Antiviral Research 99 (2013) 292–300

Contents lists available at SciVerse ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Development of specific dengue virus 2′-*O*- and N7-methyltransferase assays for antiviral drug screening

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ARTICLE INFO

Article history:
Received 21 November 2012
Revised 31 May 2013
Accepted 3 June 2013
Available online 12 June 2013

Keywords:
Dengue virus methyltransferase
Cap RNAs synthesis
N7-methyltransferase inhibition assay
Antiviral drug screening

ABSTRACT

Dengue virus (DENV) protein NS5 carries two mRNA cap methyltransferase (MTase) activities involved in the synthesis of a cap structure, ^{7Me}GpppA$_{2'OMe}$-RNA, at the 5′-end of the viral mRNA. The methylation of the cap guanine at its N7-position (N7-MTase, ^{7Me}GpppA-RNA) is essential for viral replication. The development of high throughput methods to identify specific inhibitors of N7-MTase is hampered by technical limitations in the large scale synthesis of long capped RNAs. In this work, we describe an efficient method to generate such capped RNA, GpppA$_{2'OMe}$-RNA$_{74}$, by ligation of two RNA fragments. Then, we use GpppA$_{2'OMe}$-RNA$_{74}$ as a substrate to assess DENV N7-MTase activity and to develop a robust and specific activity assay. We applied the same ligation procedure to generate ^{7Me}GpppA-RNA$_{74}$ in order to characterize the DENV 2′-*O*-MTase activity specifically on long capped RNA.

We next compared the N7- and 2′-*O*-MTase inhibition effect of 18 molecules, previously proposed to affect MTase activities. These experiments allow the validation of a rapid and sensitive method easily adaptable for high-throughput inhibitor screening in anti-flaviviral drug development.

1. Introduction

Flaviviruses belong to the family *Flaviviridae* and include more than a hundred viruses. Mosquito-borne flaviviruses such as dengue, West Nile and yellow fever viruses can cause life-threatening diseases. The four viral serotypes of dengue virus (DENV1–4) cause 50–100 million human infections annually. In about 1% of cases, the resulting dengue fever (DF) evolves to dengue hemorrhagic fever (DHF) and/or dengue shock syndrome (DSS), leading to about 30,000 annual deaths. Despite the recent clinical trial of a tetravalent dengue vaccine showing a global protection around 30% (Sabchareon et al., 2012), there is no available antiviral compound to treat or prevent DHF–DSS. Potent antiviral therapies would thus be of great benefit.

Possible targets for inhibitor development comprise the DENV NS5 mRNA cap methyltransferase (MTase) involved in post- or co-transcriptional RNA capping of viral RNA (Egloff et al., 2002; Ferron et al., 2012; Ray et al., 2006). The flavivirus genome is indeed a single-stranded positive RNA carrying a cap-1 structure (^{7Me}GpppA$_{2'OMe}$-RNA) at its 5′-end. Such RNA cap protects the viral RNA from 5′-exoribonucleases and promotes binding to eIF4E for translation (Decroly et al., 2012; Filipowicz et al., 1976). DENV NS5 MTase catalyzes two consecutive methylation reactions involved in the formation of the cap structure: methylation of the cap guanine at its N7-position to yield ^{7Me}GpppA-RNA and methylation of the first transcribed nucleotide at its 2′-*O*-position to yield ^{7Me}GpppA$_{2'OMe}$-RNA (Dong et al., 2010, 2008). Both N7- and 2′-*O*-methylations use *S*-adenosyl-L-methionine (AdoMet) as the methyl donor, and generate *S*-adenosyl-L-homocysteine (AdoHcy) as a by-product. Reverse genetics, together with structural and biochemical characterization of DENV NS5MTase demonstrated that a mutation abolishing both methylations is lethal, whereas mutants annihilating 2′-*O*-methylation specifically showed an

Abbreviations: DENV, dengue virus; NS, non-structural; MTase, methyltransferase; SLA, stem-loop A; DEAE, diethylaminoethyl; DF, dengue fever ; DHF, dengue hemorrhagic fever; DSS, dengue shock syndrome; AdoMet, *S*-adenosyl-L-methionine; AdoHcy, *S*-adenosyl-L-homocysteine; SPA, scintillation proximity assay; HTS, high throughput screening; VV, vaccinia virus; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; SDS–PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; TLC, thin layer chromatography ; TBDMS, *tert*-butyldimethylsilyl ; ATP, adenosine triphosphate; GTP, guanosine triphosphate; IC_{50}, inhibitory concentration that causes 50% reduction in enzyme activity; SIBA, *S*-isobutylthio-5′-deoxyadenosine; ATA, aurintricarboxylic acid; SD, standard deviation.

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http://dx.doi.org/10.1016/j.antiviral.2013.06.001

attenuated phenotype (Dong et al., 2010). Together with the involvement of N7-methylation in the translation, these results suggest that N7-methylation, but not 2′-*O*-methylation, is essential for viral replication.

To characterize specific inhibitors of each methylation reaction, it is necessary to devise enzymatic assays able to assess independently these two activities. N7- and 2′-*O*-methylations require different features in the RNA substrate. N7-methylation requires RNA of at least 74 nucleotides in length, having a wild-type 5′-end sequence and forming an intact 5′-stem-loop hairpin structure named SLA (Chung et al., 2010; Dong et al., 2007). In contrast, 2′-*O*-methylation can be observed using either short RNAs ($\geqslant$4 nucleotides) (Selisko et al., 2010) or longer RNAs bearing an SLA structure (Dong et al., 2007). Scintillation proximity and DEAE-filter binding assays were previously developed to follow DENV NS5 2′-*O*-MTase activity on short RNAs (Dong et al., 2010; Lim et al., 2008; Selisko et al., 2010) and allowed the selection of inhibitors (Chung et al., 2010; Luzhkov et al., 2007; Milani et al., 2009; Podvinec et al., 2010).

However, the development of high throughput methods to identify specific inhibitors of N7-MTase has not been reported so far, probably due to technical limitations in large-scale chemical or enzymatic synthesis of adequate RNA substrates. The only existing N7-MTase assay was performed using an RNA previously capped with Vaccinia virus guanylyltransferase (GTase) and radiolabeled GTP. This N7-MTase assay quantified the conversion of G*pppA-RNA$_{>70}$ into ^{7Me}G*pppA-RNA$_{>70}$ using thin-layer chromatography (TLC) coupled to cap hydrolysis using P1 nuclease (Chung et al., 2010; Dong et al., 2007; Milani et al., 2009).

