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

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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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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