Fluorescence-Based Activity Screening Assay Reveals Small Molecule Inhibitors of Vaccinia Virus mRNA Decapping Enzyme D9

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ABSTRACT: Vaccinia virus (VACV) represents a family of poxviruses, which possess their own decapping machinery as a part of their strategy to eliminate host mRNAs and evade the innate immune response. D9 is one of the two encoded VACV decapping enzymes that is responsible for cap removal from the 5′ end of both host mRNA transcripts and viral double-stranded RNAs. Little is known about the structural requirements for D9 inhibition by small molecules. Here, we identified a minimal D9 substrate and used it to develop a real-time fluorescence assay for inhibitor discovery and characterization. We screened a panel of nucleotide-derived substrate analogues and pharmacologically active candidates to identify several compounds with nano- and low micromolar IC₅₀ values. m7GpppCH₂p was the most potent nucleotide inhibitor (IC₅₀ ∼ 0.08 μM), and seliciclib and CP-100356 were the most potent drug-like compounds (IC₅₀ 0.57 and 2.7 μM, respectively). The hits identified through screening inhibited D9-catalyzed decapping of 26 nt RNA substrates but were not active toward VACV D10 or human decapping enzyme, Dcp1/2. The inhibition mode for one of the compounds (CP-100356) was elucidated based on the X-ray cocrystal structure, opening the possibility for structure-based design of novel D9 inhibitors and binding probes.

INTRODUCTION

Eukaryotic mRNAs are co-transcriptionally modified by the addition of a 7-methylguanosine triphosphate moiety at the 5′ end. This modification, referred to as the 5′ cap, protects mRNA from premature degradation by 5′-exonucleases and is necessary for canonical translation initiation. Only specialized decapping enzymes can remove the 7-methylguanosine cap from an mRNA body, thereby exposing it to 5′-to-3′ degradation. Hence, mRNA decapping is an important process, aiding the control of gene expression. Eukaryotic cells produce several decapping enzymes that participate in bulk mRNA decay, mRNA quality control, and specialized degradation pathways.¹⁻⁴ Two major decapping enzymes involved in bulk mRNA degradation are Nudix hydrolase, Dcp2, which is involved in the 5′−3′ degradation pathway and scavenger decapping enzyme, DcpS, which cleaves m⁷G-capped structures released as the products of 3′−5′ mRNA decay.⁵,⁶ Moreover, other proteins possessing intrinsic decapping activity have been identified and include Nudix family hydrolases (e.g., Nudt3 and Nudt16) and DXO decapping exoribonuclease protein, which is responsible for degrading aberrantly capped RNAs.⁷ Precise control of the activity of those enzymes is crucial to maintain the cell homeostasis.

Some viruses express their own decapping machinery as a part of their strategy to inhibit the cellular translation and promote the synthesis of viral proteins. Till date, four virus-encoded decapping enzymes have been described: D9 and D10 from vaccinia virus (VACV), g5R from African swine fever virus, and L375 Nudix enzyme from mimivirus.⁸⁻¹¹ All three viruses belong to the family of nucleocytoplasmic large DNA viruses.

The viral decapping enzymes differ greatly in the substrate specificity but share the catalytic motif and the ability to cleave m⁷GpppN-RNAs to release m⁷GDP as a product (Figure 1A). They represent a class of phosphohydrolases that cleave nucleoside diphosphates linked to another moiety X (Nudix). This Nudix motif consists of a 23 amino acid characteristic sequence GX₅EX₅(UA)XREX₂EEXGU, wherein U represents an aliphatic, hydrophobic residue, and X represents any amino acid.¹² Albeit VACV is not pathogenic to humans, it represents
a family of large DNA viruses, including zoonotic viruses, which can jump to humans and/or are pathogenic to livestock and humans. D9 and D10 from VACV are therefore prototype viral decapping enzymes, the studies of which may aid in elucidating how viruses use their decapping machinery to avoid the innate immune response and influence the host translation.

To ensure mRNA stability and translational activity, VACV adds 5′ caps onto viral mRNAs using the virus-encoded capping machinery. Early VACV mRNAs are modified to form either m′GpppA m or m′GpppG m caps, whereas intermediate and late transcripts carry only m′GpppA m caps. D9 is expressed in the early stage of infection and together with D10, it prevents accumulation of dsRNAs, thereby minimizing cellular antiviral response mediated by PKR and OAS/RNase L. D9 exhibits specificity for N7-methylated cap structures and a preference for longer RNA substrates (>30 nt). Unmethylated (Gppp-capped) RNAs are not cleaved. The enzyme is strongly inhibited by uncapped RNA, whereas N7-methylated guanine nucleotides such as m′GpppG, m′GTP, and m′GDP are less potent D9 inhibitors. Both D9 and D10 enzymes trigger the degradation of multiple cellular and viral mRNAs to promote the selective translation of viral mRNAs and reduce the levels of dsRNAs, which are the main trigger of innate immune response (Figure 1B). Interestingly, VACV deficient in either D9 or D10 is more immunogenic due to dsRNA accumulation, replicates more efficiently in cancerous than in healthy cells, and thus acts as an oncolytic virus. Significant effort has been made to understand the role of D9 and D10 in VACV replication; nonetheless, the differential functions of these enzymes, their regulation, and interdependence remain elusive.