We report here the production of two 74-mer capped RNAs: GpppA$_{2'OMe}$-RNA$_{74}$ through ligation of a GpppA$_{2'OMe}$-RNA$_{12}$ (**F-1**) with a 5′-phosphorylated 62-mer fragment **F-3** and ^{7Me}GpppA-RNA$_{74}$ through ligation of a ^{7Me}GpppA-RNA$_{12}$ (**F-2**) with the 5′-phosphorylated 62-mer fragment **F-3**. The **F-1** and **F-2** substrates are chemically synthesized using the 2′-*O*-pivaloyloxymethyl phosphoramidite method followed by the capping reaction on solid-supported RNA as recently described by Thillier et al. (2012). The **F-3** substrate is ligated to **F-1** or **F-2** using T4 RNA Ligase. In the former case, the resulting GpppA$_{2'OMe}$-RNA$_{74}$ is then used as a substrate to characterize the N7-methylation activity specifically, since its 2′-*O*-methyl position is already methylated. In the latter case, the resulting ^{7Me}GpppA-RNA$_{74}$ is used as a substrate to characterize the 2′-*O*-methylation activity specifically on a long capped RNA. This robust DEAE filter-binding assay, based on the transfer of a [^{3}H]-methyl group from radiolabeled *S*-adenosyl-L-methionine (AdoMet) to GpppA$_{2'OMe}$-RNA$_{74}$ or ^{7Me}GpppA-RNA$_{74}$ allows comparing the N7- and 2′-*O*-MTase activities of DENV3 NS5. We set up this assay, validated it with a set of 18 known MTase inhibitors, and demonstrated the compounds with specific inhibition potential on either N7- or 2′-*O*-MTases activities.

2. Materials and methods

2.1. Materials

T4 RNA Ligase 1 (ssRNA Ligase), T4 RNA Ligase 2 (dsRNA Ligase), T4 RNA Ligase reaction buffers (10×) and ATP were purchased from New Englands BioLabs Inc. ScriptCap™ 2′-*O*-Methyltransferase, Vaccinia Virus Cap 1 methyltransferase (VV2′-*O*-MTase), was purchased from Epicentre® Biotechnologies. Human N7-MTase used in this study was cloned and purified as previously described (Peyrane et al., 2007). [^{3}H] AdoMet (80.7 Ci/mmol) was purchased from PerkinElmer. DENV3 NS5 MTase potential inhibitors were purchased from Sigma–Aldrich (**1**–**3**, **6**, **7**, **9**, **12**, **13**, **14** and **17**), Life Chemicals Inc. (**4**), InterBioScreen Ltd. (**5**), Enamine (**16**), Trilink Biotechnologies (**8**), New Englands BioLabs Inc. (**10** and **11**) and Chembridge (**15** and **18**). These compounds were dissolved in H_2O or DMSO and stored as 2 mM and 10 mM stock solutions, or as 8 mM stock solution in 0.1 mM NaOH (ATA, **9**), at −20 °C.

2.2. Production of NS5 DENV3 MTase

The DNA fragment coding for the DENV3 MTase (amino acid region 1–277) was synthesized by Geneart. The coding sequence was cloned in pMcox20A by Gateway recombination, downstream a cleavable Hexahistidine–Thioredoxin tag using a two step PCR protocol. The protein was expressed in *Escherichia coli* Rosetta (DE3) pLysS strain (Novagen) at 25 °C in terrific broth. The purification of the protein and the tag removal was performed in non denaturing conditions as previously described (Lantez et al., 2011). The final size exclusion chromatography step was performed in 20 mM Tris, 200 mM NaCl, glycerol 10%, 2 mM DTT, pH 7.5.

2.3. RNA synthesis and purification

2.3.1. Capped 13-mer **F-1** (GpppA$_{2'OMe}$GUUGUUAGUCUA) and 13-mer **F-2** (^{7Me}GpppAGUUGUUAGUCUA)

*2.3.1.1. Chemical synthesis of GpppA$_{2'OMe}$-12mer **F-1** and ^{7Me}GpppA-12mer **F-2** on solid support.* Chemical synthesis of the 13-mers was performed on an ABI 394 synthesizer (Applied Biosystems) from commercially available (Link Technologies) long chain alkylamine controlled-pore glass (LCAA-CPG) solid support with a pore size of 1000 Å derivatized through the succinyl linker with 5′-*O*-dimethoxytrityl-2′-*O*-Ac-N^6-Pac adenosine. RNA sequence was assembled on a 1-μmol scale in a Twist oligonucleotide synthesis column (Glen Research) using the 2′-*O*-pivaloyloxymethyl amidites (5′-*O*-DMTr-2′-*O*-PivOM-[U, C^{Ac}, A^{Pac} or G^{Pac}]-3-*O*-(*O*-cyanoethyl-*N*,*N*-diisopropylphosphoramidite) (Lavergne et al., 2008) and the 5′-*O*-DMTr-2′-*O*-Me-A^{Bz}-3′-*O*-(*O*-cyanoethyl-*N*,*N*-diisopropyl-phosphoramidite) in the case of **F-1** (Chemgenes). RNA assembly followed by 5′-functionnalization of solid-supported 13-mer with cap structure (Gppp). Deprotection and release of 5′-GpppA$_{2'OMe}$-RNA$_{12}$ **F-1** and 5′-GpppA-RNA$_{12}$ were performed following a previously described procedure (Thillier et al., 2012).

*2.3.1.2. Analysis and purification of **F-1** and GpppA-RNA$_{12}$ by IEX-HPLC.* Analytical and semi-preparative HPLC were performed on a DIONEX bio-inert Ultimate 3000 Titanium HPLC system equipped with anion-exchange DNAPac PA200 column (4 × 250 mm) or DNAPac PA100 column (9 × 250 mm) (Dionex). The crude Gppp13-mers were analyzed using a 0–50% linear gradient of buffer B (400 mM $NaClO_4$ in buffer A) in buffer A (5% CH_3CN in 25 mM Tris–HCl buffer, pH 8) for 20 min at 25 °C at a flow rate of 1.2 mL min^{-1}. **F-1** was purified using a step gradient of 0–25% for 10 min then 25–45% for 20 min of buffer B at 30 °C at a flow rate of 5 mL min^{-1}. GpppA-RNA$_{12}$ was purified using a step gradient of 0–25% for 10 min then 25–65% for 20 min of buffer B at 40 °C at a flow rate of 5 mL min^{-1}. They were characterized by MALDI-TOF spectrometry (Thillier et al., 2012). The pure fractions of **F-1** and GpppA-RNA$_{12}$ were pooled in 100 mL round bottomed flask and concentrated to 0.5 mL under reduced pressure at 30 °C. The residues were dissolved in 1 mL of water and loaded on a NAP-10 cartridge containing Sephadex G-25 (GE-Healthcare). Elution was performed with 2 × 0.7 mL of water and the fraction containing the desired **F-1** or GpppA-RNA$_{12}$ was collected and freeze-dried. Ninety three nanomoles of pure **F-1** and 91 nmol of pure GpppA-RNA$_{12}$ were obtained.

