OPINION ARTICLE

REVISED Human respiratory syncytial virus methyltransferase: a potential antiviral target? [version 2; peer review: 1 approved, 1 approved with reservations]

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Abstract

Human respiratory syncytial virus (HRSV) causes bronchiolitis and pneumonia. The role of methyltransferase (MTase) activity of HRSV polymerase in viral replication is unknown. Literature reviews of similar viral MTases and homology- modeling of RSV MTase bound to GTP and S-adenosylmethionine (SAM) have shown sequence similarity and the conserved catalytic residues (K-D-K-E) and the SAM-binding (GXGXXG) domain. Combined with the recent reports of the importance of 2'O methylation of viral RNAs in the host innate immune response evasion, and its proposed role in viral replication, HRSV MTase holds promise as a potential antiviral target. Further biological validation of HRSV MTase could facilitate the discovery of novel HRSV antivirals targeting MTase enzyme activity.

Keywords

RSV, methyltransferase, antiviral target, replication, broad spectrum, polymerase, methylation, MTase, innate immunity, homology modeling

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Any reports and responses or comments on the article can be found at the end of the article.

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REVISED Amendments from Version 1

The purpose of our opinion article is to draw a wider attention to the unmet medical need for treating HRSV infections and to highlight MTase as a potential target. In the past decade, targeting protein methyltransferases has led to many successful clinical candidates for cancer treatment (*Philos Trans R Soc Lond B Biol Sci.* 2018 Jun 5; 373(1748): 20170080). We also use this article to highlight key areas where drug discovery research needs to focus its attention in order to advance the field and aid in the identification of novel agents to treat HRSV infection. Our goal is not to be exhaustive but to make sure that the appropriate references are included so that the readers could be directed to those to find mechanistic details. As such we believe our article is timely and serves this intent.

In the revised version of the manuscript, we have updated the virus classification terminology, polymerase "regions" instead of "domains" and added 2 new references (Rima *et al.* 2017 and Decroly *et al.* 2011). Distinction in the capping mechanisms and Li Lab references (for HMPV work) have been added. As per the reviewer's suggestion, we have added a figure (Figure 1) to illustrate the vRNA capping. We have mentioned that SAM is the methyl donor and SAH is a byproduct of MTase activity in Figure 1 and removed redundancies.

Late elongation and polyadenylation reactions happen subsequent to methylation of viral RNA cap per Liuzzi *et al.* 2005. Hence it appears that inhibition of viral cap methylation leads to reduction in transcripts with late elongation and polyadenylation. Since viral MTase have multiple catalytic residues required for enzyme activity, mutation at multiple contact residues in the catalytic site might be needed to overcome antiviral activity of an inhibitor.

Any further responses from the reviewers can be found at the end of the article

Human respiratory syncytial virus (HRSV) causes lower respiratory tract infections (bronchiolitis and pneumonia) in premature babies, young children, immuno-compromised adults and bone marrow transplant patients^{1,2}. It is a significant risk factor for asthma, wheezing³ and progression of chronic obstructive pulmonary disease (COPD)^{3,4}. However, the molecular pathogenesis of HRSV in asthma, wheezing and COPD is not clear. HRSV-associated infections result in significant disease and mortality. In children under 5-years of age, 33.4 million cases of HRSV-associated acute lower respiratory tract infections which result in 3.4 million hospitalizations and between 53,250 and 199,000 deaths, are reported annually worldwide⁵.