Structural and functional studies on cellular decapping enzymes, both in vitro and in vivo, can be greatly facilitated by the design of specific small-molecule ligands and inhibitors. As such, in this work, we aimed to design molecular tools that could find utility in studying the activity of recombinant D9 and aid discovery of small-molecule inhibitors of this enzyme. Little has been determined about cap-related substrate specificity of D9 or design of specific inhibitors. The so-far-reported inhibition studies relied on isotopically labeled RNA substrates, the use of which entails relatively high cost and low throughput. Here, we sought to design a new simple approach that can be used to study the activity of VACV D9 and discover inhibitors via high-throughput screening (HTS). To this end, we developed fluorescence intensity-based (FLINT) assay with a small-molecule fluorescent probe (Figure 2A). The designed method was adopted to the HTS format to screen an in-house library of nucleotide-derived compounds and a commercially available library of pharmacologically active compounds (LOPAC1280, Sigma Aldrich). The identified hits were verified on short RNA substrates and cross-examined against VACV D10 and human decapping enzyme, Dcp1-Dcp2. Finally, one of the most potent inhibitors was crystalized in complex with D9, providing a deeper insight into the inhibition mode.

RESULTS

Development of Activity-Based Assay for D9 Enzyme.

We first synthesized a set of fluorescently labeled nucleotides as potential fluorogenic substrates for D9. Knowing that D9 specifically cleaves 7-methylguanosine-capped transcripts, all probes were equipped with a 7-methylguanine nucleotide moiety as a recognition element and a fluorescent tag sensitive to m7G presence as a cleavage-responsive element (Figure 2A). 7-Methylguanosine has been shown to act as a quencher of electron-rich fluorescent dyes, giving the basis for the development of fluorescent turn-on probes. To select the most suitable probe in terms of substrate and fluorescent properties, we tested several fluorescent dyes [pyrene (Py), perylene (Pe), and boron dipyrromethene (BODIPY-FL)] and different oligophosphate chain lengths (from tri- to pentaphosphate; Figure 2B). The compounds were subjected to enzymatic cleavage by D9, and emission spectra were recorded over time (Figure 2C,D). In all cases, the gradual increase in fluorescence intensity was observed, consistent with cleavage by D9 enzyme. The greatest changes were observed for probes labeled with pyrene, but the efficiency varied depending on the length of the oligophosphate chain. The system response decreased with the length of the oligophosphate (over 14-, 4-, and 3-fold enhancement of the fluorescence signal for tri-, tetra-, and pentaphosphate, respectively); triphosphates were also the most rapidly cleaved substrates (Figure S1).

![Figure 1.](https://doi.org/10.1021/acschembio.2c00049)
Therefore, the pyrene-labeled m\textsuperscript{7}GTP analogue (1b) was selected for further studies as a turn-on probe with the optimal substrate and emission properties. As m\textsuperscript{7}GTP-Py is structurally different from natural D9 RNA substrates, we tested whether the hydrolysis mechanism remains similar. The probe was subjected to D9 cleavage, and the reaction progress was monitored using RP-HPLC (Figure S2A,B). Appropriate fractions containing hydrolysis products were collected and identified by mass spectrometry (Figure S2C−F), proving conclusively that D9 enzyme cleaves the probe liberating m\textsuperscript{7}GDP and monophosphate of the rest of the molecule, that is, with the same regioselectivity as observed for capped RNA.

Probe 1b was then characterized in more detail and employed to develop high-throughput screening assay in 96-well plates (Figure 3A). Buffer compositions, concentrations of divalent metal ions, and protein-stabilizing agents were adjusted to optimize the reaction conditions. Manganese (Mn\textsuperscript{2+}) ions were essential for efficient enzyme activity (Figure S3A), whereas addition of BSA as a stabilizing protein significantly improved reproducibility (Figure S3B). Initial hydrolysis rates for varying 1b concentrations were next determined by measuring fluorescence intensity changes as a function of time. Plotting the initial rates as a function of substrate concentrations enabled the determination of Michaelis–Menten kinetic parameters for the probe (K\textsubscript{M}).
Figure 3. Validation of fluorescence-based assay for D9 activity and evaluation of an in-house library of nucleotide-based compounds. The assay was performed at 30 °C in a 96-well format using 3 μM 1b, in the presence of the putative inhibitor (half-logarithmic dilution from 100 μM) and S
KOAc and 2 mM MgCl₂, 0.3 mM MnCl₂, 2 mM DTT, and 0.1% BSA at 30 °C. In the presence of 5 nM D9, data shown are means from three independent experiments ±SEM. (C) Determination of the Z-factor for D9 inhibition assay. Reaction conditions: 3 μM 1b, 5 nM D9 in the absence (positive control) or presence (negative control) of the 100 μM nucleotide inhibitor. The experiment was carried out in a 96-well plate reader in 10 mM MOPS·HCl, pH 7.0 buffer containing 100 mM KOAc and 2 mM MgCl₂, 0.3 mM MnCl₂, 2 mM DTT, and 0.1% BSA at 30 °C, and (D) determined IC₅₀ values. Only compounds with IC₅₀ < 60 μM are shown. All IC₅₀ values, along with compound structures, are shown in Table S1; (E) representative inhibition curves and (F) structures of the most potent compounds from mono-, di-, and trinucleotide groups. Data are shown as mean values from triplicate experiments ±SEM.