N7-methylation of the purified GpppA-RNA$_{12}$ (74 nmol) to give ^{7Me}GpppA-RNA$_{12}$ **F-2** was carried out using 0.25 μM N7-hMTase and 0.4 mM *S*-adenosylmethionine (New England Biolabs) in 40 mM Tris–HCl pH 8 with 5 mM dithiothreitol in a 1.75 mL

reaction volume (GpppA-RNA at a final concentration of 40 μM) at 30 °C (Thillier et al., 2012). N7-methylation was monitored using IEX-HPLC analysis of an aliquot from the reaction mixture passed through ZipTip C_{18} prior to analysis. Crude **F-2** was analyzed with a linear gradient of 0–60% of solvent B for 25 min at 40 °C. After 5 h incubation, the reaction was complete. Proteic material and remaining AdoMet and the *S*-adenosylhomocysteine (AdoHcy) product were then removed as follows: the reaction mixture was loaded onto a Sephadex G-25 gel filtration column (12 g, h 120 × d 25 mm). **F-2** was eluted using 30 mL of 12.5 mM TEAAc applied in batches of 5 mL each and fractions were analyzed by IEX-HPLC. Then batches containing the pure ^{7Me}GpppA-RNA_{12} **F-2** were pooled together in a 100 mL round-bottom flask, and lyophilized from water. The residue was dissolved in 0.8 mL, lyophilized twice with sequential dissolution in 0.4 mL, and finally 0.3 mL of water, and transferred to a 2 mL Eppendorf tube and freeze-dried. 51 nmol of ^{7Me}GpppA-RNA_{12} **F-2** was obtained with 81% purity.

2.3.2. 5′-Phosphorylated and 3′-biotinylated 62-mer RNA oligonucleotide *F-3*

2.3.2.1. Chemical synthesis. Chemical synthesis of the **F-3** was performed as for **F-1** but with controlled-pore glass derivatized through the succinyl linker with TEG biotin. RNA sequence was assembled on a 200 nmol-scale in an ABI3900-oligonucleotide synthesis column using standard TBDMS phosphoramidites (Chemgenes, USA). Exocyclic amino groups of riboC and riboG were acetyl-protected. Phosphorylation of 5′-hydroxy was done on column using the Chemical Phosphorylation Reagent (Link Technologies, UK) according to the manufacturer's instruction.

The cleavage from solid support of **F-3** and the deprotection of phosphate and exocyclic amino groups were achieved by incubation with AMA reagent (methylamine in ethanol/conc. ammonia; 1:1, v/v) for 30 min at 60 °C. The solid support was removed by centrifugation and the AMA solution was evaporated. In order to remove the 2′-*O*-TBDMS protecting groups, the residue was dissolved triethylamine trihydrofluoride in DMSO (1:1, v/v) and incubated for 3 h at 65 °C. Crude RNA oligonucleotide **F-3** was precipitated (butanol precipitation) and the precipitate was washed twice with cold acetone to remove residual organic compounds from the deprotection steps. The precipitate was subsequently dried under vacuum at 30 °C.

*2.3.2.2. Analysis and purification of 5′-phosphorylated 62-mer **F-3**.* The crude **F-3** was redissolved in nuclease-free water and desalted using a HiTrap SEC column on an ÄKTA Explorer FPLC system (both GE Healthcare, Sweden). Collected salt-free RNA fractions were combined and evaporated in a vacuum concentrator. The yield was determined to be 110.4 nmol (10.6%). **F-3** was analyzed by 7% PAGE, GelRED™ stained (Biotium, USA) and bands visualized under UV light (Decon Science Tec., Germany). LC/ESI-MS analysis was performed using a HP1100 (Hewlett Packard) HPLC system connected to a Qq-ToF mass spectrometer (QSTAR pulsar i, ABSciex) configured for electrospray ionization. For reversed phase chromatography a C18 Xbridge 2.5 μm column, 1 × 50 mm (Waters) was employed at a flow rate of 50 μL min^{-1}. The mobile phases were 5 mM tributylammonium acetate pH = 6 (TBAA) in water/acetonitrile 95/5 (v/v) (mobile phase A) and 5 mM tributylammoniumacetate (pH = 6) in water/acetonitrile 15/85 (mobile phase B). After injection, the sample was desalted at 30 % B during 5 min, and a gradient from 30% B to 100% B in 17.5 min was applied at 60 °C. The MS spectra were deconvoluted using the BioAnalyst 1.1 software (ABSciex).

2.3.3. Synthesis and purification of $GpppA_{2'OMe}$-RNA_{74} and ^{7Me}GpppA-RNA_{74}

$GpppA_{2'OMe}$-RNA_{74} was obtained by ligation of **F-1** and **F-3** fragments using T4 RNA Ligase 2 and ^{7Me}GpppA-RNA_{74} was obtained by ligation of **F-2** and **F-3** fragments in similar conditions. The ligation was performed as follows: 10 nmol of **F-1** and 10 nmol of **F-3** RNA were dissolved in 200 μl of H_2O, heated for 5 min at 70 °C and stored on ice. The ligation was performed at 37 °C for 1 h in 50 mM Tris–HCl pH 7.5, 2 mM $MgCl_2$, 1 mM DTT, 400 μM ATP supplemented with 100 U of T4 RNA Ligase 2. The ligase was removed by treatment with 10 μL of StrataClean resin (Stratagene) and the buffer exchanged on a Microspin™ G-25 column (GE-Healthcare) against water and lyophilized. At this stage, the ligated product can be used as crude (>90% pure as judged by PAGE analysis) or purified product after IEX-HPLC performed on the same DIONEX HPLC system as described above equipped with the DNAPac PA200 column. Elution was performed at a 1.2 mL min^{-1} flow rate using a 0–60% linear gradient of buffer B (700 mM $NaClO_4$ in A) in buffer A (10% CH_3CN in 25 mM Tris–HCl buffer, pH 8) at 25 °C in 30 min. Fractions of pure $GpppA_{2'OMe}$-RNA_{74} were pooled and concentrated to 0.5 mL under reduced pressure. The residue was dissolved in 1 mL of water, desalted through Sephadex G-25 (GE-Healthcare) and lyophilized. $GpppA_{2'OMe}$-RNA_{74} was >99% pure as judged by HPLC.

2.4. DENV3 N7- and 2′-O-MTase assays

For characterization of *in vitro* DENV3 N7- and 2′-*O*-MTase activities, pH dependence and time course activities were tested. pH dependence was determined over the range of 7–9. DENV3 2′-*O*-MTase activity was assayed by incubating DENV3 MTase with two different capped RNA substrates, the small capped RNA substrate $^{7Me}GpppAC_4$ (Milani et al., 2009; Selisko et al., 2010) or the long capped RNA substrate ^{7Me}GpppA-RNA_{74} in the presence of [^{3}H] AdoMet. Briefly, the MTase activity assay was performed in 20 μL samples containing 40 mM Tris–HCl pH 7.5, 5 mM DTT, 10 μM AdoMet (0.2–2 μCi [^{3}H] AdoMet), 1 μM of DENV3 MTase and 1 μM $^{7Me}GpppAC_4$ (or 0.5 μM ^{7Me}GpppA-RNA_{74}). The enzyme and the buffer were first mixed together and the reaction was started with a premix of AdoMet and capped RNA substrates. Reactions were incubated at 30 °C for 30 min and stopped by 20-fold dilution in ice-cold 100 μM AdoHcy solution. Samples were then transferred onto DEAE membrane (DEAE Filtermat; Wallac) by a Filtermat Harvester (Packard Instruments) washed with 0.01 M ammonium formate (pH 8.0), water and ethanol, and the radioactivity transferred onto RNA was measured using a Wallac 1450 MicroBeta Trilux Liquid Scintillation Counter. DENV3 N7-MTase activity was performed as described above but using of 0.5 μM $GpppA_{2'OMe}$-RNA_{74} as substrate.