HRSV, now formally known as human orthopneumovirus, is an enveloped RNA virus with non-segmented negative sense RNA (nsNS) genome, belonging to the *Pneumoviridae* family⁶. There are two subtypes of HRSV, subtype -A and -B, circulating in the population. HRSV causes significant health problems but at present there is no effective treatment or vaccine. The U. S. Food and Drug Administration (FDA) has approved palivizumab⁷, for prophylactic therapy in premature infants with less than 29-weeks of gestation and children with congenital lung or heart disease. Ribavirin, a broad-spectrum antiviral agent is the only FDA approved drug for the treatment of severe HRSV disease⁸. However, the use of ribavirin is limited due to the potential side effects, high cost, difficulty in administration and lack of demonstrated benefit in decreasing hospitalization and mortality⁹. Currently, the few drug

candidates in clinical development (GS-5806, ALS-8176, EDP-938) are directed towards a limited number of viral targets (HRSV F protein, polymerase and N protein respectively)¹⁰⁻¹². Thus, novel targets in HRSV replication are needed to address the clinically unmet medical need¹³.

mRNA Capping and methylation functions of HRSV polymerase

HRSV produces mRNAs, which are co-transcriptionally capped and polyadenylated¹⁴⁻¹⁶. Viral mRNA methyltransferases (MTases) catalyze the transfer of methyl groups from SAM (S-adenosylmethionine; methyl donor) to viral mRNA caps comprised of a guanosine nucleotide linked *via* a unique 5'-5' linkage¹⁷. These processes of capping and methylation are distinct in different viruses, even though the end products are chemically equivalent and contribute to mRNA stability and translation efficiency¹⁸. Previous analysis of the L-protein sequence of non-segmented negative strand (nsNS) RNA viruses revealed the presence of 6-conserved regions (I-VI)¹⁹. These regions were implicated in viral genome replication, transcription, cap methylation and 3' polyadenylation^{19,20}. Fearn and Deval have graphically illustrated these regions using the VSV RNA polymerase as an example¹³. Using computational analyses of L protein of the Mononegavirales family viruses alongside site-directed mutagenesis, the MTase domain has been previously mapped to region VI, with a putative K-D-K-E catalytic tetrad and a glycine-rich motif (GxGxG) (SAM binding site)^{20,21}. A hallmark of the SAM-dependent MTase superfamily is the conserved sequences (segregated into motifs I-X) responsible for two-conserved functions- SAM binding and MTase catalytic reaction. Motifs I, III and IV are shown to be involved in SAM binding, whereas motifs IV, VI, VIII and X play a major role in the catalytic reaction¹⁹. Ribose 2'-O MTase is typically shown to have K-D-K-E tetrad residues at its core and these residues participate in the catalytic methylation reaction.

Viral mRNA cap methylation process for vesicular stomatitis virus (VSV) has been well understood (a prototype for nsNS group of viruses) model (graphically illustrated in Figure 1¹³). As per this model, viral mRNA capping process is thought to involve three different enzymatic steps (1) GTPase (2) GDP polyribonucleotidyltransferase (PRNTase) and 3) MTase. This viral mRNA cap methylation model, based on published literature, has revealed some unique features that are distinct from the host mechanism²⁰. These include (1) dual specificity of MTase activity on both the N-7 guanosine and 2'-O ribose positions encoded in a single conserved region (CR VI) of L-protein; (2) sharing of the same binding site for S-adenosylmethionine (SAM), that acts as the methyl donor²²⁻²⁴ (3) 2'-O methylation preceding and facilitating G-N-7 methylation²⁵, and (4) requirement of *cis*-elements in viral RNA for cap methylation. Mechanisms involved in mRNA capping functions of HRSV L-protein are not clearly understood. Ogino and coworkers²⁶ have shown that the mRNA cap for VSV and other rhabdoviruses is added by a PRNTase activity, rather than by guanylation. Due to the similarities within their capping domains, it is likely that HRSV uses the same mechanism as the rhabdoviruses, although this has not yet been experimentally demonstrated.

