Binding of the methyl donor SAM to MERS-CoV 2'-O-methyltransferase nsp16 promotes the recruitment of the allosteric activator nsp10.

Short title: MERS-CoV 2'-O-methyltransferase allosteric regulation

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Abstract:

The Middle East respiratory syndrome coronavirus (MERS-CoV) non-structural protein 16 (nsp16) is an S-adenosyl-L-methionine (SAM)-dependent 2′-O-methyltransferase (MTase) that is thought to methylate the ribose 2′-OH of the first transcribed nucleotide (N1) of viral RNA cap structures. This 2′-O MTase activity is regulated by nsp10. The 2′-O methylation prevents virus detection by cell innate immunity mechanisms and viral translation inhibition by the interferon-stimulated IFIT-1 protein.

To unravel the regulation of nsp10/nsp16 2′-O-MTase activity, we used purified MERS-CoV nsp16 and nsp10. First, we showed that nsp16 recruited N7-methylated capped RNA and SAM. The SAM binding promotes then the assembly of the enzymatically active nsp10/nsp16 complex that converted 7mGpppG (cap-0) into 7mGpppG2′Om (cap-1) RNA by 2′-OH methylation of N1 in a SAM-dependent manner. The subsequent release of SAH speeds up nsp10/nsp16 dissociation that stimulates the reaction turnover. Alanine mutagenesis and RNA binding assays allowed the identification of the nsp16 residues involved in RNA recognition forming the RNA binding groove (K46, K170, E203, D133, R38, Y47 and Y181) and the cap-0 binding site (Y30, Y132 and H174). Finally, we found that nsp10/nsp16 2′-O-MTase activity is sensitive to known MTase inhibitors, such as sinefungin and cap analogues. This characterization of the MERS-CoV 2′-O-MTase is a preliminary step towards the development of molecules to inhibit cap 2′-O methylation and to restore the host antiviral response.
MERS-CoV codes for a cap 2'-O-methyltransferase that converts cap-0 into cap-1 structure in order to prevent virus detection by cell innate immunity mechanisms. We report the biochemical properties of MERS-CoV 2’O-methyltransferase, which is stimulated by nsp10 acting as an allostERIC activator of the nsp16 2’-O-methyltransferase possibly through enhanced RNA binding affinity. In addition, we show that SAM promotes the formation of the active nsp10/nsp16 complex. Conversely, after cap methylation, the reaction turnover is speeded up by cap-1 RNA release and nsp10/nsp16 complex dissociation, at the low intracellular SAH concentration. These results suggest that SAM/SAH balance is a regulator of the 2’-O-methyltransferase activity and raises the possibility that SAH hydrolase inhibitors might interfere with CoV replication cycle. The enzymatic and the RNA binding assays developed in this work were also used to identify nsp16 residues involved in cap-0 RNA recognition and to understand the action mode of known methyltransferase inhibitors.
Introduction:

Middle East respiratory syndrome coronavirus (MERS-CoV) is an emerging zoonotic betacoronavirus that was initially isolated from a 60-year-old Saudi Arabian man in September 2012 (1). Since then, MERS-CoV is steadily spreading in the Arabian Peninsula and contained secondary outbreaks have occurred in Europe, Africa, Asia and North America with more than 1728 confirmed infected patients up to April 2016 and a 36% fatality rate (2). MERS-CoV infection may be asymptomatic or result in clinical symptoms ranging from mild respiratory illness to severe acute pneumonia, renal failure, systemic disorder (1) and severe neurological syndrome (3). MERS-CoV belongs to the lineage C of the genus betacoronavirus and is phylogenetically related to the bat coronaviruses HKU4 and HKU5 (4–7). The bats are the host of a variety of coronaviruses including betacoronavirus genetically very closely related to the MERS-CoV which crossed the species barrier into dromedary camels (2). The virus was detected in Camelus dromedarius milk and nasal swabs, suggesting that camels are the probable source for zoonotic transmission of the virus to humans (6, 8). Recent works found at least five lineages of MERS-CoV in camels and identified six recombination events in MERS-CoV which may raise the virus’ pathogenicity (9). Human-to-human transmission requires close contact and occurred principally in health care settings (8)(10). Currently there are no approved antiviral treatments or vaccines available against MERS-CoV infection.

Following binding of MERS-CoV spike envelope proteins to the dipeptidyl peptidase-4 receptor (DPP4, also known as CD26) (11), the virus genome
is released in the host cytoplasm. MERS-CoV genome is a polycistronic
positive-stranded RNA of 30119 nt in length organized in eleven open
reading frames (ORFs). The 3’ part of the genome contains at least nine
ORFs that encode structural and virus accessory proteins, which are
translated from a set of subgenomic RNAs. The two large ORFs (1a and b)
that encode non-structural proteins (nsp) are in the 5’ proximal two thirds of
the genome (12). After viral infection, the 5’ region of capped genomic RNA
is directly translated into the polyprotein pp1a and pp1ab, which are the
precursors of the CoV nsps. The pp1ab protein involves a ribosomal
frameshift during RNA translation. These precursors proteins are cleaved
by viral proteinases into 11 and 16 nsps, respectively, that form the
replication-transcription complex (RTC) (12, 13). RTC harbours the catalytic
activities required for the synthesis of genomic and subgenomic RNAs and
contains most of the enzymes involved in the formation of the cap structure
that decorates the 5’ end of viral mRNA.

Capping is a common modification of the 5’ end of eukaryotic mRNA. The
cap structure consists of a methylated guanosine at position 7 linked to the
first transcribed nucleotide by a 5’-5’ triphosphate bridge (\(^7\text{mGpppN}\)). The
cap is co-transcriptionally added to the 5’ end of nascent mRNA after
synthesis of the first 20-30 nucleotides (11,12). Cap structure synthesis
involves four sequential reactions catalysed by an RNA 5’ triphosphatase
(TPase), a guanylyltransferase (GTase), a guanine N7 methyltransferase
(N7-MTase) and a 2’-O-MTase (15). The cap structure plays several key
roles in mRNA functionality. First, capping is essential for mRNA stability
and limits its degradation by cellular 5’-3’ exoribonucleases, such as XRN1
In the cytoplasm, the cap-0 structure ($^{7}\text{mGpppN}$) ensures efficient mRNA recognition by the eukaryotic translation Initiation Factor 4E (eIF4E) (15). RNA capping also provides a molecular signature for the discrimination between self and non-self RNA. Indeed, viral double stranded RNA, 5'-triphosphate RNA and incorrectly capped RNA are detected by intracellular pathogen recognition receptors (PRRs). Among these PRRs, Retinoic acid-Inducible Gene (RIG)-like receptors, such as RIG-I and Melanoma differentiation-associated protein 5 (Mda5), can detect uncapped 5′-triphosphate RNA and also cap-0 RNA (17–20). Detection of erroneously capped RNA in the cytoplasm induces a signal transduction cascade that initiates an antiviral response through interferon production (21). Among the interferon-stimulated genes (ISG), interferon-induced protein with tetratricopeptide repeats 1 (IFIT 1) can recognize mis-capped RNA and inhibit its translation (22, 23).

Like other CoVs, MERS-CoV replicates in the cytoplasm of infected cells where it should produce its own capping machinery. CoVs seem to adopt the eukaryotic canonical capping pathway with four sequential events that involve several viral nsps: (i) first, the 5′-3′helicase/nucleoside triphosphatase (NTPase) nsp13 hydrolysies the γ-phosphate from nascent 5′-triphosphate RNA (24); (ii) then, a guanosine monophosphate (GMP) molecule is transferred to 5′-diphosphate RNA by a yet-unknown guanyllytransferase, thus forming a primitive cap structure (GpppN); (iii) then, the guanosine is methylated, at the N7 position, by nsp14 in the presence of the methyl donor S-adenosyl-L-methionine (SAM) to produce the cap-0 structure ($^{7}\text{mGpppN}$) and S-adenosyl-homocysteine (SAH) as by-
product (25); (iv) finally, the nsp10/nsp16 complex methylates the 2'-OH group (ribose) of the first transcribed nucleotide of the viral RNA, leading to the conversion of the cap-0 into a cap-1 structure (7mGpppN₂̂om) (26, 27).