2.5. Demonstration of the specific N7 methylation in DENV3 N7-MTase assay

N7 methylation of $GpppA_{2'OMe}$-RNA_{74} was assessed by dot blot analysis using monoclonal antibodies recognizing 2,2,7-trimethylguanosine (Bochnig et al., 1987). 1.5 μM of $GpppA_{2'OMe}$-RNA_{74} was incubated at 30 °C with 500 nM of DENV3 MTase in methylation buffer for a time course analysis. DENV3 MTase was removed by treatment with 1 μL of StrataClean resin (Stratagene). The methylated RNA was lyophilized and resuspended in 1 μL of water. Each RNA was spotted onto a Nitrocellulose membrane (Whatman) and crosslinked by $UV_{260\ nm}$ during 1 h. The membrane was saturated in Tris–HCl 20 mM pH 7.6, NaCl 150 mM containing 0.1 % Tween (TBST) and 5 % BSA, and then incubated with a 1/1000 dilution of monoclonal mouse antibody against m3G-m7G-cap (Synaptic System) in TBST-5% BSA buffer for 1 h at room tempera-

ture. The membrane was washed three times for 5 min with TBST buffer before incubation with a 1/5000 dilution of rabbit anti-mouse secondary antibody conjugated to horseradish peroxidase (Dako) for 1 h at room temperature. After subsequent washings with TBST, the presence of 7-methylguanosine linked to the RNA was detected using ECL plus detection reagent (Pierce).

2.6. Inhibition testing and data analysis

We first performed the N7 and 2′-*O*-methylation reactions in the presence of 50 μM of each inhibitor. Compounds showing MTase inhibition were next tested using decreasing inhibitor concentration (with 2-fold serial dilutions) in order to determine the inhibitor concentration at 50% activity. The DENV3 MTase assay was performed as described above except that DENV3 MTase was mixed with inhibitor candidates before addition of a pre-mix of RNA substrate and AdoMet to start the reaction. The final concentration of DMSO in reaction mixtures was below 5%, and controls reactions indicate that DMSO did not alter MTase activity. All data points were measured in duplicate. The IC_{50} values were determined using Prism software and adjusted to a logistic dose–response function: % activity = $100/(1 + [I]/IC_{50})^b$, where b corresponds to the slope factor and $[I]$ to inhibitor concentration (Delean et al., 1978).

3. Results and discussion

3.1. Synthesis of $GpppA_{2'OMe}$-RNA_{74} as a specific substrate for DENV N7-MTase and $^{7Me}GpppA$-RNA_{74} as a specific substrate for DENV 2′-*O*-MTase

We recently developed a method allowing the chemical synthesis of short capped RNA (Thillier et al., 2012) at preparative scale. In the present study, we make use of these short capped RNAs to obtain long capped RNAs of authentic dengue virus genome sequence. These RNAs serve as substrates for the AdoMet-dependent DENV NS5 MTase activities. In order to follow specifically the DENV N7-MTase activity, we synthesized $GpppA_{2'OMe}$-RNA_{74}, where the 2′-*O*-position of the ribose of the first nucleotide (A) is already methylated. For this purpose, we ligated two synthetic oligoribonucleotides, $GpppA_{2'OMe}$-RNA_{12} (fragment **F-1**, Fig. 1) and a 5′-phosphorylated 62-mer (fragment **F-3**, Fig. 1) using the T4 RNA Ligase. Since it is known that nucleotide sequences surrounding the ligated bond determine the ligation efficiency, we used a donor oligomer (**F-3**) carrying a 5′-phosphorylated cytidine and a purine-rich acceptor oligomer (**F-1** and **F-2**). The chemical synthesis of $GpppA_{2'OMe}$-RNA_{12} **F-1** and $^{7Me}GpppA$-RNA_{12} **F-2** were performed in two steps on solid support (see Section 2) leading to RNAs bearing *bona fide* cap structures, with a good yield (50% and 40%, respectively). Deprotection and release of $GpppA_{2'OMe}$-RNA_{12} **F-1** and GpppA-RNA_{12} from the support were achieved using DBU followed by ammonia treatments for 3 min and 3 h at 30 °C. The crude material **F-1** (320 nmol) and GpppA-RNA_{12} (603 nmol) were analyzed and purified by anion-exchange HPLC. **F-1** was isolated with high purity (99%; yield = 29%, 93 nmol) whereas GpppA-RNA_{12} was isolated with 89% purity (yield = 15%, 91 nmol). Both capped RNAs were characterized by MALDI-TOF mass spectrometry (**F-1**: Calc. *m/z* 4621.6; Found *m/z* 4621.64; GpppA-RNA_{12}: Calc. *m/z* 4607.60; Found *m/z* 4608.60). Subsequent N7-methylation of GpppA-RNA_{12} (74 nmol) with *h*N7-MTase in solution afforded **F-2** with 81% purity and 69% yield (51 nmol). **F-2** was characterized by mass spectrometry (Calc. *m/z* 4622.60; Found *m/z* 4622.30).

To avoid self-oligomerization or RNA cyclisation of **F-3** RNA during ligation, its 3′-end was protected by a biotin moiety (see Fig. 1). The 5′-phosphorylated and 3′-biotinylated RNA oligonucleotide **F-3** was synthesized, purified, and characterized by denaturing PAGE and LC/ESI-MS (M_{found} = 20558.2 g/mol, $M_{calcd.}$ = 20558.9 g/mol).

The chemically synthesized fragments **F-1** and **F-3** were joined using T4 RNA Ligase. Various parameters were tested in order to determine optimal ligation conditions. We first compared ligation efficiency at increasing incubation times and different temperatures. Reactions were run with different **F-1/F-3** ratios at 16 °C or 37 °C in the presence of 10% DMSO (v/v) to limit RNA secondary structures. The ligation yield remained below 65 % when using T4 RNA Ligase 1. T4 RNA Ligase 2 allowed higher ligation yields when reactions were run with equimolar concentrations of acceptor **F-1** and donor **F-3**. Under optimal reaction conditions (see Section 2), about 92% of **F-3** RNA was converted into $GpppA_{2'OMe}$-RNA_{74} confirming that T4 RNA Ligase 2 is much more active joining nicks on double stranded RNA (Ho and Shuman, 2002; Nandakumar et al., 2004). A typical ligation reaction analyzed by PAGE is shown in Fig. 2 after SYBR Green II RNA staining. We note that maximum efficiency of ligation is reached after 30 min of incubation at 37 °C with a ligase concentration of 0.5 U/μL. We also applied the same ligation procedure to the $^{7Me}GpppA$-RNA_{12} (**F-2**) and 5′-phosphorylated 62-mer (**F-3**) fragments to generate $^{7Me}GpppA$-RNA_{74} with similar ligation rates.

