Supplementary Data

Probing the SAM Binding Site of SARS-CoV-2 nsp14 in vitro Using SAM Competitive Inhibitors Guides Developing Selective bi-substrate Inhibitors

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**Protein Expression and Purification**

The expression clone for nsp14 with a 6xHis-tag and tobacco etch virus (TEV) protease cleavage site was codon optimized, synthesized, and cloned into the NdeI-BamH1 sites of pET11b by Bio Basic Inc. (Markham, ON, Canada). A DNA fragment encoding full-length SARS-CoV-2 nsp14 (A1 to Q527) was amplified from this synthetic template by PCR and sub-cloned into p28BIOH-LIC (Addgene plasmid # 62352). This added a N-terminal Avi-tag for in vivo biotinylation and C-terminal His-tag. Following transformation into E. coli BL21 (DE3) containing a BirA expression plasmid (BPS Bioscience) the cells were cultured in LB broth overnight at 37 °C supplemented with kanamycin and spectinomycin. Terrific Broth was inoculated with overnight culture of nsp14 supplemented with 50 µg/mL Kanamycin and 100 µg/mL spectinomycin and the cells were grown using LEX system (https://www.thesgc.org/science/lex). At OD₆₀₀ of 0.8-1.5, the temperature was lowered to 18°C and the culture was supplemented with 1 mM IPTG (isopropyl-1-thio-D-galactopyranoside). D-Biotin was added at 10 µg/mL final concentration to allow in cell biotinylation of the protein. The cells were incubated overnight before being harvested (5000 xg for 15 minutes at 4°C) using a Beckman Coulter centrifuge (model: Avanti J-20 X PI, rotor JLA-8.1000).

Harvested cells were re-suspended in binding buffer containing 20 mM Tris-HCl pH 8.5, 500 mM NaCl, 10 mM MgCl₂, 5% glycerol, and 0.5 mM TCEP. The mixture was then treated with 1x protease inhibitor cocktail (100X protease inhibitor cocktail in 70% ethanol (0.25 mg/ml Aprotinin, 0.25 mg/ml Leupeptin, 0.25 mg/ml Pepstatin A and 0.25 mg/ml E-64)) and Roche complete EDTA-free protease inhibitor cocktail tablets. The cells were lysed chemically by rotating 30 min with CHAPS (final concentration of 0.5%) and 5 µl/L Benzonase Nuclease (in house) followed by sonication at frequency of 8.0 (10” on/7” off) for 10 min (Sonicator 3000, Misoni). The crude extract was clarified by high-speed centrifugation (45 min at 36,000 xg at 4°C) by Beckman Coulter centrifuge (rotor: JLA 16.250).

The clarified lysate was passed through pre-equilibrated Monomeric Avidin agarose resin (Pierce) following the manufacturer’s instructions. Briefly, the column was washed with PBS followed by blocking the resin with 2 mM Biotin solution in PBS. Subsequently, the excess biotin was removed by 0.1 M Glycine, pH 2.8 followed by a full column wash with PBS. The lysate was continuously
passed through the beads for 3 hours at 4°C. The resin was then washed with PBS to remove the non-specific proteins until Bradford dye remained unchanged. The biotinylated nsp14 was then eluted using 2 mM biotin solution in PBS. Finally, the purity of the nsp14 protein was confirmed by SDS-PAGE (Suppl. Fig. S17a).

The eluted protein was then dialyzed into the final buffer containing 50 mM Tris-HCl pH 8.5, 200 mM NaCl, 5% glycerol, 0.5 mM TCEP for 60 min while spinning at 4°C.

To evaluate the stability of nsp14 protein after freeze and thaw, an analytical SEC was performed by first thawing the protein on ice and centrifuging it at 18,900 ×g for 10 min at 4°C. Then, the nsp14 was loaded onto Superdex200 10X 300 column (GE Healthcare) after equilibration with 50 mM Tris-HCl pH 8.0 buffer containing 200 mM NaCl, 5% glycerol, 0.5 mM TCEP and the molecular weight of the protein was estimated based on the standard peaks used to plot the calibration curve. The molecular weight of the nsp14 was confirmed by running 10 μg of proteins on mass spectrometer (Agilent Technologies, 6545 Q-TOF LC/MS) (Suppl. Fig. S17b).