Figure 1. Viral RNA Cap methylation process. A proposed model for HRSV MTase in the methylation and capping of RNA based on similarities to VSV and other rhabdoviruses: GTPase hydrolyzes Guanylyl Triphosphate (GTP) to Guanylyl Diphosphate (GDP). GDP polyribonucleotidyltransferase (PRNTase) facilitates the covalent linkage of the 5'-end of viral RNA with viral polymerase (L-protein) after removal of pyrophosphate. GpppRNA:cap product is formed after GDP attacks the phosphodiester bond between L-protein and RNA. Viral MTase methylates the viral RNA cap at 2'-position of ribose of the first nucleotide and N7 of the first guanine in viral RNA cap in a sequential manner. For the methylation reaction, MTase uses S-adenosyl methionine (SAM) as substrate, with S-adenosyl-L-homocysteine (SAH) as a byproduct.

Sequence similarity of HRSV MTase domains with other viruses

Alignment of the L-protein sequences from different members of the *Pneumoviridae* and other nsNS RNA viruses has demonstrated conserved residues in the MTase domains²¹. As previously reported, conserved motifs within the HRSV MTase domain could be predicted between amino acid sequences F1821-N2025, with catalytic tetrad at K1831, D1936, K1973, E2004 and a putative SAM-binding GxGxG...D motif at positions G1853-E-G1855-A-G1857 and D1912 in HRSV L protein. MTase catalytic tetrad (K-D-K-E) and SAM binding domains (GxGxG...D) are conserved in CR VI of HRSV and VSV (Vesicular Stomatitis Virus), suggesting a similar mechanism of cap methylation^{26–28}.

Structure of HRSV MTase

An X-ray crystal structure of HRSV MTase domain is not available in the literature. Previously, a 3.8 Å resolution structure of VSV L-protein and the methyl transferase domain using electron cryomicroscopy has demonstrated that the MTase contacts both the connector and the capping domains, without direct contact with the RNA dependent RNA polymerase (RdRp)²⁹. However, the authors also predicted the substantial conformational change in the L protein following initial polymerization. The lack of a high resolution X-ray crystal structure of the HRSV MTase is a major caveat for a structure based drug design effort.

In the absence of an X-ray crystal structure, homology modeling provides an alternative approach to model the protein structure using the crystal structure of related protein(s) where significant sequence identity/similarity exists. Sequence alignment of HRSV polymerase suggested an overall low protein sequence similarity among the members of different nsNS viruses. Similarly, low sequence homology (8–13%) was

observed between HRSV MTase domain and those for which X-ray structures were available namely dengue virus NS5, vaccinia virus VP39 and *E. coli RrmJ* that precluded the building of a homology model of HRSV MTase. Fortunately, crystal structure of human metapneumovirus (HMPV) methyltransferase were reported recently³⁰. HMPV is a pneumovirus of the *Pneumovirinae* subfamily closely related to respiratory syncytial virus (HRSV). The MTase domains of these viruses have about 35% sequence identity and close to 60% sequence similarity allowing homology-based modeling of the RSV MTase domain. The suitability of crystal structure of human metapneumovirus (HMPV) methyltransferase for homology modeling of HRSV MTase and structure-based drug discovery needs to be further explored.

Genetic support for HRSV MTase role in viral replication

Recombinant VSVs (a prototype of nsNS RNA viruses) with point mutations in the methyltransferase catalytic site (rVSV-K1651A, -D1762A, and -E1833Q) were reported to be defective in cap methylation and demonstrated reduced growth in cell culture and mice³¹. Though SAM binding site point mutations (rVSV-G1670A, G1672A, G1674A and G4A), were attenuated *in vitro*, low level virulence was still observed *in vivo*²⁰. In contrast, mutations in the SAM binding site of L-protein in recombinant flaviviruses and metapneumoviruses attenuated viral replication in cell culture and animal models (cotton rats and turkeys)^{27,28}, supporting the importance of MTase for viral replication and virulence³².