Thus, in CoVs, RNA cap methylation might involve at least three proteins (nsp10, nsp14 and nsp16) and it is known that both nsp14 and nsp16 can interact with nsp10 (28).

CoV 2'-O-MTase activity is mediated by nsp16 that contains both a conserved K-D-K-E catalytic tetrad, which is characteristic of SAM-dependent 2'-O-MTases, and a conserved SAM-binding site (29). CoV 2'-O-MTase activity was first demonstrated using in vitro biochemical assays with purified feline CoV (FCoV) nsp16 (30). However, recombinant SARS-CoV nsp16 was inactive using similar experimental conditions. It was then demonstrated that SARS-CoV nsp16 needs to interact with nsp10 to become catalytically active (26). The nsp10/nsp16 complex MTase activity was demonstrated using synthetic capped N7-methylated RNA and longer RNAs that mimic the 5' end of the SARS-CoV genome (26). In contrast, RNA with an unmethylated cap structure (Gppp-RNA) was not recognized by the nsp10/nsp16 complex and no enzymatic activity was detected.

Several mutagenesis studies of SARS-CoV nsp10 and nsp16 confirmed the key role of the K-D-K-E catalytic tetrad for the 2'-O-MTase activity (31) and demonstrated that the interaction between nsp10 and nsp16 is absolutely required for this activity (31, 32). The molecular basis governing nsp16 stimulation by nsp10 was partially elucidated by the crystal structure determination of the SARS-CoV nsp10/nsp16 complex (27, 31). Nsp16 adopts the catechol O-MTase fold containing alternating β strands (β1-β7).
and α helices (αZ and αA- αE) that form a seven-stranded β sheet with three α helices on each side (31, 33). In addition, nsp10 binds to nsp16 through a 930 Å² activation area on nsp10 and stabilizes nsp16 (26, 27, 31, 34). Moreover, structural and biochemical analyses also suggest that nsp10 binding extends and narrows the RNA binding groove to accommodate the RNA substrate and enhances nsp16 RNA- and SAM-binding properties. Although cap 2'-O-MTases are present in different virus families (16), the exact role of this RNA-cap modification was only recently unravelled through reverse genetic studies performed, among others, on CoV (17–20). Single point mutations in the conserved K-D-K-E tetrad of nsp16 have revealed a barely attenuated phenotype in infected cells (17, 35). In contrast, infection of small animal models by viruses expressing nsp16 with active-site substitution showed a robust reduction of viral titers and less severe infection symptoms (weight loss, lung histology and breathing function) concomitantly with a strong antiviral response, possibly linked to stimulation of the innate immunity. Indeed, it was shown that incompletely capped RNA can be detected by RIG-I or Mda5 (17, 19, 20), leading to the initiation of a signalling cascade that stimulates the secretion of type-I IFN and induces an antiviral response in neighbouring cells. Altogether these observations indicate that the inhibition of the viral 2'-O-MTase activity might stimulate the detection of viral RNAs by RIG-I or Mda5 and restore the host antiviral response mediated by ISG such as IFIT proteins (36). In this study, we assessed the biochemical activity of the MERS-CoV nsp10/nsp16 complex MTase. We show that MERS-CoV nsp16 possesses 2'-O-MTase activity. As previously shown nsp16 is stimulated by nsp10,
and leads to 2'-O-methylation of cap-0 RNA (34). Biochemical assays were used to decipher the fine-tuning of the reaction turnover. Mutagenesis combined with RNA binding assays identified the residues essential for cap-0 recognition and those forming the RNA binding groove. Finally, using our radioactive enzymatic assay we screen a small subset of MTase inhibitors blocking nsp10/nsp16 MTase activity and the compounds blocking RNA recognition were identified using the fluorescence polarization assay.
Materials and methods

Plasmid constructs

The expression vectors pDEST-14/6 His-nsp10, pDEST-14/6 His-nsp16 containing the coding sequence of the human betacoronavirus 2c EMC/2012 (GenBank: JX869059.2), a MERS-CoV strain, were provided by Eric Snijder’s team. For alanine scanning, nsp16 mutants were generated by single site amino acid substitution to alanine using the pDEST-14/6His-nsp16 plasmid and the Quickchange site directed mutagenesis kit (Agilent), according to the manufacturer’s instructions (mutagenic primers are listed in Table S1). The mutant numbering starts at the beginning of the nsp16 sequence: Y30A, K31A, R38A, H41A, N43A, K46A, Y47A, K76A, D99A, N101A, D130A, Y132A, D133A, T136A, K137A, F149A, F150A, K170A, H174A, Y181A, E203A. All constructs were verified by DNA sequencing (Eurofins MWG operon).

Expression and purification of the MERS-CoV nsp10, nsp16 and nsp10/nsp16 proteins

MERS-CoV nsp10 and nsp16 fusion proteins (N-terminal hexahistidine tag) were expressed in E. coli C2566 cells that contain the pRARE-2 plasmid. Transformed bacteria cells were grown at 37°C in LB and 2YT medium, containing 100 µg/ml ampicillin and 17 µg/ml chloramphenicol. Protein expression was induced by addition of 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG). After overnight incubation at 17°C, cells expressing nsp10, nsp16 or a mixture of nsp10 and nsp16 (equal volume) were pelleted by centrifugation (13,000 x g, 10 min) and frozen before
resuspension in lysis buffer (50 mM Hepes pH 7.5, 300 mM NaCl, 30 mM imidazole, 10% glycerol supplemented with 1 mM PMSF, 0.25 mg/ml lysozyme and 10 µg/ml DNase I). After sonication and clarification (80,000 x g, 4°C, 30 min), the supernatant were incubated with HisPur™ Cobalt resin (Thermo Scientific) at 4°C with gentle shaking for 30 min. After washing in buffer W (50 mM Hepes pH 7.5, 40 mM imidazole, 10% glycerol and 1 mM TCEP) containing 300 mM or 500 mM NaCl, bound proteins were eluted with buffer W supplemented with 250 mM imidazole. Recombinant nsp10 and nsp16 proteins were then concentrated by ultrafiltration devices with molecular weight cut-offs (MWCO) of 5 kDa (Millipore) and 10 kDa (Sartorius), respectively. Nsp10/nsp16 complex was purified on a Superdex 200 column 16/60 (GE) equilibrated with buffer W containing 500 mM NaCl (GE ÄKTApurifier). After peak collection, fractions containing the nsp10/nsp16 complex were concentrated by ultrafiltration using 10 kDa Vivaspin 15 (Sartorius). All purified proteins were analysed by SDS-PAGE followed by Coomassie blue staining. Purified enzymes were stored at -20°C in 50% glycerol for enzymatic assays.

Methyltransferase assays

Assay to test the MTase activity were carried out in a reaction mixture (40 mM Tris-HCl, pH 8.0, 1 mM DTT, 1 mM MgCl₂, 2 µM SAM and 0.33 µM ³H-SAM (Perkin Elemer) in the presence of 0.7 µM synthetic RNA corresponding to the 5’ extremity of the MERS-CoV genome with various 5’ end modifications (tri-phosphorylated: pppGAUUAA, cap:...
GpppGAUUUAA, cap-0: \( \text{mGpppGAUUUAA} \), or 2'-O-methylated cap: GpppG\(_{2'\text{OM}}\)AUUUAA). Purified nsp10 was added at a final concentration of 2 µM in presence of 1.2 µM of nsp16 as the apparent interaction \( K_d \) is about 2 µM (Figure 2A). The purified nsp10/nsp16 complex was used at 1 µM. Reaction mixtures were incubated at 30°C and stopped at the indicated time points by diluting the reaction mixture with a ten-fold excess of 20 microM ice-cold SAH (AdoHcy, New England Biolabs). Samples were then transferred to diethylaminoethylcellulose (DEAE) filters (Perkin Elmer) by using a Filtermat Harvester apparatus (Packard Instruments). Unincorporated \( ^3\text{H}-\text{SAM} \) was removed from the DEAE filters by several washes with 0.01 M ammonium formate, pH 8.0, H₂O, and absolute ethanol. After drying, filters were incubated with BetaplateScint (Wallac) scintillation fluid before quantification of the \( ^3\text{H} \) methylation of the RNA substrates using a Wallac 1450 MicroBetaTriLux liquid scintillation counter (results were expressed as counts per minute, c.p.m.).