Vₚmax and kₚas (Figure 3B). Based on those results, we established optimal conditions (Table 1) that ensured sufficient fluorescence signal response, proper reaction kinetics, high protein stability, and good reproducibility. Z' factor, determined under the optimized conditions was 0.75 (Figure 3C), indicating that the quality of the assay is sufficient for the application in high-throughput experiments.

Inhibition Studies. To identify the structural preferences for D9 inhibition by substrate analogues, the optimized D9 assay was employed to evaluate an “in-house” library consisting of unmodified mono-, di-, and trinucleotides and their analogues. To assess whether such approach is feasible, we first tested susceptibility of selected nucleotides to D9 cleavage. A set of 15 compounds with different modifications were incubated with D9 for 2 h under assay conditions. Most of the compounds were unsusceptible or did not undergo significant hydrolysis during initial 30 min of the analysis (Figure S4).

Next, the half-maximal inhibitory concentrations (IC₅₀) were determined for all nucleotides from the library by observing the degradation of 1b in the presence of different concentrations of the putative inhibitor (Figure 3D, Table S1). The determined IC₅₀ values varied between 0.077 and over 10 μM. The strongest D9 inhibitors (IC₅₀ below 1 μM), 26-nt long RNA substrate co-transcriptionally capped with m’GpppCH₂pG (IC₅₀ = 2.6 ± 0.4 μM) and m’Gppp, m’G D3 (IC₅₀ = 3.6 ± 0.4 μM), the latter has been previously identified as an inhibitor of yeast decapping enzyme SpDcp1/Depc2. The most potent compound in the group of trinucleotides was m’GpppGanG with IC₅₀ value of ~4 μM. Interestingly, both in the group of dinucleotides and trinucleotides, 2′-O methylation of 7-methylguanosine notably increased inhibitory properties, suggesting that this modification might increase binding affinity for D9. At the same time, compounds that were 2′-O methylated either at 7-methylguanosine or within the second nucleotide showed the highest susceptibility to hydrolysis by D9 (Figure S4), which might suggest that this is a binding affinity-related effect.

In addition, we screened a commercially available compound library consisting mostly drug-like compounds (LOPAC1280, Sigma-Aldrich). Compounds were screened at 30 μM under conditions given in Table 1 (Figure 4A). The relative reaction progress for each compound was calculated by normalizing the initial rate of the reaction in the presence of the tested compound to the initial rate in the absence of any inhibitor. The criterion of the cutoff for inhibitor selection for further evaluation was set to 30% of the maximal initial rate (dotted line), which yielded six potential hits. The hits were then further evaluated, including determination of their IC₅₀ values (Figure 4B,C). Three most potent compounds had IC₅₀ value below 10 μM, and they included an adenine derivative seliciclib (IC₅₀ = 0.57 ± 0.05 μM), quinazolinamine derivative CP-100356 (IC₅₀ = 2.7 ± 0.7 μM), and a purine derivative SCH 58261 (4.5 ± 0.8 μM) (Figure 4D).

Verification of Inhibitory Properties toward D9 for the Identified Hits. To further validate the hits, we checked whether the three most potent inhibitors from the LOPAC library and the best hit from nucleotide-derived library inhibit D9-catalyzed decapping of short capped RNA transcripts. A 26-nt long RNA substrate co-transcriptionally capped with m’GpppApG (cap 0 structure) was subjected to D9-catalyzed hydrolysis in the presence of the evaluated compound at three different concentrations (1, 10, and 100 μM). The reaction progress was analyzed electrophoretically in reference to the reaction performed in the absence of the inhibitor (Figure S5A). The band intensities corresponding to capped and uncapped RNAs were quantified densitometrically, and the decay of the substrate was plotted as a function of time (Figures S5B,C, S5). All compounds showed inhibitory activity toward RNA decapping, and their relative potencies were in good agreement with IC₅₀ values determined by the fluorescence assay using probe 1b. Finally, we tested the specificity of the compounds in the context of VACV D10 and human decapping enzyme Dcp1/2, which are also m’GpppRNA hydrolases belonging to the Nudix family. We did not observe any inhibitory activity toward Dcp1/2 for the tested compounds at concentrations up to 100 μM, thereby confirming their selectivity toward D9 (Figures S5D,E, S6). m’GpppCH₂P and CP-100356 showed weak inhibitory activity toward D10 (Figure S7). The most potent drug-like D9 inhibitor identified in this study, seliciclib, showed very good selectivity for D9.