Previously, HRSV transcription was shown to be independent of cap methylation, where S-adenosyl-L-homocysteine (SAH), a byproduct of MTase activity, did not affect HRSV transcription despite SAM-dependent inhibition of methylation³³. However, this observation was based only upon *in vitro*

transcription using infected cell extracts without evaluation of the quality/stability of the HRSV transcripts. For VSV (a prototype nsNS RNA virus), SAH was shown to affect the quality of mRNA (aberrant polyadenylation) without an apparent effect on transcription^{34,35}. Thus, evaluation of the quality of transcribed viral mRNAs in the presence of SAH might reveal a previously uncharacterized effect on HRSV MTase activity. The HRSV minigenome assay provides an opportunity to address this possibility, as it is performed in cells with measurement of both transcription and translation³⁶. Based on the model suggested for HRSV transcription, methylation of viral RNA cap occurs before late elongation and poly-adenylation¹⁶. Hence, it is possible that inhibition of viral cap methylation might lead to reduction in late elongation and polyadenylation of viral RNAs. However, additional studies are needed to confirm this hypothesis for HRSV MTase.

Recent reports suggest that amino acid substitutions in the conserved SAM binding site and MTase domain of metapneumoviruses result in defective mRNA cap methylations and attenuate viral replication *in vivo*^{27,28}. Since metapneumoviruses also belong to the *Pneumovirinae* subfamily as HRSV, it is possible that inhibition of HRSV MTase and SAM binding functions will negatively affect viral mRNA transcription and consequently, viral replication.

HRSV MTase as an antiviral target

Several small molecule inhibitors of viral MTase such as Sinefungin (SIN) and S-adenosyl-L-homocysteine (SAH) derivatives have been reported. SIN, a natural SAM-analog and a potent inhibitor of MTase, shows antiviral activity against VSV, Newcastle disease and vaccinia viruses³⁷. Similarly, derivatives of SAH, a byproduct of mRNA cap methylation, have shown selective inhibition of MTase of dengue virus³⁸. Key residues in HRSV MTase catalytic motif and SAM binding domain seem to be conserved between different subtypes A and B of HRSV, pneumoviruses and negative sense non-segmented viruses except *Bornaviridae* family members^{21,39}. Such a conserved sequence could provide a basis for structure-based design for pan-antiviral inhibitors targeting viral MTase²⁸. The recently established X-ray structure of the MTase domain of HMPV might allow us to build a homology model for HRSV MTase³⁰. This new information could facilitate structure-guided drug design.

The 2'O methylation of viral RNA is reported to be important for viral evasion of host innate immunity. The interferon-induced

proteins with tetratricopeptide repeats (IFIT) are a part of the innate immune response needed to defend against viruses, recognize unmethylated mRNAs as “non-self” and target them for degradation, thus underscoring the importance of mRNA capping in replication of West Nile virus (WNV), Poxviruses, Coronaviruses, and HMPV^{28,32}. Although additional studies are needed to confirm this phenomenon for HRSV, one could envision a potential “double whammy” effect of HRSV MTase inhibition on viral replication; on one hand the inhibition of MTase activity may attenuate or block viral transcription and replication whilst the generation of unmethylated viral mRNA caps will result in its degradation by the cellular innate immune response machinery. Since viral MTase have multiple (4) catalytic residues required for enzyme activity, mutation at multiple contact residues in the catalytic site might be needed to overcome antiviral activity of an inhibitor. Moreover, targeting HRSV MTase at the SAM binding site might offer a high barrier to drug resistance, as mutations at key inhibitor-contact residues required for SAM-binding would attenuate interactions with SAM and result in reduced catalytic efficiency, eventually leading to poor replicative fitness. Thus, targeting HRSV MTase for antiviral activity might offer multiple benefits. Mechanistic distinctions between nsNS viral MTases (using VSV as a prototype) in comparison to host MTases could enable the discovery of antivirals that selectively target viral MTases but spare host MTases thus minimizing potential toxicity²⁵.