Figure S1. Crystal structure of SARS-CoV nsp10-nsp14 in complex with GpppA and SAH. Exonuclease domain of nsp14 is in light gray, methyltransferase domain of nsp14 is in dark gray, SAH in red stick, and GpppA with green stick. Nsp10 is shown in blue. (PDB ID: 5C8S)\(^1\)
Figure S2. Sequence alignment of nsp14 from 7 coronaviruses that are currently known to infect humans. The amino acid sequence of nsp14 is highly conserved among these coronaviruses. The alignment indicates 95.1%, 62.7%, 57.8%, 58.5%, 52.9% and 53.7% identity of SARS-CoV-2 nsp14 sequence with SARS-CoV, MERS-CoV, OC43, HKU1, 299E and NL63, respectively. Total of 184 amino acids are conserved among all 7 coronaviruses. The sequences have been aligned using Clustal Omega.² The image was prepared using Jalview.
Figure S3. Nsp14 activity assay optimization. Effect of (a) Buffers, (b) pH, (c) DTT, (d) Triton X-100, (e) DMSO and (f) MgCl$_2$ on nsp14 activity was assessed. All values are mean ± standard deviation of three independent experiments (n=3).
Figure S4. Kinetic characterization of nsp14 activity. Linear initial velocities at various concentrations of (a) RNA substrate at fixed concentration of SAM (1 µM) and (b) various concentrations of SAM at fixed concentration of RNA (1 µM) were evaluated. The values calculated within the linear period for each reaction were used to plot figure 1a and 1b and calculate the $K_m$ and $k_{cat}$ values. All values are mean ± standard deviation of three independent experiments (n=3).

Figure S5. Effect of nsp10-nsp14 complex formation on methyltransferase activity of nsp14. Complex preparations of nsp10: nsp14 ratios up to 20 (nsp10): 1 (nsp14) were tested for methyltransferase activity. No significant effect on activity of nsp14 was observed at any concentrations of nsp10.
Figure S6. Dose response for nsp14 inhibition by sinefungin. All values are mean ± standard deviation of three independent experiments (n=3). IC\textsubscript{50} value (0.019 ± 0.01 μM) was determined for sinefungin at optimized conditions as described in methods.

Figure S7. Orthogonal confirmation of nsp14 screening hits. The screening hits, (a) SS148, (b) WZ16, (c) DS0464, (d) DS0466, (e) JL27-56A1, (f) Compound 8, (g) SAH and (h) MTTR025495 were tested by Surface Plasmon Resonance (SPR). For each compound, the Sensorgram (solid green) is shown with the kinetic fit (black dots), and the steady state response (black circles) with the steady state 1:1 binding model fitting (red dashed line). KD values are presented in Table 1.
Figure S8. Amino acid homology of nsp14 from SARS-CoV-1 and SARS-CoV-2. Sequence alignment of SARS-CoV-1 nsp14 from PDB code 5C8S and SARS-CoV-2 nsp14 downloaded from Uniprot. Amino acids within 4.0 Å of the cofactor or GpppA in structure 5C8S are boxed in blue.

Figure S9. MOA determination. Dose Response Curves for DS0464 against nsp14 at varying concentrations of (a) RNA at 1.25 µM of SAM and (b) SAM at 250 nM of RNA (saturating concentration). The IC$_{50}$ values from these experiments were plotted in figure 5 for determining the mechanism of action of DS0464.
Figure S10. Selectivity of the nsp14 screening hits against G9a, a protein lysine methyltransferase (PKMT). Dose response data are presented for indicated compounds. None of the compounds inhibit G9a activity up to 50 µM of compound.

Figure S11. Selectivity of the nsp14 screening hits against ALKBH5, an RNA demethylase. Dose response data are presented for indicated compounds. None of the compounds inhibit ALKBH5 activity up to 50 µM of compound.
Figure S12. Selectivity of the nsp14 screening hits against BCDIN3D, an RNA methyltransferase (RNMT). Dose response data are presented for indicated compounds. Experiments were performed in triplicate only for SS148 and WZ16.

Figure S13. Selectivity of the nsp14 screening hits against SETD3, a PKMT. Dose response data are presented for indicated compounds. Experiments were performed in triplicate for SS148 and WZ16 (n=3).
Figure S14. Selectivity of the nsp14 screening hits against METTL3-METTL14, an RNA methyltransferase. Dose response data are presented for nsp14 screening hits. Experiments were performed in triplicate for SS148 and WZ16 (n=3).
Figure S15. Selectivity of SS148. Selectivity of SS148 against protein arginine methyltransferases (PRMT1, PRMT3, PRMT4, PRMT5, PRMT6, PRMT7, PRMT8, and PRMT9), Protein Lysine methyltransferase (DOT1L and SETD3), DNA methyltransferases (DNMT1, DNMT3a, and DNMT3b) and RNA methyltransferase (BCDIN3D and METTL3-METTL14) were assessed in dose response. Experiments were performed in duplicate (n=2).
**Figure S16. Selectivity of DS0464.** Dose response analysis of DS0464 against selected methyltransferases. Experiments were performed in duplicate (n=2).
Figure S17. Nsp14 purification and quality assessment. (a) SDS-PAGE demonstrating biotinylated nsp14 after affinity purification on Avidin agarose resin. The lanes next to the molecular-weight size marker from left to right correspond to (1) pellet, (2) supernatant, (3) flow-through, (4) wash, (5) 10 μL elution, and (6) 15 μL elution. The arrowhead indicates the nsp14 at approximately 63 kDa. (b) Mass spectrum of nsp14 confirms the molecular weight of the protein.

References

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