For the MTase inhibition assays, 0.5 µM nsp10/nsp16 complex was mixed with 50 µM of each candidate inhibitor before addition of SAM (AdoMet, New England Biolabs) and \( \text{mGpppGAUUUAA} \) to start the reaction. The final DMSO concentration in the reaction mixtures was lower than 5%, and control reactions contained a similar DMSO concentration. Reaction mixtures were incubated at 30 °C for 30 min and then analysed by filter binding assay, as described above.

The IC\(_{50}\) values of SAH (AdoHcy), sinefungin, cap analogues (\( \text{mGpppA} \) and \( \text{mGpppG} \)) were determined with GraphPad Prism using a log (inhibitor) vs response variable slope equation.
Fluorescence polarization assays

RNA labelling: 10 µM RNA was labelled by ligation of 12.5 µM pCp-Cyanine-5 (pCp-Cy5) (Jena bioscience) at the 3' extremity using 1 mM T4 RNA ligase-1 (New England Biolabs) in 50 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 1 mM DTT and 1 mM ATP at 16°C overnight, according to the manufacturer's instruction. T4 RNA ligase-1 was removed using StrataClean Resin (Agilent) and labelled RNA was collected after centrifugation in a Micro-Spin G-25 column (GE healthcare) to eliminate the free pCp-Cy5 dye.

Polarization assay: pCp-Cy5-labelled RNA was mixed with increasing concentrations of freshly purified nsp10/nsp16 complex, nsp10 or nsp16 in binding buffer (50 mM Tris-HCl, pH 8, and 1 mM DTT) in a 384-well opaque microplate (Greiner bio-one). Fluorescence polarization (FP) was measured using a microplate reader (PHERAstar) with the optic module FP 590/675 (590 nm excitation and 675 nm emission wavelengths). The dissociation constant values (K_d) were determined using non-linear regression analysis: site-specific binding with Hill slope equation (GraphPad Prism). The competitive effect of cap analogues on 7mGpppGAUUUAA-cy-5 binding by 20 µM nsp10/nsp16 complex was determined using increasing concentrations of GpppG or 7mGpppG (New England Biolabs) in binding buffer. Data were plotted in GraphPad Prism 5.0 and a nonlinear sigmoidal dose response curve fitted in order to determine IC50 values of RNA binding inhibition.
Interferometry for nsp10/nsp16 interaction

Nsp10 biotinylation: nsp10 was biotinylated (at room temperature for 30 min) using EZ-Link NHS-PEG4-biotin (Thermofisher) at a molar ratio of 1:1 in biotinylation buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM TCEP). Biotinylated nsp10 was separated from free biotin using NAP-5 columns equilibrated with biotinylation buffer.

Octet analysis: Assays were performed using streptavidin sensors pre-incubated in 50 mM Hepes pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM TCEP and 0.5 mg/ml BSA. Biotinylated nsp10 (100 nM) was loaded on streptavidin-coated biosensors for 5000s to reach 0.8 nm binding. Various nsp16 concentrations (0 to 7.8 µM) were used for the association step (300s) performed in 50 mM Hepes pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM TCEP and 0.5 mg/ml BSA. The dissociation step was followed for 500s. Real time nsp10-nsp16 interaction kinetics were recorded by bio-layer interferometry (BLI: Octet RED96). The $K_d$, $K_{on}$ and $K_{off}$ values were calculated using the forteBio software after subtraction of the reference sensor from the sample traces. All curves were fitted using a 1:1 interaction model. The steady state curve of nsp10/nsp16 interaction was traced using one site-specific binding with the Hill slope equation (GraphPad Prism).

Synthesis of RNA substrates

RNAs were chemically synthesized on a solid support using an ABI 394 synthesizer. After RNA elongation with 2'-O-pivaloyloxymethyl phosphoramidite monomers (Chemgenes, USA) (37), the 5'-hydroxyl group was phosphorylated and the resulting $H$-phosphonate derivative oxidized and activated into a
phosphoroimidazolidate derivative to react with pyrophosphate (pppRNA) (38) or guanosine diphosphate (GpppRNA) (39). After deprotection and release from the solid support, pppRNAs and GpppRNAs were purified by IEX-HPLC and their purity (>95%) confirmed by MALDI-TOF spectrometry. N7-methylation of purified GpppRNAs was then performed enzymatically using an N7-hMTase (39).

**Mass spectrometry and IEX-HPLC analysis**

Crude pppRNAs and GpppRNAs were analysed and purified by IEX-HPLC on a Dionex Ultimate 3000 apparatus equipped with an UV detector at the wavelength of 260 nm and an anion exchange DionexDNAPac® PA200 column (4 x 250 mm for analysis or 9 x 250 mm for semi-preparative purpose) with a flow rate of 1.5 ml/min for analysis or 5 ml/min for semi-preparative purposes. The following eluents were used: Buffer A (20% CH$_3$CN in 25 mM Tris-HCl, pH 8), Buffer B (20% CH$_3$CN containing 200 mM NaClO$_4$ in 25 mM Tris-HCl, pH 8). After purification, pure fractions containing the desired RNA were pooled in a 100 ml round-bottomed flask and concentrated to dryness under reduced pressure. Residues were desalted on a C$_{18}$ cartridge (Sep-Pak® Classic). MALDI-TOF mass spectra were recorded on a Voyager-DE (PerSeptive Biosystems, USA) or on an AXIMA Assurance spectrometer (Shimadzu corp., Japan) equipped with a N$_2$ laser (337nm) using 2,4,6-trihydroxyacetophenone as saturated solution in a mixture of acetonitrile/0.1M ammonium citrate solution (1:1, v/v) for the matrix. The ionization mode was a linear negative ion mode. Analytical samples were mixed with the matrix in a 1:5 (v/v) ratio, crystallized on a 100-well stainless steel plate and analysed.
**Results**

MERS-CoV *nsp16* assembles with *nsp10* to constitute an active SAM-dependent cap-0 2'-O-methyltransferase.

The CoV nsp16 2'-O-MTase contains both a conserved K-D-K-E catalytic tetrad, which is characteristic of SAM-dependent 2'-O-MTase, and a conserved SAM-binding site (29). While purified feline CoV (FCoV) nsp16 shows a weak 2'-O-MTase activity in absence of nsp10, SARS-CoV nsp16 2'-O-MTase activity is strictly dependent on its interaction with nsp10 (30, 34). MERS-CoV nsp16 shows ~65% and ~53% amino acid residue similarity with SARS-CoV and FCoV nsp16, respectively; and requires nsp10 for nsp16 2'-O-MTase activity (34). To assess the mechanism of fine-tuning of MERS-CoV nsp16 2'-O-MTase activity by nsp10, recombinant nsp10 and nsp16 with an N-terminal His$_6$-tag were produced and purified, and SDS-PAGE analysis showed that nsp10 and nsp16 migrating at the expected molecular weight (Figure 1A, 16 kDa and 37 kDa, respectively) and their identity was confirmed by mass spectrometry after trypsin digestion (not shown). The nsp10/nsp16 complex was obtained by co-lysis of an equal volume of His$_6$-nsp10 and His$_6$-nsp16 expressing bacteria and purification by metal affinity chromatography (IMAC) and size-exclusion chromatography. The chromatogram (Figure 1B) indicated that the nsp10/nsp16 complex was detected as one major peak eluting at a volume corresponding to an nsp10/nsp16 heterodimer (~ 50 kDa).