The high degree of sequence conservation of the HRSV MTase catalytic residues and the fundamental differences between viral and host capping mechanisms combined with the potential for the restoration of innate immune response that could specifically degrade viral mRNAs makes HRSV MTase a logical target for HRSV drug discovery efforts. Due to the sequence similarity of HRSV MTase with other members of the paramyxoviridae family, it is likely that an HRSV MTase inhibitor also have activity against paramyxoviruses. Such an antiviral spectrum might be added value for empirical treatment, especially due to unavailability/delay of virus-specific diagnostics and short time to treatment initiation. HRSV MTase inhibitors should be counter screened against viral panels to determine the antiviral specificity as is the norm for antiviral drug discovery efforts during lead optimization.

Data availability

No data are associated with this article.

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A number of changes have been made to the article, but a number of issues remain, as follows (in no particular order):

1. Even after revision, virus classification remains incorrect in many parts of the article. There is no subfamily *Pneumovirinae* (used at least twice) and HMPV is not a member of the family *Paramyxoviridae*.
2. It is stated multiple times that VSV is a prototype for the nsNS group of viruses. This only needs to be stated once. Also, the abbreviations VSV and HRSV are introduced multiple times.
3. The first sentence in the paragraph beginning "Viral mRNA cap methylation process..." is not grammatically correct.
4. In the section "HRSV MTase as an antiviral target" the statement that the MTase catalytic motif and SAM binding domain seems to be conserved between RSV subtypes, pneumoviruses and nsNS viruses is redundant: if it is conserved in nsNS viruses, then it will be conserved in RSV subtypes and pneumoviruses.
5. In the same section, in the discussion regarding the "double whammy" effect of MTase inhibition, there should be a more accurate explanation of what happens when a non-methylated RNA is recognized as non-self.
6. In the same section, lack of a methylated cap would be expected to result in poor translation, rather than having an impact on transcription.
7. In the same paragraph, last sentence, it is not clear why VSV would be used as a prototype for studies to develop antivirals. It would be much more logical to perform experiments with HRSV or HMPV polymerase.

8. A number of statements lack citations, e.g. "The processes of capping and methylation are distinct in different viruses" and "requirement of cis-elements in viral RNA for cap methylation", or the citations are incorrect or do not cite the most relevant paper e.g. "The interferon-induced proteins with tetratricopeptide repeats (IFIT) are a part....."
9. Overall, the impression given is that the article has been hurriedly composed, without attention to existing literature and current taxonomy and without careful proofreading. As the authors note, this is a really interesting and timely topic. I think that this could be a very useful article, but it needs further editing.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: RSV transcription and genome replication mechanisms

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 30 September 2019

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Dirk Jochmans

Laboratory of Virology and Chemotherapy, Department of Microbiology and Immunology, Rega Institute for Medical Research, KU Leuven, Leuven, Belgium

Manuscript has sufficiently improved and covers well the potential of RSV MTase as a target for antiviral therapy. In figure 1 Gulanylyl and Guanylyl should be replaced by guanosine.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Antiviral research.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 02 July 2019

<https://doi.org/10.5256/f1000research.20595.r49405>

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Kalkeri *et al.* investigate the potential of the human RSV MTase as target for antiviral drug discovery. This is a very relevant topic, as RSV remains an important human pathogen for which it has been very challenging to find potent therapies and/or vaccines. MTase of several other viruses have been investigated and the authors link this information to RSV with the hope to find new starting points for RSV drug discovery.

In general, we do not have the impression this review brings a lot of value. Several of the hallmark papers in the field (example Nature Communications volume 6, Article number: 8749 (2015)¹ or Nature Reviews Microbiology volume 10, pages 51–65 (2012)²) describe many of the arguments of the authors in a more understandable/graphical way.

The value of the manuscript is also limited as it is challenging to read. It lacks a clear explanation of the different biochemical reactions involved in vRNA capping. A reaction scheme would be very helpful for the reader (like for example in Nature Reviews Microbiology volume 10, pages 51–65 (2012)²). The paper also lacks an explanation on what is SAM and how SAH is a by-product from MTase activity that has SAM-dependent inhibition.