Crystallographic Structure of Vaccinia Virus D9 with CP-100356. To better understand the mechanism of inhibition, we determined the crystal structure of wild-type D9 bound to inhibitor CP-100356 (P3_B7) to 1.78 Å (Figure 5A). The structure was solved to high resolution in the presence of the inhibitor, which occupied the active site cleft. D9 bound to inhibitor CP-100356 (P3_B7) to 1.78 Å (Figure 5A). The structure was solved to high resolution in the presence of the inhibitor, which occupied the active site cleft.
6A, Table 3). This crystal form has two copies of D9 in the asymmetric unit (all-atom RMSD 1.81 Å), each bound to 1 molecule of CP-100356. The conformation of CP-100356-bound D9 is nearly identical to a recently published structure of D9 bound to m7GDP product in the post-catalytic, inactive conformation (all-atom RMSD 2.55 Å). Our crystal structure reveals that the CP-100356 is positioned in the cap-binding pocket by continuous stacking of the 6,7-dimethoxyquinazoline moiety between aromatic residues F54 and Y158 in a manner similar to m7G cap recognition in the product-bound structure, PDB 7SEZ (Figure 6B,C). These conserved residues are essential for efficient cap hydrolysis.28 However, in the complex with CP-100356, Y158 is significantly reoriented compared to the position occupied in the complex with m7GDP (Figure 6B,C), presumably to allow more efficient π−π stacking interaction with the aromatic quinazoline system.

Figure 4. Identification of D9 inhibitors by LOPAC1280 library screening. (A) Relative reaction progress of D9-catalyzed (5 nM) hydrolysis of probe 1b (3 μM) with 1280 compounds from the LOPAC library (30 μM). Data are means from the duplicate experiment; (B) IC_{50} values for six selected hits from the screening experiment (30% cutoff for inhibitor selection). Numerical values are shown in Table 2; (C) inhibition curves for identified inhibitors—one compound had to be tested separately due to its spectroscopic properties. Data are mean values ± SEM from three independent experiments; (D) structures of six most potent hits, and their IC_{50} values are shown in Table 2.
Figure 5. Verification of inhibitory properties and selectivity of the identified hits in a decapping assay on short RNA substrates. (A) Idea of RNA decapping assay based on short m7Gppp-RNA substrates (26 nt) and electrophoretic analysis and (B) representative gel (right) from single experiment with compound CP-100356 and densitometric analysis (left) of gel band intensities. m7Gppp-capped RNA (20 ng) was incubated for 1 h at 37 °C with D9 (3 nM) without or with the presence of the tested inhibitor at 1, 10, or 100 μM. Samples from different time points were analyzed by polyacrylamide gel electrophoresis (PAGE) and stained with SYBR Gold. The data for all tested compounds are shown in Figure S5; (C) results from triplicate experiments ±SEM for four identified hits confirming their inhibitory properties toward D9 decapping enzyme; and (D,E) analogous experiments with human Dcp1/2 complex (11 nM)—data show no inhibitory properties of tested compounds toward this enzyme. More details are given in the Supporting Information.
of the ligand. Unlike the m7G cap in PDB 7SEZ, which is oriented by hydrogen bond base pair mimicry between the guanine base and residues D151 and E16, the CP-100356 inhibitor is not oriented by hydrogen bonding. Rather, the 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline moiety extends into a small cavity past D151 and E16 (Figure 6C). It is clear from our crystal structure that CP-100356 inhibits D9 cap hydrolysis by stabilizing the enzyme in an inactive conformation, preventing conserved aromatic residues F54 and Y158 from binding capped mRNA substrate.

Attempts to crystallize D9 with the other most potent inhibitors identified from our screen, seliciclib (P4_A6) and SCH 58261 (P16_F10), were unsuccessful. Nonetheless, based on the similarity of these compounds to natural nucleobase moieties, we hypothesize that they likely bind in a similar manner, that is, by stacking between F54 and Y158.