3.2. Design of reaction conditions for N7- and 2′-*O*-methylation assays

N7- and 2′-*O*-MTase activities in DENV were previously characterized (Chung et al., 2010) and data indicated that both N7- and 2′-*O*-methylation could be carried out under the same buffer conditions (Tris–HCl pH 7.5 with or without NaCl). To ascertain the efficiency of the N7- and 2′-*O*-methylation on the $GpppA_{2'OMe}$-RNA_{74} and $^{7Me}GpppA_{2'OMe}$-RNA_{74} as substrates, respectively, we first followed the time-course of a preliminary filter-binding assay (Fig. 3A) and explored the effects of pH on DENV3 N7- and 2′-*O*-MTase activities (Fig. 3B). For this purpose, RNA substrates were tested over a pH range of 7–9 using 40 mM Tris–HCl and 5 mM DTT and incubated with DENV3 MTase in the presence of [^{3}H] AdoMet. MTase activity was followed by measuring the incorporation of [^{3}H]-methyl into RNA by the filter-binding assay. Since our goal was to compare N7- and 2′-*O*-MTase activities in a similar assay, we sought to identify assay conditions that could support both activities. Fig. 3A shows that the best incubation time at 30 °C is 30 min since N7-MTase reactions longer than 1 h reach a plateau. Fig. 3B shows that the enzyme is most active at pH 8.5 but also remains 80% active at pH 7.5. Therefore, for subsequent experiments, we selected the buffer at pH 7.5 to keep it as close as possible to physiological conditions.

To further confirm the specificity of methylation, we next compared DENV3 MTase activity with that of *h*N7-MTase and VV2′-*O*-MTase on **F-1**, **F-2**, **F-3**, $^{7Me}GpppA$-RNA_{74}, $GpppA_{2'OMe}$-RNA_{74} and $^{7Me}GpppAC_4$ substrates. Fig. 3C shows that $GpppA_{2'OMe}$-RNA_{74} is efficiently methylated by *h*N7-MTase and to a lesser extent by DENV MTase. Similar results were obtained using $GpppA_{2'OMe}$-RNA_{74} directly from the ligation mix, and from $GpppA_{2'OMe}$-RNA_{74} purified using IEX-HPLC (data not shown). As expected, the VV2′-*O*-MTase is not active on this substrate since the 2′-*O* position of the first nucleotide is already methylated (Zhou et al., 2007). Conversely, the $^{7Me}GpppA$-RNA_{74} substrate is methylated in the presence of VV2′-*O*-MTase and DENV3 MTase, but not of *h*N7-MTase, since the N7 position of its capped guanine was already methylated. For the same reason, $^{7Me}GpppAC_4$ is efficiently methylated by VV2′-*O*-MTase and to a lesser extent by DENV MTase, but *h*N7-MTase is not active on this substrate. We also observe that **F-1** capped RNA is methylated in the presence of *h*N7-MTase, but not DENV3 MTase confirming that the N7-methylation mediated by DENV3 MTase requires a long RNA mimicking the SLA hairpin structure. We also observe that the uncapped **F-3** RNA is

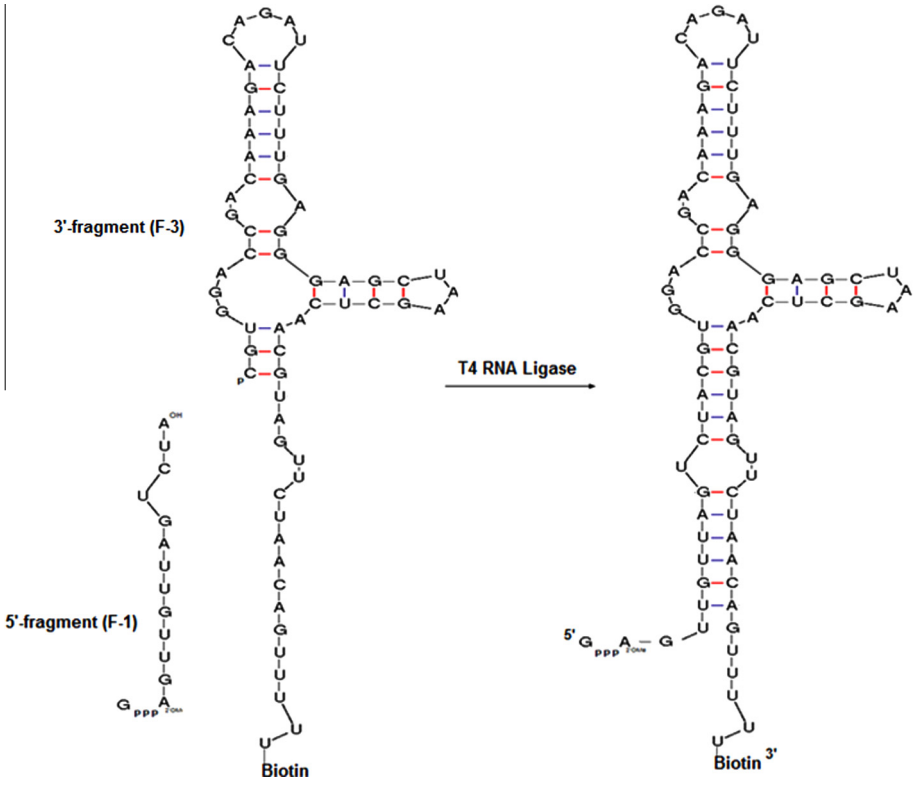

Fig. 1. Design of the long capped 5′-end DENV mRNA by fragments condensation using T4 RNA Ligase.

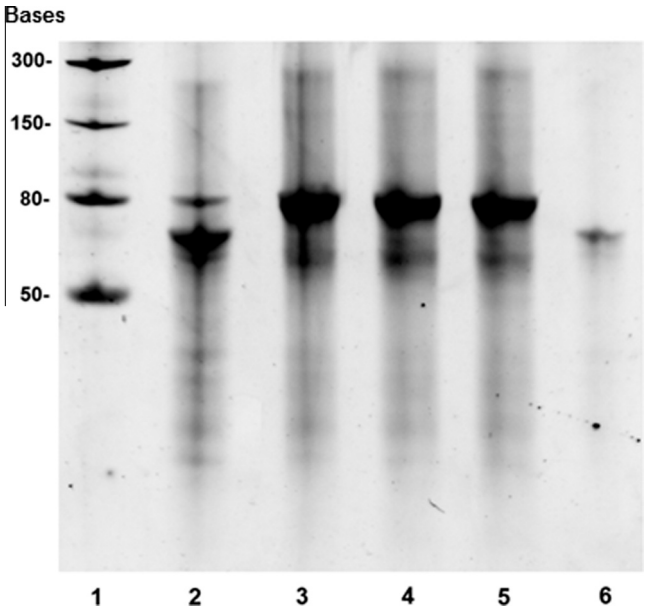

Fig. 2. Kinetics of RNA ligation followed on 14% denaturing PAGE (gel stained with SYBR green II RNA gel stain). Lane 1 consists of RNA Mw marker; Lanes 2–5 are the ligation reaction with T4 RNA Ligase 2 at 1 min, 30 min, 2 h and 6 h, respectively; Lane 6 is the 5′-phosphorylated 62-mer **F-3**.

barely methylated by DENV MTase and VV2′-*O*-MTase (≈5% compared to the GpppA$_{2'OMe}$-RNA$_{74}$). The **F-2** capped RNA is efficiently methylated in the presence of VV2′-*O*-MTase, but not in the presence of *h*N7-MTase since the N7 position of the cap guanine was already methylated. Further, we observe that DENV3 MTase is not able to methylate **F-2** in contrast to the shorter ^{7Me}GpppAC$_4$. This observation suggests that there is an optimal length and sequence allowing the 2′-*O* methylation on sequence-specific RNAs, whereas shorter non-natural molecules, such as the ^{7Me}GpppAC$_{4-8}$ can be accommodated and be effective substrates (Selisko et al., 2010). Therefore, we suggest that even for 2′-*O* inhibition assays, long sequence-specific RNAs might provide more relevant substrates.