Then, *in vitro* MTase assays were performed using RNA oligonucleotides with sequences corresponding to the 5'-end of the MERS-CoV genome with
various 5'-end modifications (pppGAUUUAA, GpppGAUUUAA, 7mGpppGAUUUAA, GpppG_2'omAUUUAA, 7mGpppG_2'omAUUUAA) in the presence of nsp10 or nsp16 alone or as a complex and of 3H-SAM as methyl donor. Quantification of the amount of [3H]-CH3 transferred to RNA over time using a DEAE filter-binding assay indicated the absence of MTase activity when nsp10 or nsp16 alone was used (Figure 1C). Conversely, RNA carrying a cap-0 structure (7mGpppGAUUUAA) was methylated in a time-dependent manner when incubated with both nsp16 and nsp10 or with the purified nsp10/nsp16 complex. As MTase activity was detected only using 7mGpppGAUUUAA but not GpppG_2'omAUUUAA or 7mGpppG_2'omAUUUAA, we hypothesized that the MERS-CoV MTase converts cap-0 into cap-1 RNA. The addition of a single methyl group on 7mGpppGAUUUAA was confirmed by comparing the molecular weight of 7mGpppGAUUUAA before and after incubation with nsp10/nsp16 and SAM. We detected a mass increase of about \( \approx 14.2 \) Da by MALDI-TOF mass spectrometry analysis indicating that one methylation event has occurred. We conclude that MERS-CoV nsp16 carries the cap 2'-O-MTase activity and is stimulated by nsp10. The nsp10 stimulation effect on nsp16 was confirmed by assessing nsp16 2'-O-MTase activity in the presence of increasing concentrations of nsp10. An apparent dissociation constant for the nsp10/nsp16 complex could be derived from the stimulation curves (K_d = 2 ± 0.1 μM, Figure 2A). Moreover, the nsp10/nsp16 MTase activity required the addition of divalent ions (Mg^{2+} or Mn^{2+}) and was inhibited by EDTA (Figure 2B). The optimum of activity peaked between pH 8 and 8.5 (Figure 2C). The optimal concentration of methyl-donor (SAM) (K_d = 4.3 ±
0.6 µM) was also determined by measuring the MTase activity of the complex in the presence of increasing SAM concentrations (Figure 2D). Finally, MTase assays performed using the optimal conditions deduced from these biochemical assays and cap-0 RNAs with sequences corresponding to the 5'-end of various viruses showed that the nsp10/nsp16 complex methylated cap-0 RNAs that started with either an A or a G (Figure 2E). Taken together, these results suggest that MERS-CoV nsp16 acts as an nsp10-dependent 2'-O-MTase that converts cap-0 into cap-1 RNA structures with no obvious specific recognition mechanism of the MERS-CoV sequence. Moreover, the absence of 2'-O-MTase activity using GpppRNA suggests that nsp16 can discriminate cap-0 structure from an unmethylated cap structure.

*The MERS-CoV nsp10/nsp16 complex recognizes specifically a cap-0 structure; and downstream RNA nucleotides promote loading of cap-0 substrate and their 2'O methylation*

RNA binding assays were then developed to determine why the nsp10/nsp16 complex was active only on cap-0 RNA. For this purpose, the 3'-end of various 5'-end-modified RNA was labelled with pCp-Cy5 and then their binding to freshly purified MERS-CoV nsp10/nsp16 complex was measured in fluorescence polarization assays. The nsp10/nsp16 complex recognized only cap-0 RNA (7mGpppRNA) with a KD of ~0.35 µM (Figure 3A). No significant binding was detected using the other RNAs (pppRNA, or GpppRNA, GpppG 2'omRNA, 7mGpppG 2'omRNA). This observation is
consistent with the MTase assay results (Figure 1C) and indicates that the absence of nsp10/nsp16 2'-O-MTase activity using GpppRNA is due to lack of recognition of such capped RNA. It also suggests that the nsp10/nsp16 complex has an RNA binding site that recognizes specifically cap-0 RNA. Moreover, the ability of this binding site to recognize cap-0 RNA is strongly regulated by the 2'-O-methylation status of the substrate RNA. Indeed, 7mGpppG2'omRNA, which corresponds to the nsp16 2'-O-MTase reaction product, did not bind to the nsp10/nsp16 complex (Figure 3A). This indicates that cap-1 RNA generated by the nsp10/nsp16 2'-O-MTase activity is actively released, promoting the reaction turnover.

As nsp16 2'-O-MTase activity strictly depends on its interaction with nsp10 (Figures 1C and 2A), we then asked whether nsp10 regulates nsp16 RNA-binding properties. Comparison of nsp10, nsp16 and nsp10/nsp16 complex binding to cap-0 RNA (7mGpppRNA) using a fluorescence polarization assay showed that nsp10 barely interacted with cap-0 RNA (Figure 3B). Conversely, both nsp16 and nsp10/nsp16 complex bound to cap-0 RNA with an apparent K_d of ~1.4 and ~0.33 µM, respectively. The polarization signal detected after nsp16 binding is higher than that obtained in the presence of the nsp10/nsp16 complex. This suggests that the RNA might bind to nsp16 dimers. Our result indicates that only nsp16 harbours the cap-0 binding site and that RNA binding is enhanced by nsp16 interaction with nsp10. As nsp16 alone binds cap-0 RNA (Figure 3B) but is not methylated (Figure 1C), we suggest that nsp10 is an allosteric regulator of nsp16 2'-O-MTase activity. In addition, the methyl donor SAM and, to a lesser extent, the reaction by-product SAH increased the affinity for the RNA substrate.
Conversely, magnesium ions (Mg$^{2+}$), which stimulate nsp10/nsp16 MTase activity (Figure 2B), did not seem to affect RNA binding (Figure 2F) in these experimental conditions, indicating that magnesium ions are not required for RNA recognition.

The MTase assay showed that the nsp10/nsp16 complex is able to methylate cap-0 RNA of variable RNA sequences (Figure 2E), starting with either $^\text{m}$GpppA or $^\text{m}$GpppG. However, the MTase activity is weaker using short RNA substrates (Figure 3E). Therefore, we tested whether nucleotides downstream the cap structure contributed to nsp10/nsp16 complex binding to RNA. To this aim, fluorescence polarization assays were performed using 3'-end labelled RNA sequences of increasing length that mimicked the 5'-end of the MERS-CoV genome sequence. Quantification of their interaction with the nsp10/nsp16 complex (Figure 3D and Table 1) showed that the cap analogue $^\text{m}$GpppG-cy5, which was not methylated by nsp10/nsp16 (Figure 3E), barely interacted with the nsp10/nsp16 complex. In contrast, a significant interaction was observed using $^\text{m}$GpppGAUU-cy5. The affinity increased then with the RNA length and the optimal affinity was observed with $^\text{m}$GpppGAUUUAAGUG-cy5 (Figure 3D). The MTase activity measured using a filter-binding assay followed the same trend (Figure 3E).

**Identification of nsp16 residues playing a key role in RNA recognition and MTase activity**

The crystal structure of the SARS-CoV nsp10/nsp16 complex with SAH has been reported at 2.0 Å resolution (31). As no structure with a cap analogue or substrate RNA is available yet, the nsp16 residues involved in RNA binding were tentatively inferred by modelling the RNA in the catalytic site.
Based on the structural model, alanine mutagenesis was performed and the effects of nsp16 mutations on MTase activity (Figure 5B) and on RNA binding properties (Figure 5A) were determined by filter binding assay and fluorescent polarization assay, respectively. Alanine substitution of the conserved residues in the catalytic tetrad (K-D-K-E) almost completely abolished the 2’-O-MTase activity, as expected. With the exception of D130A, these mutations also strongly reduced RNA binding, indicating that these catalytic residues also participated in RNA binding process. Mutation of the residues adjacent to the putative SAM binding site (H41, N43, D99, F149 and F150) drastically reduced MTase activity, but had no significant effect on RNA binding. Mutation of K46, K170, E203, D133, R38, Y47 or Y181 concomitantly reduced MTase activity (80-100%) and RNA binding. These residues are localized in the putative RNA binding groove of nsp16 (Figure 4). In addition, mutation into alanine of Y30 and Y132, which are within two mobile α helices (26-38) and (130-148) in close proximity with the cap in the nsp10/nsp16 model (Figure 4), and of H174, which is close to the 130-148 loop, also decreased nsp16 RNA binding properties and MTase activity. These observations confirm that the cap-0 RNA binding site involves these three aromatic residues.