**CONCLUSIONS**

Literature reports that VACV D9 requires RNA body for decapping activity, and that the decapping efficiency increases with the length of the RNA.\(^8\) Surprisingly, in this work, we discovered a set of minimal substrates (activity probes) for D9 that consist of m7GTP molecule connected to a fluorescent tag via the terminal phosphate moiety. The activity probes were evaluated as D9 substrates to select the most efficient and responsive internally quenched probes suitable for high-throughput assay development. Pyrene-labeled m7GTP (probe 1b) had the most favorable properties, both in terms of hydrolysis kinetics and fluorescent response upon cleavage. Probably both the m7G nucleotide and the pyrene moiety contribute to recognition by D9 because the corresponding unlabeled compounds such as m7GTP and m7GpppG are very poor D9 substrates (as determined by RP HPLC). The probe was used to develop fluorescent HTS assay to screen for D9 inhibitors. The addition of Mn\(^{2+}\), beside Mg\(^{2+}\) ions, and the presence of BSA were necessary to maintain high protein activity and stability. The optimized assay was characterized by a \(Z'\) factor of 0.75, that is, suitable for HTS. The screening experiments were performed on two distinct compound libraries. The first library consisted of nucleotide derivatives, most of which were substrate analogues of varying sizes (mono-, di-, and trinucleotides), and the second was

**Table 2. IC\(_{50}\) Values for Inhibitors Identified by HTS Screening**

| LOPAC ID | compound                     | IC\(_{50}\) (μM) |
|----------|------------------------------|-----------------|
| P2_H5    | amscarine hydrochloride (A 9809) | 12.6 ± 1.7     |
| P3_B7    | CP-100356 monohydrochloride (P20171) | 2.7 ± 0.7      |
| P4_A6    | seliciclib                    | 0.57 ± 0.05     |
| P5_A5    | CGS-15943 (C-199)             | 20.1 ± 5.2      |
| P10_F4   | BIO (B 1686)                  | 95 ± 18         |
| P16_F10  | SCH 58261 (S4568)             | 4.5 ± 0.8       |

| Reference Compounds | m7GpppCH\(_2\)P | m7GpppG |
|---------------------|-----------------|---------|
|                     | 0.18 ± 0.01     | 15 ± 2  |

**Table 3. Data Collection and Refinement Statistics**

|                         | D9 + CP-100356 (PDB: 7T7H) |
|-------------------------|-----------------------------|
|                         | Data Collection             |
| space group             | P21_21_21                   |
| Cell Dimensions         |                             |
| a, b, c (Å)             | 56.77, 82.77, 96.31         |
| α, β, γ (deg)           | 90, 90, 90                  |
| resolution (Å)          | 62.77–1.78 (1.844–1.78)     |
| R\(_{merge}\)           | 0.04373 (0.9509)            |
| I/σ(I)                  | 30.35 (2.39)                |
| completeness (%)        | 99.79 (97.95)               |
| redundancy              | 12.9 (12.0)                 |
|                         | Refinement                  |
| resolution (Å)          | 62.77–1.78                  |
| no. unique reflections  | 44,130                      |
| R\(_{work}\)/R\(_{free}\)| 0.1864/0.2182               |
|                         | No. Atoms                   |
| protein                | 3294                        |
| ligand/ion             | 84                          |
| water                  | 194                         |
|                         | B-Factors                   |
| protein                | 48.3                        |
| ligand/ion             | 61.3                        |
| water                  | 50.8                        |
|                         | RMS Deviations              |
| bond lengths (Å)        | 0.019                       |
| bond angles (deg)       | 1.97                        |
|                         | Ramachandran (%)            |
| favored                | 98                          |
| allowed                | 1.74                        |
| outliers               | 0.26                        |

\(^{a}\)Values in parentheses are for the highest resolution shell, and the data set was collected from a single crystal.

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commercially available LOPAC). The nucleotide screening revealed that highly negatively charged 7-methylguanosine mononucleotides are the most potent inhibitors of D9. The LOPAC screening revealed several heteroaromatic compounds as potential hits, which were then further evaluated to show good IC₅⁰ values and verified inhibitory properties on a longer set of substrates. The most interesting hits identified in the screening were seliciclib and CP-100356. Seliciclib, a 2,6-diaminopurine derivative, was the most potent D9 inhibitor, which combined with its very low inhibitory activity against D10 and hDcp1/2 making it a good candidate for future development. Albeit we were not able to determine the crystal structure of D9 in complex with this inhibitor, its structure suggests that the recognition may be mediated by amino acid-forming nucleic acid-binding sites. CP-100356 was another potent and selective inhibitor of D9 with high potential of future development. The crystal structure of recombinant D9 in complex with CP-100356 was determined, which revealed that the compound targets the m’GDP-binding site and forms stacking interactions similar to those observed for 7-methylguanine, suggesting a competitive inhibition mode. This, combined with the observed selectivity of the compound upon cross-examination with VACV D10 and human PNRC2-Dcp1/Dcp2 complex, indicates that the cap-binding site in D9 is distinctive enough from other decapping Nudix family hydrolases to enable the development of small molecules selectively targeting this chemical space.