To confirm that DENV3 MTase thoroughly converted GpppA$_{2'O-Me}$-RNA$_{74}$ into ^{7Me}GpppA$_{2'OMe}$-RNA$_{74}$, we next analyzed the RNA methylation using a monoclonal antibody recognizing 2,2,7-trimethylguanosine as well as a N7-methylated cap structure (Bochnig et al., 1987). GpppA$_{2'OMe}$-RNA$_{74}$ was incubated with DENV3 MTase, and the resulting methylated RNA was cross-linked to a nitrocellulose membrane and probed with the anti-N7-methylated guanosine monoclonal antibody. The results, shown in Fig. 4, firstly confirm that unmethylated and 2′-*O* methylated capped RNAs (negative controls) are not recognized by the antibody. The time course performed on GpppA$_{2'OMe}$-RNA$_{74}$ leads to the time-dependent increase of N7 and/or N3 methylated RNA by DENV3 MTase. Taken together with studies showing that flaviviral MTases are able to perform only 2′-*O* and N7 methylations (Selisko et al., 2010), our results demonstrate that DENV3 MTase methylates the N7 position of the cap structure present at the 5′-end of the GpppA$_{2'OMe}$-RNA$_{74}$, although we cannot entirely rule out the presence of methylation at position N3 of the cap.

3.3. Validation of N7- and 2′-O-methylation assay with compound testing

To further validate the specificity of our DENV N7-MTase assay, we tested 18 compounds, some having documented viral mRNA cap MTase inhibition potency, such as the reaction product, *S*-adenosyl-homocysteine **3** (AdoHcy) (Bheemanaik et al., 2003; Patnaik et al., 2004; Pugh and Borchardt, 1982), Sinefungin **1**

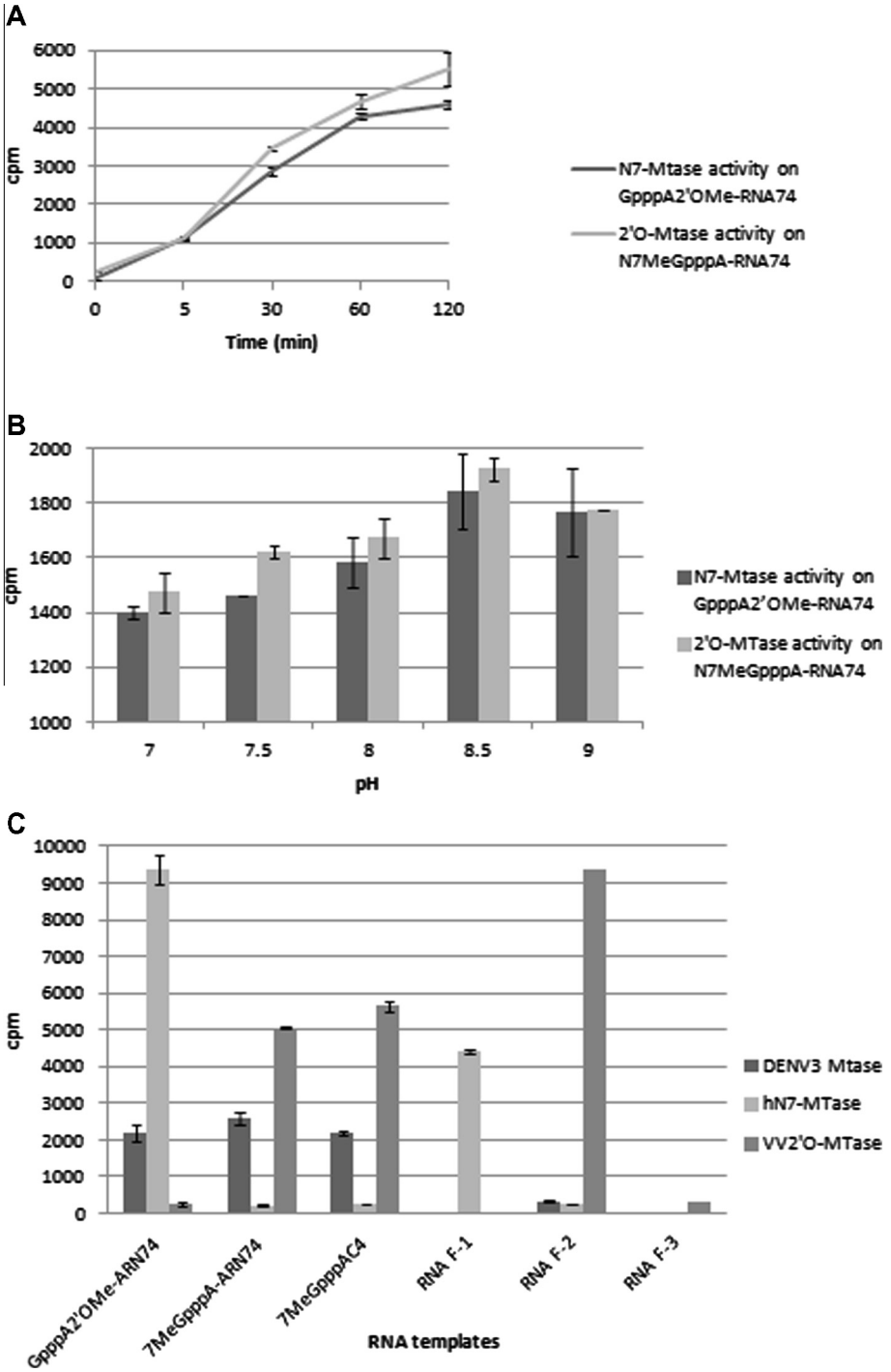

Fig. 3. Methylation activities catalyzed by DENV3 MTase, hN7MTase and VV2′OMTase on RNA templates **F-1**, **F-2**, **F-3**, $GpppA_{2'OMe}$-RNA_{74}, $^{7Me}GpppA$-RNA_{74} and $^{7Me}GpppAC_4$. (A) Time course of DENV3 N7- and 2′-*O*-MTase activities measured by filter binding assay at 30 °C (pH 7.5) in the presence of $GpppA_{2'OMe}$-RNA_{74} and $^{7Me}GpppA$-RNA_{74}, respectively. (B) Optimal pH conditions for DENV3 N7- and 2′-*O*-MTase activities using substrates $GpppA_{2'OMe}$-RNA_{74} and $^{7Me}GpppA$-RNA_{74}, respectively, were obtained by filter binding assay at 30 °C for 30 min. (C) Filter binding assay analysis of methylations on **F-1**, **F-2**, **F-3**, $GpppA_{2'OMe}$-RNA_{74}, $^{7Me}GpppA$-RNA_{74} and $^{7Me}GpppAC_4$ using DENV3 MTase (500 nM), hN7MTase (500 nM) and VV2′OMTase (150 U) at 30 °C for 30 min.