**SAM and SAH levels regulate nsp10-nsp16 interaction**

Nsp16 2’-O-MTase activity requires interaction with nsp10. To determine how co-substrate (SAM) availability might regulate the RNA-capping reaction pathway, the nsp16 interaction with nsp10 was analysed using
biolayer interferometry. Nsp10 was biotinylated before immobilization on a streptavidin-coated biosensor. Binding of nsp16 was then monitored in the presence of various SAM/SAH concentrations, and the apparent affinity constants were determined (Figure 6A and Table 2). Nsp16 interaction with biotinylated nsp10 increased in the presence of 100 µM SAM or SAH. The interaction kinetics was characterized by the same association rate (same $k_{on}$) in the presence or in the absence of SAM or SAH. Conversely, the addition of SAM or SAH increased nsp10/nsp16 complex stability by decreasing the dissociation kinetics ($k_{off}$). This suggests that both SAM and SAH induce nsp10/nsp16 complex assembly. To further analyse how SAM or SAH regulated nsp10-nsp16 interaction, the dissociation of the nsp10/nsp16 complex, formed in the presence of 100 µM SAM, was assessed using dissociation buffers containing various concentrations of SAM or SAH (Figure 6B). The dissociation rate of the nsp10/nsp16 complex depended on SAM or SAH concentration. In the presence of 100 µM SAM, a concentration that mimics its intracellular level (40), the nsp10/nsp16 complex stability was highest, as indicated by the slow nsp16 release. In contrast, after MTase reaction, which converts SAM into SAH, the latter is released from the enzyme at the lower intracellular concentration of SAH ($\approx 20$ µM (41)). In turn, the release of SAH will speed-up the dissociation of the nsp10/nsp16 complex. Altogether, these data indicate that nsp10 and nsp16 form a dynamic complex the association of which is promoted by SAM binding. The complex will dissociate when SAH, the reaction by-product, is released, thus favouring the reaction turnover.
It was previously shown that 2'-O-methylation of viral RNA cap structures limited the infected host's antiviral response (42), suggesting that molecules blocking nsp16 2'-O-MTase activity might favour viral clearance in infected animals (36). In MTase inhibition assays, we tested 18 molecules known to block MTase activity (26, 43). Four of these compounds (Figure 7A) inhibited nsp10/nsp16 2'-O-MTase activity (between 45 and 100%) at a concentration of 50 μM. Among them, two SAM analogues (sinefungin and SAH: number 2 and 3 in Figure 7A) and showed the most potent inhibition. Cap-0 analogues (7mGpppG and 7mGpppA: number 18 and 16) also inhibited nsp16 2'-O-MTase activity (40-45%), whereas GpppG and GpppA (number 17 and 15) did not. This suggests that cap analogues block the binding of the RNA substrate in nsp16 RNA or cap binding site, in agreement with the weak interaction (Kd >20 μM) of these compounds with the nsp10/nsp16 complex (Figure 3D).

To better define the 2'-O-MTase activity inhibition of sinefungin, SAH, 7mGpppG and 7mGpppA, the nsp10/nsp16 complex was pre-incubated with increasing concentrations of each inhibitor, and then, the MTase reaction was started by addition of 3H-SAM and the RNA substrates. The dose-response curves (Figure 7B) indicated that sinefungin, and SAH inhibited nsp10/nsp16-mediated 2'-O-MTase activity with IC_{50} values in the micromolar range (7.4 and 7.0 μM, respectively, Table 3). The IC_{50} values of the cap analogues 7mGpppG and GpppG (45 and 274 μM, respectively) (Table 3) suggest that 7mGpppG is a competitive inhibitor that blocks specifically the RNA cap-0 binding site. This was confirmed by measuring
the effect of these compounds on nsp10/nsp16 RNA binding activity. 7mGpppG blocked RNA binding with an IC₅₀ value of 45 µM, which is in the same range of that of the inhibition of the MTase activity (Figure 7C). Conversely, the SAM analogues (SAH and sinefungin), which are supposed to enter in the nsp16 SAM binding site, barely affected RNA binding properties, as expected (Figure 7D and Table 4). Thus, the radioactive assay provides an efficient method to screen molecules to identify inhibitors of SAM-dependent MTase activity of the MERS-CoV nsp10/nsp16 complex. The fluorescence polarization assay developed here represents a tool to analyse their mode of action.
Discussion:

MERS-CoV is the most recently discovered zoonotic virus that causes pathology in humans. Upon cell infection, MERS-CoV starts its replication by translation of the ORF 1a and 1b to produce 16 nsps of the replication/transcription complex (RTC)(12). In this study, we characterized the MERS-CoV 2'-O-MTase activity of nsp16 and show that nsp10 is a cofactor required for nsp16 2'-O-MTase enzymatic activity, as previously reported for SARS and MERS-CoV (26, 34). The substrate of the reaction is RNA with a cap-0 structure. This observation is consistent with RNA binding assays showing that N7-methylated cap RNA is recognized by the nsp10/nsp16 complex, whereas unmethylated RNA is not. Thus, MERS-CoV cap methylation follows an obligatory sequence in which 2'-O-methylation might occur after N7 methylation mediated by nsp14. Consequently, it is likely that RNA cap synthesis by MERS-CoV follows the canonical capping pathway observed in eukaryotic cells and in some viruses, such as SARS-CoV, dengue virus, and West Nile virus (21).

Although the cap-0 structure gives RNA binding specificity, cap analogues barely bind to the nsp10/nsp16 complex at the micro molar concentration of \(^{7}\text{mGpppG-cy5}\) used in our assay, and are not a good substrate for the 2'-O-MTase. The presence of additional nucleotides downstream of the cap structure increases the substrate recognition and 2'-O-MTase activity. These nucleotides are thus required for substrate stabilization in the RNA binding groove. The optimal recognition is reached with ten nucleotides capped RNA. This is consistent with the model proposed for the SARS-CoV
nsp10/nsp16 complex with 7mGpppA-RNA in which the first nucleotides are held by nsp16 and the following nucleotides might be positioned in a RNA binding groove stabilized by nsp10 (27, 31). In addition, it is likely that the MERS-CoV 2'-O-MTase recognizes and methylates cap-0 RNA with no obvious sequence specificity. We also observed that the nsp10/nsp16 complex methylated similarly cap-0 RNA with A or G as the first nucleotide. This observation is in contrast to SARS-CoV 2'-O-MTase that preferentially binds and methylates RNA with A at N1 position ((7mGpppA-capped RNA, (27)). Using synthetic RNAs that mimic the 5'-end of the MERS-CoV genome, we did not observe any additional methylation (N2) or internal methylation, as indicated by the absence of enzymatic activity on cap-1 RNA or pppRNA sequences. The absence of internal methylation was also confirmed using longer (about 500 nucleotides) pppRNA substrates (not shown). This observation contrasts with findings on other viral MTases (flavivirus NS5-MTase) that can perform internal methylation of adenosines (44). Nevertheless, the absence of internal methylation with the nsp10/nsp16 complex is consistent with the fact that cap-1 RNAs or pppRNAs do not interact with the nsp10/nsp16 complex.

To further understand the mechanism whereby nsp10 promotes the stimulation of nsp16 2'-O-MTase activity by nsp10, we determined whether nsp10 is required for substrate recognition process. Our findings indicate that nsp16 can recognize cap-0 RNA even in the absence of nsp10 as already reported for FCoV nsp16 (30). Nevertheless, nsp10 increases nsp16 RNA binding properties of nsp16 (3-fold) and in turn the 2'-O-MTase...
activity can be detected only in the presence of both proteins under this condition. These observations suggest that nsp10 acts as an allosteric regulator of nsp16 2'-O-MTase activity rather than as an RNA binding module. A similar allosteric effect was reported for the vaccinia virus and human RNA N7-MTases. Indeed, in the vaccinia virus family, the D1 catalytic subunit alone is unstable and inactive and its stability and MTase activity are enhanced by the presence of D12 through the increase of GTP, SAM and GpppA binding affinity (45, 46). Similarly, the human RNA N7 MTase (RNMT) is allosterically activated by RNMT Activating Miniprotein (RAM) that stabilizes its structure and favours the recruitment of methyl donors (47).