**METHODS**

**Expression and Purification of Recombinant Viral D9 and D10 Proteins.** Recombinant viral D9 decapping enzyme with additional C-terminal His-tag sequence was expressed, as previously described. The concentration of the protein was determined spectrophotometrically by assuming ε₂₈₀ = 14,900 M⁻¹ cm⁻¹. The enzyme was stored at −80 °C in a storage buffer [50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM DTT, and 20% glycerol].

**Fluorescent Probe Synthesis.** All solvents and chemical reagents for non-fluorescent nucleotide synthesis were purchased from Sigma-Aldrich and used without any pre-treatment, unless otherwise indicated. BODIPY FL iodoacetamide was acquired from ThermoFisher Scientific. Pyrene azide and perylene azide were purchased from Lumiprobe. Guanosine and guanosine S’-monophosphate disodium salts were purchased from Carbosynth. m’GTP’S triethylammonium salt was synthesized, as described previously. Probes 1b, 1d, and 1e were synthesized, as described previously.

Fluorescent nucleotides were purified using analytical or semi-preparative HPLC. Analytical HPLC was performed on Agilent Tech. Series 1200 using the (RP)Supelcosil LC-18-T HPLC column (4.6 × 250 mm, flow rate 1.3 mL/min) with a linear gradient of acetonitrile (0–100% from 0 to 15 min) in 0.05 M ammonium acetate buffer (pH 5.9). UV detection was performed at 260 nm, as well as fluorescent label absorption and fluorescence maximum. Semi-preparative RP HPLC was performed on the same apparatus equipped with the Grace Vision HT C18 HL column (250 × 2 mm, 10 μm, flow rate 5.0 mL/min) with a linear gradient of acetonitrile (0–100% from 0 to 120 min) in 0.05 M ammonium acetate buffer (pH 5.9) and UV detection at 260 nm and at the fluorescent tag absorption and emission maximum. The structure and homogeneity of each final product were confirmed by RP HPLC and high-resolution mass spectrometry HRMS (ESI+).

**m’GTP’S-BODIPY-FL (1a).** m’GTP’S triethylammonium salt (7.5 mg, 8.8 μmol) was mixed with BODIPY-FL iodoacetamide (1.0 mg, 2.4 μmol) in 113 μL of DMSO. The reaction was carried out at room temperature for 1 h and stopped by addition of 0.85 μL of H₂O. The precipitated fluorescein dye remains were centrifuged. The reaction product was purified using analytical RP HPLC (Method A3). The collected eluate was lyophilized repeatedly to afford 0.5 mg (0.6 μmol) of 1a as an orange solid. HPLC yield: 58%. Yield after purification: 25%.

**HRMS (--)ESI m/z; found, 841.9796, calcld for C₅₅H₉₅BF₅N₃O₇P₅S₈: 841.3535.**

**m’GTPC₈Pe (1c).** Aqueous solution of m’Gp₃C₈H₈S triethylammonium salt (1.5 mg, 1.9 μmol) was mixed with a solution of perylene azide (2.0 mg, 4.4 μmol) in DMSO (80 μL), followed by addition of 1 M aqueous solutions of CuSO₄·SH₂O (0.8 mg, 3 μmol, 3 mL) and 1 M sodium ascorbate (1.2 mg, 6 μmol, 6 μL). The reaction was carried out at 37 °C for 24 h with shaking (350 rpm) and quenched by addition of Na₂EDTA (1.8 mg, 4.8 μmol) and water (100 μL). The precipitated perylene dye was separated from the mixture by centrifugation. The reaction product was purified by semi-preparative RP HPLC to give 1c (0.15 mg, 0.14 μmol) as an ammonium salt. HPLC yield: 81%. Yield after purification: 7.3%.

**HRMS (--)ESI m/z; found, 1024.2221, calcld for C₄₂H₅₃N₄O₈P₅S₈: 1024.2203.**

**Spectroscopic Measurements.** Fluorescence emission spectra were recorded using the Agilent Cary Eclipse fluorescence spectrophotometer equipped with the xenon lamp under temperature-controlled conditions. All experiments were performed at 37 °C in the Hellma quartz cuvette (10 × 4 mm) with a sample volume 1000 μL in 10 mM Tris-HCl (pH 7.5), 100 mM KOAc, 2 mM MgCl₂, 0.5 mM MnCl₂, and 2 mM DTT. Before each measurement, the buffer was degassed. Emission spectra were registered for all excitation maxima (detailed wavelengths for each probe: 1a—exc. 490/em. 471 nm, 1b, 1d, and 1e—435/378 nm; and 1c—420/489 nm).