(Chrebet et al., 2005; Pugh et al., 1978; Smith and Norton, 1980), but also other AdoMet-dependent MTases inhibitors, such as 3-deaza-adenosine **2** (Kloor et al., 2004; Woyciniuk et al., 1995), 5′-*S*-isobutylthio-5′-deoxyadenosine **6** (SIBA) (Selisko et al., 2010) or an analog of adenosine, *S*-(5′-adenosyl)-L-cysteine **17**. We also included four putative cap analogs (GTP **13**, ^{7Me}GTP **12**, the broad-spectrum antiviral ribavirin **7** and its triphosphate form **8**; and two other GTP-based RNA cap analogs, GpppA **10** and $^{7Me}GpppA$ **11**) and six inhibitors (selected *in silico* and showing *in vitro* inhibition) of flavivirus mRNA cap MTase activities: a substituted adamantine derivative **4**, an oxobenzo[c]chromene derivative **5** (compounds **7** and 3, respectively from Luzhkov et al. (2007), aurintricarboxylic acid **9** (ATA) (Milani et al., 2009), 5,5′-methylenedisalicylic acid **14**, 3-[2-(4-anilinophenyl)hydrazinyl]benzenesulfonic acid **15** and 2-[(*E*)-[[2-(furan-2-yl)quinoline-4-carbonyl]hydrazinylidene] methyl]benzoic acid **16** (compounds 8, **10** and 35, respectively from

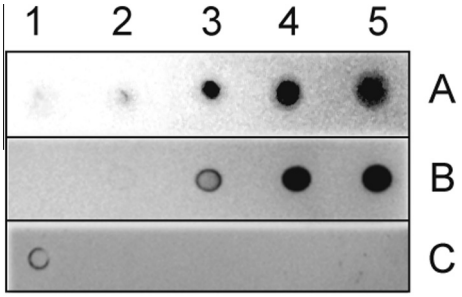

Fig. 4. Dot blot of the specific N7 methylation in DENV3 *N7*-MTase assay on ^{7Me}GpppA-RNA$_{74}$. (A) DENV3 MTase kinetic assay of ^{7Me}GpppA-RNA$_{74}$ (25 nmol) at 0 min (A1), 5 min (A2), 1 h (A3), 2 h (A4) and 4 h (A5). (B) Concentration range of ^{7Me}GpppA-RNA$_{12}$ (**F-2**) at 1 nmol (B1), 5 nmol (B2), 10 nmol (B3), 15 nmol (B4) and 25 nmol (B5). (C) Positive and negative controls: ^{7Me}GpppA-RNA$_{12}$ at 10 nmol (C1), ^{7Me}GpppA-RNA$_{12}$ at 3 nmol (C2), GpppA$_{2'OMe}$-RNA$_{12}$ (**F-1**) at 10 nmol (C3), GpppA-RNA$_{12}$ at 10 nmol (C4) and pppA-RNA$_{12}$ at 10 nmol (C5).

Podvinec et al. (2010). Finally, we tested 2-thioxothiazolidin-4-one derivative **18** recently identified by HTS, which showed potent inhibition of GTP binding and of the guanylyltransferase activity of the DENV capping enzyme (Stahla-Beek et al., 2012). We compared the DENV 2′-*O*-MTase inhibition (methylation of substrate ^{7Me}GpppAC$_4$ resulting in ^{7Me}Gppp$_{2'OMe}$AC$_4$ and substrate ^{7Me}GpppA-RNA$_{74}$ resulting in ^{7Me}Gppp$_{2'OMe}$A-RNA$_{74}$) to DENV N7-MTase inhibition (methylation of substrate GpppA$_{2'OMe}$-RNA$_{74}$ resulting in ^{7Me}GpppA$_{2'OMe}$-RNA$_{74}$). As shown in Fig. 5, **12** out of **18** compounds weakly inhibit both N7- and 2′-*O*-MTase activities (between 0% and 45%) at a concentration of 50 μM except compounds **1**, **3**, **9** and **12**–**14**. Sinefungin **1** and ATA **9** show very potent inhibition of both N7- and 2′-*O*-MTase activities. Interestingly, co-product AdoHcy **3** and compound **14** inhibit more efficiently 2′-*O*-MTase activity (on ^{7Me}GpppAC$_{4>}$ 90%) but show limited (30–40%) inhibition of N7-MTase activity. Likewise, compounds **12** and **13** can also be considered as more potent inhibitors of 2′-*O*-MTase than of the N7-MTase activity.

The IC$_{50}$ of two AdoMet analogs (Sinefungin **1** and AdoHcy **3**), two GTP-pocket binders (^{7Me}GTP **12** and GTP **13**), compound **14** and ATA **9** were determined for both N7- and 2′-*O*-MTase activities (Table 1). DENV3 MTase was pre-incubated with increasing concentrations of inhibitor for 5 min, and the reaction was started with [^{3}H] AdoMet and appropriate RNA substrates. As expected, dose response curves indicate that Sinefungin **1** and ATA **9** strongly inhibit both N7- and 2′-*O*-MTase activities. Sinefungin **1** shows a 8- to 10-fold stronger inhibition of N7-MTase compared to 2′-*O*-MTase activity with IC$_{50}$ values in the submicromolar to 4 micromolar range, respectively (Table 1 and Fig. 6). ATA **9** inhibits both activities with IC$_{50}$ values in the range of 2–8 μM, whichever substrate may be. Milani et al. (2009) described a comparable IC$_{50}$ value (2.3 μM) for ATA using a filter-binding assay to measure the 2′-*O*-MTase activity, but they determined a higher IC$_{50}$ value of 127 μM for the N7-MTase activity which was monitored through a TLC assay. Interestingly, compound **14** can be considered as an ATA derivative (missing one salicylic acid) and exhibits, as ATA, a low IC$_{50}$ value for 2′-*O*-MTase activity on ^{7Me}Gppp AC$_4$. However,