As nsp10 is not the main player involved in RNA binding, we mapped the nsp16 residues involved in cap-0 RNA recognition. We could not determine the structure of nsp10/nsp16 with RNAs or cap analogues, although we performed many attempts (not shown). Therefore, we used alanine scanning mutagenesis to identify the residues involved in cap-0 RNA specific recognition and nsp16 MTase activity. Our results suggest that the cap-0 RNA structure is stacked between two flexible loops (26-38) and (130-148) by the aromatic residues Y30, Y132 respectively and the H174 residue in close proximity. The alanine scanning results also indicate that the RNA chain of the substrate might be held by K46, K170, E203, D133, R38, Y47 and Y181 because both RNA binding and MTase activity decreased upon mutation of these residues. Interestingly, three residues belonging to the catalytic tetrad (K46, K170 and E203) might directly...
participate in RNA recognition. These results corroborate the model of MERS-CoV nsp10/nsp16 (Figure 4) in which the RNA was positioned using vaccinia MTase VP39 structures (PDB entry: 1AV6) (27, 31, 48). The mutagenesis study also allowed identifying residues that strongly reduce MTase activity with a weak effect on RNA recognition (D99, F149, F150, H41 and N43). On the basis of their position in the structural model, we suggest that these residues might participate in SAM binding.

We then demonstrated that SAM/SAH balance plays an important role in nsp10-nsp16 interaction. The methyl donor SAM, strengthens the interaction between nsp10 and nsp16 at the physiological intracellular concentration ((100 µM, (40)) and in turn, the RNA binding properties of nsp16 are enhanced. This suggests that SAM stabilizes or induces small conformational changes of the enzyme leading to an increase in both RNA affinity and methylation by nsp10/nsp16. This observation is also consistent with thermal shift assay indicating that the nsp10/nsp16 complex is stabilized in the presence of SAM ($\Delta T_m \approx 1$ deg). During RNA methylation, SAM is converted into SAH. The SAH will be next released by nsp16 stimulating the dissociation of the nsp10/nsp16 complex. In addition, the reaction product (cap-1 RNA) barely interacts with the nsp10/nsp16 complex and is released. Overall, these observations suggest that cap-0 RNA methylation is linked to the association and dissociation of the nsp10/nsp16 complex (Figure 8). These data also suggest that the SAM/SAH balance in infected cells is a key factor for cap RNA methylation. As, SAH hydrolase is a key enzyme for the maintaining of low levels of SAH
in cells, inhibitors of cellular SAH hydrolases, might have an antiviral effect on CoV replication. Interestingly, the SAH hydrolase inhibitor 3-deazaneplanocin A, has previously been shown to inhibit several viruses (49). It was recently demonstrated to limit Ebola virus replication and to lead to IFN-α production in virus-infected mice (50, 51). The SAH hydrolase inhibitors may thus confer a selective antiviral activity that differ from one virus to another depending on their RNA methylation levels (52). The inhibition may result to a direct effect of SAH concentration increase on the viral MTase or to indirect effects on cellular mRNA cap methylation which may then be recognized as "non-self" and, thus trigger an IFN response.

The viral 2'-O-MTase was highlighted as potential antiviral target (42). Indeed, such RNA methylation could be a marker of "self", thus avoiding detection of viral RNA by Mda5/RIG-I sensors (17, 19, 20). In addition, one interferon-stimulated gene products, IFIT1, sequesters 2'O-unmethylated viral RNA and thereby blocks their translation, which results in inhibition of viral replication (53). Thus, compounds blocking the viral 2'-O-MTase could limit viral replication in infected animals and elicit a strong antiviral response, favouring viral clearance. Here, we show that SAM analogues, such as SAH and sinefungin, limit 2'-O-MTase activity with IC₅₀ values in the micromolar range (7.0 and 7.4 µM, respectively) with no inhibition of RNA binding, as expected. Conversely, a N7-methylated cap analogue (7mGpppG) inhibits both 2'-O-MTase activity and RNA recognition with an IC₅₀ value of 45 µM. This indicates that 7mGpppG acts as a competitive...
inhibitor that blocks cap-0 RNA recognition by the nsp10/nsp16 complex. This was confirmed by the absence of inhibition in the presence of unmethylated cap analogues. Altogether, these results might help developing an efficient assay to screen compounds that inhibit the SAM-dependent MERS-CoV nsp10/nsp16 complex. The biochemical tools and the RNA binding assay allow determining whether inhibitors are competing directly with the RNA substrate. It remains to be tested whether targeting the 2'-O-MTase activity can limit viral replication in cells or animal models that harbour the Mda5/RIG-I antiviral pathway (34).

Acknowledgments

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References:


milk collected according to local customs from dromedary camels, Qatar, April 2014. Euro Surveill 19:1–5.


and 2′-O-methyl discrimination in capped RNAs by the innate immune


unconventional mechanisms for capping viral mRNA. Nat Rev Microbiol

22. Diamond MS. 2014. IFIT1: A dual sensor and effector molecule that
detects non-2′-O methylated viral RNA and inhibits its translation.

23. Daugherty MD, Schaller AM, Geballe AP, Malik HS. 2016. Evolution-
guided functional analyses reveal diverse antiviral specificities encoded
by IFIT1 genes in mammals. eLife 5:1–22.

protein 13: characterization of duplex-unwinding, nucleoside

Functional screen reveals SARS coronavirus nonstructural protein nsp14

26. Bouvet M, Debarnot C, Imbert I, Selisko B, Snijder EJ, Canard B,
Decroly E. 2010. In vitro reconstitution of SARS-coronavirus mRNA cap

insights into the mechanisms of SARS coronavirus RNA ribose 2′-O-


49. **Universiteit K.** 1987. WI4 The importance of SAH hydrolase as a potential 36.


**Figure 1: MERS-CoV nsp16 2'-O-MTase activity is promoted by nsp10**

Recombinant MERS-CoV nsp10, nsp16 and the nsp10/nsp16 complex were expressed in *E. coli* and purified by affinity chromatography on IMAC columns. A) After SDS-PAGE separation of purified proteins (0.2 µg), gels were stained with Coomassie blue. MW, molecular size markers. B) The nsp10/nsp16 complex was purified by gel filtration using a S200-16/60 column. The elution chromatogram (OD: 280 nm) shows one main peak that eluted at 90 ml and corresponded to the nsp10/nsp16 complex. C) The methyltransferase activity (MTase) of nsp16 (1.2 µM) and nsp10 (2 µM) alone or together and of the nsp10/nsp16 complex (1 µM) was determined by monitoring the transfer of $^3$H-CH$_3$ from SAM to RNA oligonucleotides with sequences corresponding to the 5'-end of the MERS-CoV genome with various cap modifications (pppGAUUUAA, GpppGAUUUAA, $^{7m}$GpppGAUUUAA GpppG$_{2'om}$AUUUAA and $^{7m}$GpppG$_{2'om}$AUUUAA). Assays were stopped after 5, 30, 60 and 180 min and the radioactivity associated with the RNA was determined by DEAE-filter binding assay. The bar graph presents the mean value and the standard deviation of three independent experiments.