**Susceptibility to D9 Hydrolysis/Probe Hydrolysis Experiments.** The enzymatic activity of D9 was assayed at 30 °C with mixing at 300 rpm in 10 mM Tris-HCl (pH 7.5) containing 100 mM KOAc, 2 mM DTT, 2 mM MgCl₂, and 0.3 mM MnCl₂. Reaction mixtures contained 30 μM (nucleotide analogue) or 100 μM (probe 1b) of the tested compound and 25 nM of the recombinant protein. Enzymatic reaction progress was examined after 5, 15, 30, 45, and 60
min. Aliquots were collected, and the reaction progress was terminated by heat inactivation for 3 min at 95 °C, followed by centrifugation. The samples were analyzed by RP HPLC (Agilent Technologies Series 1200). Appropriate fractions from probe 1b hydrolysis were collected and identified based on the mass measurements. Mass spectra were recorded on the AB Sciex API 3200 spectrometer.

Point Fluorescence Measurements—General Information. Point fluorescence measurements were performed using the BioTek Synergy H1 microplate reader. Before each measurement, the reaction buffer was degassed. Experiments were performed in the Greiner 96-well, black, non-binding plates at 30 °C. Before the measurement, the plate was preincubated for 15 min at 30 °C with mixing (300 rpm). The point fluorescence was registered with 1 min interval and detection at 345 nm excitation wavelength and 378 nm emission wavelength.

Kinetic Parameter Determination. To determine the kinetic parameters of enzymatic hydrolysis of probe 1b by D9 enzyme, point fluorescence measurements were performed. Each well of the plate contained an appropriate buffer (10 mM MOPS-HCl, pH 7.0, 100 mM KOAc, containing 2 mM MgCl₂, 0.3 mM MnCl₂ and 2 mM DTT) with addition of 0.1% BSA, a substrate (probe 1b at a concentration range 0–15 μM) and an enzyme (5 nM of D9) to a final volume of 150 μL. The measurement was carried out on until saturation, which allowed us to convert the initial rates from au/min to mol/min/mg. The initial rates were calculated by standard hill slope from GraphPad Prism software.

\[
V = \frac{E \cdot k_{cat} \cdot C_{probe}}{K_m + C_{probe}} = \frac{V_{max} \cdot C_{probe}}{K_m + C_{probe}}
\]

where \( V \) is the initial rate of the reaction, \( E \) is the concentration of enzyme sites, \( k_{cat} \) is the turnover number indicating the catalytic efficiency, \( C_{probe} \) is the concentration of the probe 1b, \( K_m \) is the Michaelis–Menten constant, and \( V_{max} \) is the maximum enzyme velocity.

Z’ Factor Determination. To determine the Z’ factor of the method, point fluorescence measurements were performed. The positive control contained a mixture of probe 1b (3 μM) and enzyme D9 (5 nM) in 10 mM MOPS-HCl, pH 7.0, 100 mM KOAc, containing 2 mM MgCl₂, 0.3 mM MnCl₂ and 2 mM DTT. The negative control additionally included a nucleotide inhibitor (30 μM of m7GpppApG), RNA Polymerase Buffer (Thermo Fisher Scientific), 1 μM of annealed oligonucleotides as DNA template (CAGTATACGACTCTATTAGGGAGCGGCATCGGCC-CAGCCATAGCGGCTTCCCTAATAGTGAGTCGTATTACTG), 1 U/μL RiboLock RNase inhibitor (Thermo Fisher Scientific), 20 mM MgCl₂, and T7 RNA polymerase (20 U/μL Thermo Fisher Scientific). Uncapped transcripts (IVT) were obtained as mentioned above but in the presence of 5 mM ATP and without addition of cap analogue. After 4 h incubation, 7 U of DNase I (Thermo Fisher Scientific) was added, and the mixture was incubated for another 30 min at 37 °C. The reaction mixture was stopped by the addition of equal molar amounts of Na₂EDTA water solution (EDTA to Mg²⁺) and RNA–phenois–chloroform extraction, followed by RNA precipitation (3 M NaOAc pH 5.2, ethanol). The crude RNA sample was next purified using the HPLC and Phenomenex Clarity 3 μm Oligo-RP column (150 × 4.6 mm) (the method is given below) and precipitated as before. To obtain a homogeneous RNAs at 3’ end, appropriate sample underwent treatment by DNAnyme 10–23 (TGATCGCGTACTCATATATTAGGGAGGCGGCATCGGCC-CAGCCATAGCGGCTTCCCTAATAGTGAGTCGTATTACTG) and heated up at 95 °C for 5 min.

High-Throughput Screening of the LOPAC1280 Library. To screen the LOPAC1280 Library, point fluorescence measurements were performed. Each plate well contained an appropriate buffer (10 mM MOPS-HCl, pH 7.0, 100 mM KOAc, containing 2 mM MgCl₂, 0.3 mM MnCl₂ and 2 mM DTT) with addition of 0.1% BSA, a fluorescent substrate (3 μM of probe 1b), an inhibitor (in half-logarithmic dilution, \( C_{inh} \) ∈ [0, 100 μM]), and an enzyme (5 nM of D9) to a final volume of 150 μL. To determine the IC₅₀ values, a three parameter dose–response formula with standard hill slope from GraphPad Prism software was fitted

\[
\frac{V}{V_0} = \frac{I_{max} - I_{min}}{1 + \frac{C_{inh}}{IC_{50}}}
\]

where \( V/V_0 \) is the relative initial rate of the reaction, \( I_{max} \) and \( I_{min} \) are bottom and top plateaus, and \( C_{inh} \) is the concentration of the inhibitor.