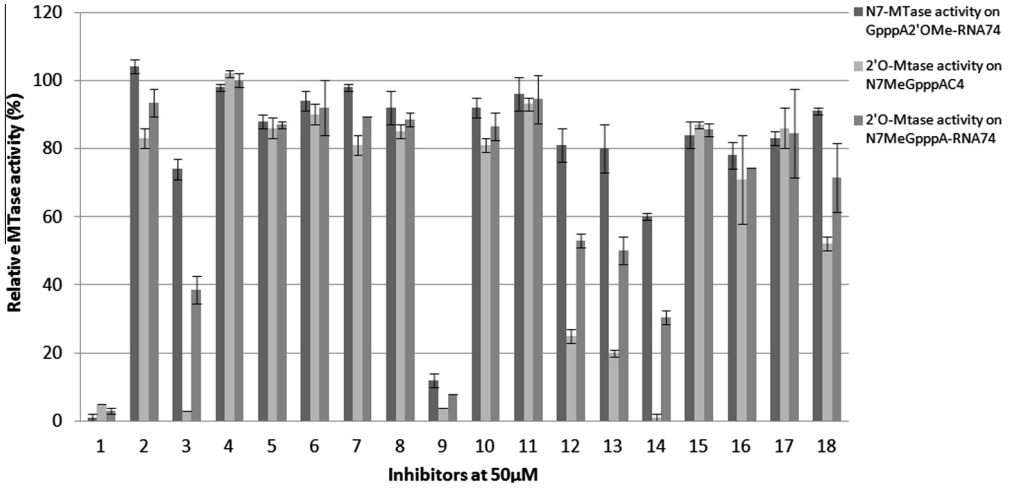

Fig. 5. Inhibition of DENV3 N7- and 2′-*O*-MTase activities by 18 putative inhibitors. DENV3 MTase was incubated with GpppA$_{2'OMe}$-RNA$_{74}$ (for N7-MTase activity), ^{7Me}GpppAC$_4$ or ^{7Me}GpppA-RNA$_{74}$ (for 2′-*O*-MTase activity) and [^{3}H]AdoMet in the presence of 50 μM of each inhibitor candidate as given in Methods. Error bars represent SD values of two to three independent experiments.

Table 1
IC$_{50}$ determination of competitive inhibitors (Sinefungin **1**, AdoHcy **3**, ATA **9** and compound **14**) and GTP-pocket binders (^{7Me}GTP **12** and GTP **13**) on DENV3 N7- and 2′-*O*-MTase activities.

Compounds	N7-MTase activity on GpppA$_{2'OMe}$-RNA$_{74}$ IC$_{50}$ (μM)	2′*O*-MTase activity on ^{7Me}GpppAC$_4$ IC$_{50}$ (μM)	2′*O*-MTase activity on ^{7Me}GpppA-RNA$_{74}$ IC$_{50}$ (μM)
Sinefungin **1**	0.49 ± 0.04	3.75 ± 0.35	4.37 ± 0.29
AdoHcy **3**	94.26 ± 8.06	1.91 ± 0.13	19.33 ± 1.46
ATA **9**	3.27 ± 0.19	2.10 ± 0.15	8.17 ± 0.89
^{7Me}GTP **12**	138.80 ± 17.25	4.58 ± 0.72	75.57 ± 6.01
GTP **13**	77.51 ± 8.11	3.04 ± 0.42	68.90 ± 11.17
5,5′-Methylene-disalicylic acid **14**	51.12 ± 5.54	6.93 ± 0.52	40.11 ± 2.89

The results shown are the average of two independent experiments and the average of *Z*-factor obtained was ⩾0.65.

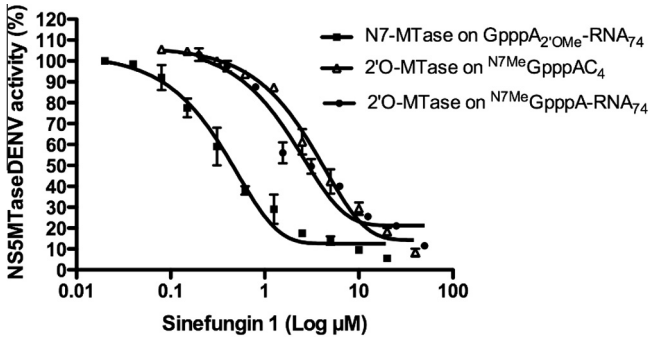

Fig. 6. Example of dose-dependent inhibition curves of DENV3 N7- and 2′*O*-MTase activities by Sinefungin **1**.

it exhibits higher IC_{50} value for both N7 and 2′-*O*-MTase activities on longer RNAs (around 50 μM), suggesting that compound **14** is less potent than ATA. A similar observation was made for ^{7Me}GTP (compound **12**) or GTP (compound **13**) with IC_{50} below 5 μM on the 2′-*O*-MTase assay using the short non homologous RNA and with IC_{50} over 50 μM for the 2′-*O*- and N7-MTases activities on longer RNA substrates. This suggests that the size or the hairpin structure present in the substrate RNA favored its specific recognition by DENV3 MTase. AdoHcy **3** efficiently inhibits 2′-*O*-methylation on short non homologous RNA (IC_{50} = 1.9 μM) and to a lower extend on long homologous RNA, but shows a limited (IC_{50} = 94 - μM) inhibition potency on N7-MTase activity. Chung et al. (2010) also reported that AdoHcy was almost 4-fold less active against DENV N7-MTase compared to 2′-*O*-MTase. This ratio is highly enhanced in the assay using ^{7Me}Gppp AC_4 but it is comparable when using long homologous sequences. The reasons for this difference may be attributed to the different assay formats used and to the presence of different RNA templates. Interestingly, it can be noticed from Fig. 6 and table 1 that all the compounds inhibiting the 2′-*O* activity on ^{7Me}GpppA-RNA_{74} have an equal or a more drastic effect when using the shorter non natural RNA substrate ^{7Me}GpppAC_4. Taking into account that the synthesis of the latter RNA is cheaper and that it can provide a more sensitive inhibition assay, its use on HTS screening can be relevant, whereas at later stages, when addressing specificity of optimized compounds, the use of ^{7Me}GpppA-RNA_{74} mimicking the 5′-end of the viral genome could be more relevant. Altogether, our results demonstrate that a sensitive assay is now available to discover and characterize inhibitors of the AdoMet-dependent N7-MTase. Moreover, N7- and 2′-*O*-Mtase activities can be discriminated under similar assay conditions and specific N7- and 2′-*O*-MTase RNA substrates.

In conclusion, we developed an efficient method to produce a long capped RNA used as substrate to assess specific DENV N7- and 2′-*O*-MTase activity *via* a sensitive filter-binding assay. Our method overcomes two important bottlenecks for the synthesis of capped RNAs. Firstly, it is now possible to get long synthetic capped RNAs in high yield without any 5′-end sequence. Secondly, this method also allows obtaining RNAs capped exclusively with a GpppA cap structure, i.e. unmethylated at its N7 position. Moreover, these synthetic RNAs constitute suitable substrates to follow the N7-MTase activity of DENV3 uncoupled from its 2′-*O*-MTase activity. The sequence/structure specificity of the long capped RNA can be easily adapted to study the enzymatic activities of a wide variety of RNA capping MTases. This assay also constitutes a robust and sensitive screening test for the identification and characterization of DENV N7- and 2′-*O*-MTase inhibitors, which was confirmed through the determination of low IC_{50} values of known AdoMet-dependent MTase inhibitors. The specificity of this assay should ease anti-flaviviral drug design.

Acknowledgements

We wish to thank Julie Lichière and Joelle Boretto-Soler for technical assistance and Dr. Karine Alvarez for helpful discussions. This work was supported by the project SILVER (Health-F3-2010-260644) of the European Union 7th Framework Program and by the French National Agency for Research (ANR-12-BSV3-007-01 and 02).

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