**Figure 2: Biochemical characterization of the MERS-CoV nsp10/nsp16 2'-O-MTase**

The 2'-O-MTase activity of MERS-CoV nsp16 was characterized by filter binding assay as described in Figure 1C. A) Nsp16 2'-O-MTase activity is
promoted by nsp10. Nsp16 (2 μM) was incubated with \(^{7m}\text{GpppGAUUUA}\) (0.7 μM) in the presence of increasing concentrations of nsp10. The apparent K\(_d\) of nsp10/nsp16 interaction (2 ± 0.1 μM; mean value and standard error of the mean, SEM, of three independent experiments) was deduced by curve fitting using Hill slope equations. B) Effect of divalent ions on nsp16 2'-O-MTase activity. MTase assays were performed in the presence or not of divalent ions (MgCl\(_2\)). Inhibition of the MTase reaction by EDTA was assessed and the activity was recovered by addition of MgCl\(_2\) or MnCl\(_2\). The methyl transfer was measured after 5, 15, 30 and 60 min at 30°C (mean value ± SD). C) Optimum pH of the 2'-O-MTase activity mediated by nsp10/nsp16. D) Nsp10/nsp16 MTase activity in the presence of increasing concentrations of SAM. The apparent K\(_d\) of SAM (4.3 ± 0.6 μM) was deduced by curve fitting using a Hill equation and the GraphPad Prism program. E) Measurement of nsp10/nsp16 MTase activity on cap-0 RNA sequences that correspond to the 5'-end of various viruses: MERS-CoV (\(^{7m}\text{GpppGAUUUAAGUGAAUA}\)), West Nile virus (\(^{7m}\text{GpppAGUAGUUCGCCUG}\)), Dengue virus (\(^{7m}\text{GpppAGUUGUUAGUCUA}\)), Ebola virus (\(^{7m}\text{GpppGAUGAAGAUUAAG}\)) after 30 min incubation at 30°C (n=3, mean value ± SD). F) Magnesium ions do not influence nsp10/nsp16 interaction with cap-0 RNA. \(^{7m}\text{GpppGAUUUA}-\text{cy-5}\) was incubated with increasing concentrations of the nsp10/nsp16 complex with or without 1 mM MgCl\(_2\). The interaction with the cy-5-labelled RNA was followed by fluorescent polarization, and the apparent calculated K\(_d\) values are indicated on the top of the graph.

**Figure 3: RNA binding properties of MERS-CoV nsp10, nsp16 and the nsp10/nsp16 complex**
A) Nsp10/nsp16 recognizes cap-0 RNA. Different short RNA oligonucleotides (pppGAUUUAA, GpppGAUUUAA, 7mGpppGAUUUAA, GpppG2omAUUUAA or 7mGpppG2omAUUUAA) corresponding to the 5’-end of the MERS-CoV genome were 3’-end-labelled with pCp-cyanine-5 and incubated with increasing concentrations of nsp10/nsp16 complex. The interaction between the nsp10/nsp16 complex and each RNA was followed by measuring the fluorescence polarization signal at 675 nm. The apparent affinity constant (K_d) was calculated by non-linear regression analysis with a Hill slope equation and is indicated on the top of the graph.

B) Nsp16 and the nsp10/nsp16 complex recognize cap-0 RNA. 7mGpppGAUUUAA-cy-5 was incubated with increasing concentrations of nsp10, nsp16 or nsp10/nsp16 complex. The interaction with cy-5-labelled RNA was followed by measuring the fluorescence polarization, like in panel “A”, and the calculated K_d is indicated on the top of the graph.

C) SAM and SAH enhance the interaction of the nsp10/nsp16 complex with cap-0 RNA. 7mGpppGAUUUAA-cy-5 was incubated with increasing concentrations of nsp10/nsp16 complex in the presence or not (buffer alone) of 100 µM SAM or 20 µM SAH. The interaction with cy-5-labelled RNAs was followed by measuring the fluorescence polarization and the calculated K_d is indicated on the top of the graph.

D) RNA length effect on nsp10/nsp16 binding was analysed using viral native 7mGpp RNAs of different lengths (7mGpppG, 7mGpppGAUU, 7mGpppGAUUUAA, 7mGpppGAUUUAAGUG and 7mGpppGAUUUAAGUGAAUA) in the presence of increasing concentrations of nsp10/nsp16. The interaction with cy-5-labelled RNAs was followed by fluorescence polarization and the calculated K_d values are indicated in Table 1.

E) Kinetics of MTase activity of the nsp10/nsp16 complex (0.25 µM) on...
7mGpppG-RNAs (0.7 µM) of increasing length was measured by filter binding assay after 2, 4, 8, 16, 32 and 64 min incubation at 30°C in the presence of 3H-SAM. The curve presents the mean value and the standard deviation of three independent experiments. All the fluorescent polarization experiments were performed twice independently and the Kₐ values of each condition were calculated using GraphPad prism (n=2, mean value ± SEM) using one site-specific binding equation with Hill slope.

**Figure 4: 3D structural model of the nsp10/nsp16 complex interaction with 7mGppp-RNA**

The nsp10/nsp16 model was built by alignment of SARS-CoV (PDB entry 3R24) and MERS-CoV nsp10/nsp16 sequences using the Swiss model and the PyMOL software. The cap-0 RNA/nsp16 model was built by aligning the MERS-CoV nsp16 sequence with that of the vaccinia MTase VP39 (PDB entry 1AV6) using the PyMol software. The surface of nsp16 residues is in blue and that of nsp10 in pink. The nsp16 catalytic residue D130 is depicted in orange, while the catalytic and putative RNA binding residues K46, K170, E203 are in red. The putative cap-0 RNA binding site (light green) is delimited by Y30 and Y132, which are localized in the two mobile α helices (26-38) and (130-148) respectively, and by H174 close to the last helix. The residues of the putative RNA binding groove are in cyan (Y181, D133, Y47 and R38). The SAM binding pocket involves the residues N43, D99, F149, F150 and H41 (in magenta); SAM is in grey and cap-0 RNA in yellow. The zoom focuses on the RNA binding groove and the SAM binding domain (ribbon structure).
Figure 5: MERS-CoV nsp16 mutations that affect RNA recognition and/or MTase activity

A) Alanine scanning mutagenesis. Mutations were introduced in the nsp16 clone and after co-lysis, the nsp10/nsp16 complex was purified by affinity chromatography on IMAC. The affinity ($K_a = 1/K_d$) of each mutant protein for $^{7m}$GpppGAUUAA-cy-5 was measured by fluorescence polarization as described in Figure 3. The affinity ($K_a = 1/K_d$) of each mutant protein for $^{7m}$GpppGAUUAA-cy-5 was measured by fluorescence polarization as described in Figure 2. The $K_a$ values were calculated from the Hill equation using GraphPad prism (n=2, mean value ± SD). The $K_a$ values were only estimated for the mutants indicated by an asterisk because the RNA binding plateau was not reached in the fluorescence polarization experiment. B) The MTase activity of each mutant (1 µM) was determined after incubation with $^{7m}$GpppGAUUAA at 30°C in the presence of $^3$H-SAM for 60 min. The bar graph presents the mean value and the standard deviation of three independent experiments.

Figure 6: SAM and SAH modulate nsp10-nsp16 interaction

A) Nsp10/nsp16 steady state assembly: real-time nsp16 binding to biotinylated nsp10 was measured by Octet biolayer interferometry (BLI). Streptavidin biosensors coated with 100 nM biotinylated nsp10 were used to measure nsp16 (0 to 7.8 µM) association and dissociation. The reference sensor (0 µM nsp16) values were subtracted from the sample traces. All curves were fitted with a 1:1 model by using the Octet biolayer program and then the steady state curve was traced using the site-specific binding equation on GraphPad Prism. The
different K_d values of nsp10-nsp16 interaction in the presence or not of 100 µM
SAM or 20 µM or 100 µM SAH are presented in Table 2. B) Effects of SAM and
SAH on the dissociation kinetics of nsp16 from immobilized nsp10. 7.8 µM
nsp16 was bound to sensor-immobilized biotinylated nsp10 in the presence of
100 µM SAM. Then, the sensor was moved to dissociation buffer alone or
containing 5, 20 or 100 µM of SAM or SAH. Nsp16 binding to immobilized
nsp10 was normalized for each trace.