Similarly, the work describes several conserved sequences and residues but an overview in a figure of these gene/protein elements is lacking while it would be extremely helpful for the reader to keep track. Without this basic information represented in a schematic way it is impossible to link the understanding of MTase from other nsNS viruses with the potential implications for RSV.

The text also shows several language errors including many missing occasions of 'the' and repeating messages like the fact that the K-D-K-E motif is a typical 2'-O MTase fold is mentioned twice under "Guanylation and methylation functions". Also the fact that sequence similarity for homology-based modelling is important is suggested at several places in the text.

Also, some relevant arguments remain unexplained and thus difficult to understand. For example "MTase activity seems to affect late elongation or polyadenylation" or "Due to the involvement of multiple amino acid residues for enzyme activity, targeting HRSV MTase might offer a high barrier for resistance emergence."

Two aspects of the paper are certainly interesting. These are the parts on the genetic support for RSV MTase role in viral replication and the importance of 2'O methylation on viral RNA. They clearly represent the challenges and opportunities for finding novel MTase inhibitors.

References

1. Paesen GC, Collet A, Sallamand C, Debart F, et al.: X-ray structure and activities of an essential

Mononegavirales L-protein domain. *Nat Commun.* 2015; **6**: 8749 [PubMed Abstract](#) | [Publisher Full Text](#)

2. Decroly E, Ferron F, Lescar J, Canard B: Conventional and unconventional mechanisms for capping viral mRNA. *Nat Rev Microbiol.* 2011; **10** (1): 51-65 [PubMed Abstract](#) | [Publisher Full Text](#)

Is the topic of the opinion article discussed accurately in the context of the current literature?

Partly

Are all factual statements correct and adequately supported by citations?

Yes

Are arguments sufficiently supported by evidence from the published literature?

Partly

Are the conclusions drawn balanced and justified on the basis of the presented arguments?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Antiviral research.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 19 June 2019

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Rachel Fearn

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This review article focuses on the methyltransferase activity of the respiratory syncytial virus (RSV) polymerase. The review covers the role of the methyltransferase in mRNA transcription and draws parallels with related viruses to describe the features of the methyltransferase. It also makes the case that inhibition of the methyltransferase activity could have a dual impact on the virus by inhibiting viral gene expression and by eliciting innate immune responses. There are a few errors that should be addressed to make the article scientifically sound. These are listed below:

1. Throughout the review, the authors refer to RSV and HMPV as being members of the family Paramyxoviridae. This is no longer the case, they are now in a new family called Pneumoviridae. In addition, RSV is now formally termed human orthopneumovirus. The text

should be revised to accommodate the new family name.

2. The authors also refer to the RSV mRNAs being modified by guanylation, however studies by Ogino and coworkers with VSV and other rhabdoviruses have shown that the mRNA cap is added by a GDP polyribonucleotidyltransferase activity, rather than by guanylation. Given the similarities within their capping domains, it is highly likely that RSV uses the same mechanism as the rhabdoviruses (although this has not yet been shown). Therefore, the text should be modified to remove reference to guanylyltransferase activity and a sentence or two added to explain the distinctive capping mechanism that is likely used. Likewise, the phrase "This universal process of capping and methylation..." should be rewritten as the processes are distinct, even though the end products are chemically equivalent.
3. In the description of the polymerase, the authors refer to 6 conserved domains. It is more appropriate to use the term "regions" rather than "domains".
4. In the section titled "Genetic support for RSV MTase role in viral replication" there is reference to work performed on the metapneumovirus polymerase, but this work is not cited (it is cited elsewhere in the article). The work from the Li lab should be cited here.

Is the topic of the opinion article discussed accurately in the context of the current literature?

Yes

Are all factual statements correct and adequately supported by citations?

Partly

Are arguments sufficiently supported by evidence from the published literature?

Yes

Are the conclusions drawn balanced and justified on the basis of the presented arguments?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: RSV transcription and genome replication mechanisms

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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