measures, specialized facilities and training for their execution and waste management, thereby increasing costs and impairing their setup. On the other hand, antibodies have also been employed for screening purposes, but these approaches require laborious optimization to determine the appropriate amount of antibody needed for product recognition (Aouadi et al., 2017). Among this collection of methodologies, luminescence-based methods emerge as an optimal option for developing high-throughput screening platforms (Ibanez, McBean, Astudillo, & Luo 2010; Song et al., 2021).

The method developed in this study enables the measurement of WNV NS5 MTase activity in a straightforward manner. Additionally, the assay exhibits high reproducibility and robustness, as demonstrated by the Z' value of 0.53, qualifying it for high-throughput screening purposes, according with stablished standards (Zhang et al., 1999). Our method leverages the commercially available MTase-Glo™, previously successfully employed for measuring the ZIKV NS5 MTase activity (Song et al., 2021). However, in contrast to these studies, where a synthetic RNA was utilized as the acceptor molecule, we have effectively employed an unspecific poly-A homopolymer RNA. This polymer can serve as the methyl acceptor, as previously observed with DENV and ZIKV NS5s, through methylation of internal adenosines at their 2′-O position (Coutard et al., 2017; Dong et al., 2012; Mensah et al., 2023). The presence of internal 2'-O-methyladenosine has been documented in the DENV-1 wild type genomes, whereas it is absent in genomes purified from NS5 mutant viruses (Dong et al., 2012). Therefore, not only the cap 2′-O-methylation in viral RNA deceives innate immune detection mechanisms (Guo et al., 2018; Ruggieri, Helm, & Chatel-Chaix, 2021) and plays a pivotal role in counteracting the antiviral activities mediated by interferon-stimulated genes (Daffis et al., 2010), but both internal and cap 2′-O methylation might also protect the viral RNA from degradation and modulate RNA-protein interactions, thereby increasing the viral virulence (Decombe, El Kazzi, & Decroly 2023). Hence, poly-A

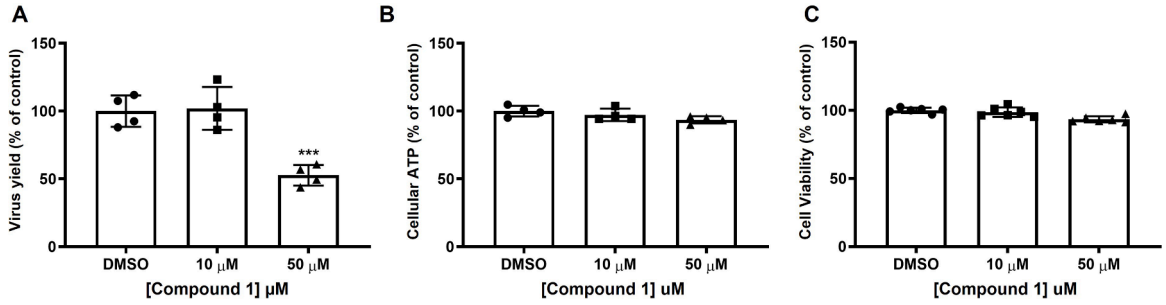

Fig. 4. Antiviral activity of compound 1 against WNV. A) Effect of compound **1** on WNV multiplication. Vero cells were infected at a MOI of 1 PFU/cell, treated with 10, 50 μM of compound **1** or the same amount of vehicle (DMSO) as a control and the virus yield released to the culture medium at 24 h post-infection was determined by plaque assay. B) Effect of compound **1** on cellular ATP levels. The cytotoxicity of compound **1** was analyzed in uninfected cells treated in parallel as in (A) by quantification of the amount of cellular ATP. C) Evaluation of the cytotoxicity of compound **1** by MTT assay. Uninfected cells treated were in parallel as in (A) and cell viability was determined analyzed by MTT assay. Averages and ±SD of four to six biological replicates are represented. *** for $P<0.0007$ for ANOVA and Dunnet's multiple comparison test in (A).

could serve as inexpensive universal acceptors for assays to detect flavivirus NS5 methylation.

Therefore, our method not only significantly reduces the cost of compound screening compared to other approaches but also might enable the development of commercially available kits, already including poly-A as the methyl acceptor, thereby greatly enhancing the utility of our microplate assay, and increasing its versatility as a general method for screening antiflaviviral compounds.

We have employed our method to screen a small series of purines and adenosine analogs. One of the samples (compound **1**) showed inhibitory capacity against MTase activity and further displayed measurable antiviral activity in cell cultures (Fig. 4A). Compound **1** is a 5′-amino-5′-deoxyadenosine derivative with a bulky trityl group link through an amide bond (Fig. 3A). It is interesting to note that pyrimidine nucleosides with a bulky silyl group at their 5′-position had been previously described as WNV MTase inhibitors (Chen et al., 2013); (Vernekar et al., 2015). Together, these results point out the possibilities that 5′-substituted nucleoside analogues offer for WNV MTase inhibition that is translated in cell culture activity.

In addition, the recombinant WNV NS5 protein, that contains both the MTase and RdRp domains, could be used to analyze the effect of compounds on the MTase activity of WNV NS5 using the bioluminescence method here described, as well as to evaluate compounds affecting the RdRp activity using real-time fluorescence assays, as we have previously stablished (Garcia-Zarandieta et al., 2023). The possibility to sequentially screen inhibitors against different viral functions might streamline the search for combination therapies and increase their effectiveness, thereby limiting the selection of treatment-escape viral mutants (Domingo & Perales 2019).

Conclusion

Altogether, we have developed a simple luminescence-based method for measuring WNV NS5 MTase activity and demonstrated its utility by discovering an adenosine analogue that inhibits the MTase and exhibits antiviral activity in cultured cells. Furthermore, our method could be implemented as a high-throughput screening platform. Thus, we believe that these characteristics, along with the unique assay conditions, open new opportunities for discovering inhibitory compounds against WNV, where effective therapeutics are currently lacking.

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Author contributions

Alejandra Álvarez-Mínguez: Investigation, Validation. **Natalia del Río**: Investigation, Validation. **Ana Belén-Blázquez**: Investigation. **Elena Casanova**: Investigation **José-María Orduña**: Investigation. **Patricia Camarero**: Investigation. **Carolina Hurtado-Marcos**: Writing – review and editing, Supervision. **Carmen del Águila**: Resources. **María–Jesús Pérez-Pérez**: Conceptualization, Writing-review and editing, Supervision, Funding acquisition, **Miguel A. Martín-Acebes**: Conceptualization, Writing - Review and editing, Supervision, Funding acquisition. **Rubén Agudo**: Conceptualization, Writing - Original Draft, Writing-review and editing, Methodology, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.crmicr.2024.100282.

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