Figure 7: Inhibition of nsp10/nsp16 complex MTase activity and RNA
binding
A) Bar graph showing the MTase inhibition activity (percentage) of each
candidate inhibitor (final concentration of 50 µM). The MTase assay was
performed as in Figure 1C by incubating 0.5 µM of nsp10/nsp16 complex with
0.7 µM 7mGpppGAUUUAA and ³H-SAM at 30°C for 30min. The methyl transfer
to RNA was measured by filter binding assay in the absence (5% DMSO) or in
the presence of each inhibitor (n=2, mean value ± SD) 1: DMSO, 2: sinefungin,
3: SAH, 4: SIBA, 5: ribavirin, 6: ribavirin-TP, 7: N-(5-chloro-2-methoxyphenyl)-
3,4-dimethoxy-N-[2-(4-methyl-1-piperazinyl)-2-oxoethyl]benzenesulfonamide, 8:
2-[[4-benzyl-5-(3-pyridinyl)-4H-1,2,4-triazol-3-yl]thiol]-N-(5-methyl-1,3,4-
thiadiazol-2-yl) acetamide, 9: N-1-cyclopropyl-N-2-(2,5-dimethoxyphenyl)-N-2-
(methylsulfonyl) glycaminide, 10: 4-bromophenyl 3-(3,5-dioxo-4-azatetracyclo
[5.3.2.0-2,6.-0-8,10-] dodec-11-en-4-yl) propanoate, 11: 1'-[(4-tert-
butylphenyl)sulfonyl]-1,4'-bipiperidine-4'-carboamide, 12: N’-(3,4-
dichlorobenzylidene)-2-(2-pyridinylthio) acetohydrazide, 13: GTP, 14: 7mGTP,
15: GpppA, 16: ³mGpppA, 17: GpppG, 18: 7mGpppG. B) Dose response of the
inhibitory effect of sinefungin, SAH and cap analogues on nsp10/nsp16 MTase activity (MTase assays performed as in panel A; n=2, mean value ± SEM). The IC₅₀ values deduced using GraphPad Prism and the log (inhibitor) vs response variable slope equation are shown in Table 3. C) The competition effect of cap analogues on ⁷mGpppGAUUAA-cy-5 binding to the nsp10/nsp16 complex was analysed by fluorescence polarization, as described in Figure 3. Measurements were performed in the presence of increasing concentrations of cap analogues. D) The effect of 20 µM SAH, 20 µM sinefungin or buffer alone on the nsp10/nsp16 complex interaction with ⁷mGpppGAUUAA-cy-5 was assessed by fluorescence polarization, as described in Figure 3C. The Kₐ values deduced from the curve fitting are presented in Table 4 (n=2, mean value ± SEM).

**Figure 8: Model of the nsp10/nsp16-⁷mGpppRNA reaction turnover regulated by the SAM/SAH balance**

The nsp10/nsp16 model was built as described previously in Figure 4. The 3D representation of nsp16 structure is in blue, ⁷mGpppGAAAAA in yellow and nsp10 in pink. According to our model, first, nsp16 binds to cap-0 RNA and loads a SAM molecule (grey, 100 µM in infected cells), thus allowing the recruitment of the nsp10 allosteric activator. Then, nsp16 catalyses cap RNA methylation and converts cap-0 into cap-1 RNA. Methylated RNA is released from the nsp16 cap-binding site. As SAH, in orange, is supposed to be at lower concentration than SAM in infected cells (20 µM vs 100 µM), SAH should be released from the nsp10/nsp16 complex and favour its dissociation and the reaction turnover.
Table 1: $K_d$ values of the interaction of the nsp10/nsp16 complex with pCp-Cy5-labelled RNA sequences of increasing length measured by fluorescence polarization (Figure 3D).

Table 2: $K_d$, $k_{on}$ and $k_{off}$ values of nsp10-nsp16 interaction measured by Octet biolayer interferometry (Figure 6A) in the presence or not of SAM or SAH.

Table 3: Measurement of nsp10/nsp16 2'-O-MTase inhibition (IC$_{50}$) by sinefungin, SAH and cap analogues, calculated from the dose response curve of Figure 7B.

Table 4: $K_d$ values of nsp10/nsp16 and $\text{m}^7$GpppRNA interaction in the presence or not of SAH or sinefungin measured by fluorescence polarization (Figure 7D).
Figure 1
Figure 2
Figure 3
Catalytic residues and putative RNA binding

Catalytic residue

Putative cap-0 binding

Putative RNA binding

Putative SAM binding

Figure 4
Figure 6
Figure 7
Figure 8
Table 1: $K_d$ values of the interaction of the nsp10/nsp16 complex with pCp-Cy5-labelled RNA sequences of increasing length measured by fluorescence polarization (Figure 3D).

<table>
<thead>
<tr>
<th>RNA</th>
<th>$K_d$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{7m}$GpppG</td>
<td>&gt;20</td>
</tr>
<tr>
<td>$^{7m}$GpppGAUU</td>
<td>1.07 ± 0.09</td>
</tr>
<tr>
<td>$^{7m}$GpppGAUUUAA</td>
<td>0.42 ± 0.01</td>
</tr>
<tr>
<td>$^{7m}$GpppGAUUUAAGUG</td>
<td>0.251± 0.02</td>
</tr>
<tr>
<td>$^{7m}$GpppGAUUUAAGUGAAUA</td>
<td>0.278 ± 0.05</td>
</tr>
</tbody>
</table>
Table 2: $K_d$, $K_{on}$ and $K_{off}$ values of nsp10-nsp16 interaction measured by Octet biolayer interferometry (Figure 6A) in the presence or not of SAM or SAH.

<table>
<thead>
<tr>
<th>Condition</th>
<th>$K_d$ interaction nsp10/nsp16 (µM)</th>
<th>$K_{on}$ (M$^{-1}$s$^{-1}$)</th>
<th>$K_{off}$ (s$^{-1}$)</th>
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<tbody>
<tr>
<td>Without SAM, SAH</td>
<td>1.24 ± 0.015</td>
<td>3.77x10$^3$ ± 4.35</td>
<td>4.68x10$^{-3}$ ± 0.016x10$^{-3}$</td>
</tr>
<tr>
<td>With 100 µM SAM</td>
<td>0.15 ± 0.001</td>
<td>3.98x10$^3$ ± 2</td>
<td>6.23x10$^{-4}$ ± 0.031x10$^{-4}$</td>
</tr>
<tr>
<td>With 20 µM SAH</td>
<td>0.48 ± 0.003</td>
<td>3.10x10$^3$ ± 1.97</td>
<td>1.51x10$^{-3}$ ± 0.004x10$^{-3}$</td>
</tr>
<tr>
<td>With 100 µM SAH</td>
<td>0.19 ± 0.0013</td>
<td>3.3 x10$^3$ ± 2.2</td>
<td>6.51x10$^{-4}$ ± 0.041x10$^{-4}$</td>
</tr>
</tbody>
</table>
Table 3: Measurement of nsp10/nsp16 2′-O-MTase inhibition (IC\textsubscript{50}) by sinefungin, SAH and cap analogues, calculated from the dose response curve of Figure 7B.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC\textsubscript{50} (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sinefungin</td>
<td>7.4 ± 0.9</td>
</tr>
<tr>
<td>SAH</td>
<td>7 ± 0.4</td>
</tr>
<tr>
<td>GpppG</td>
<td>274.4 ± 140</td>
</tr>
<tr>
<td>7\textsuperscript{m}GpppG</td>
<td>45 ± 3.5</td>
</tr>
</tbody>
</table>
Table 4: $K_d$ values of nsp10/nsp16 and $^7mGpppRNA$ interaction in the presence or not of SAH or sinefungin measured by fluorescence polarization (Figure 7D).

<table>
<thead>
<tr>
<th>Condition</th>
<th>$K_d$ nsp10/nsp16/7mGpppRNA (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer alone</td>
<td>0.47 ± 0.03</td>
</tr>
<tr>
<td>With 20 µM SAH</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>With 20 µM sinefungin</td>
<td>0.09 ± 0.009</td>
</tr>
</tbody>
</table>