All tested compounds come from LOPAC1280 (Table 2) or our in-house library of cap analogues (determined IC₅₀ values with compound’s structures and synthesis references are available in Table S1).

RNA Synthesis. Short RNA sequences were synthesized by in vitro transcription (IVT) using T7 class II promoter Δ2.5 initiated by ATP (TAATACGACTCTATATTA). The typical transcription reaction (200 μL) was carried out for 4 h at 37 °C and contained 5 mM each of UTP/GTP/CTP (Thermo Fisher Scientific), 1.25 mM ATP (Thermo Fisher Scientific), and 6.5 mM of trinucleotide cap analogue (m7GpppApG), RNA Polymerase Buffer (Thermo Fisher Scientific), 1 μM of annealed oligonucleotides as DNA template (CAGTATACGACTCTATTAGGGAGCGGCATCGGCC-CAGCCATAGCGGCTTCCCTAATAGTGAGTCGTATTACTG), 1 U/μL RiboLock RNase inhibitor (Thermo Fisher Scientific), 20 mM MgCl₂, and T7 RNA polymerase (20 U/μL Thermo Fisher Scientific). Uncapped transcripts (IVT) were obtained as mentioned above but in the presence of 5 mM ATP and without addition of cap analogue. After 4 h incubation, 7 U of DNase I (Thermo Fisher Scientific) was added, and the mixture was incubated for another 30 min at 37 °C. The reaction mixture was stopped by the addition of equal molar amounts of Na₂EDTA water solution (EDTA to Mg²⁺) and RNA–phenois–chloroform extraction, followed by RNA precipitation (3 M NaOAc pH 5.2, ethanol). The crude RNA sample was next purified using the HPLC and Phenomenex Clarity 3 μm Oligo-RP column (150 × 4.6 mm) (the method is given below) and precipitated as before. To obtain a homogeneous RNAs at 3’ end, appropriate sample underwent treatment by DNAnyme 10–23 (TGATCGCGTACTCATATATTAGGGAGGCGGCATCGGCC-CAGCCATAGCGGCTTCCCTAATAGTGAGTCGTATTACTG) and heated up at 95 °C for 5 min.

RNA Decapping Assay. 20 ng of 26-nt capped RNA transcript was incubated with an appropriate decapping enzyme (3 nM D9 or 11 nM Dcp1/2 or 200 nM D10) in an appropriate buffer with addition of 0.1% BSA at 37 °C. After being incubated, times reactions were terminated by adding equal volume of the loading dye (4.5 M urea, 50% formamide, 20 mM EDTA, 0.03% bromophenol blue, and 0.03% xylene cyanol) and flash freezing. Samples were then resolved by PAGE on denaturing 15% acrylamide/7 M urea/TBE gels and stained with SYBR Gold (Invitrogen) and visualized using a Typhoon FLA 9500 (GE Healthcare). The band intensities corresponding to capped and uncapped RNAs were quantified densitometrically using 1D Gel Image Analysis Software TotalLab CLIQS.

Buffer used in D9 experiments: 10 mM MOPS-KOH (pH = 7.0), 100 mM KOAc, 2 mM MgCl₂, 0.3 mM MnCl₂, and 2 mM DTT.

Buffer used in Dcp2 experiments: 50 mM Tris-HCl pH = 8.0, 50 mM NH₄Cl, 0.01% Igepal, 5 mM MgCl₂, 2 mM MnCl₂, and 1 mM DTT.
Buffer used in D10 experiments: 10 mM MOPS-KOH pH = 7.0, 100 mM KOAc, 2 mM MgCl₂, 0.3 mM MnCl₂, and 2 mM DTT.

**X-ray Crystallography.** The CP-100356 inhibitor was dissolved in DMSO at a concentration of 5 mg/mL. The protein–inhibitor complex was prepared by mixing D9 and CP-100356 in crystallization buffer containing 20 mM MgCl₂ to a final concentration of 10 mg/mL D9 and 3 mM cap analogue substrates and incubated at room temperature for 30 min. The protein–substrate complex was mixed with well solution at a 1:1 ratio, and crystals were grown by hanging drop vapor diffusion at room temperature. D9-CP-100356 well solution contained 20% PEG 3350 and 100 mM NaF. Crystals were flash frozen in liquid nitrogen using a cryoprotectant consisting of well solution with 25% glycerol. All data sets were collected on beamline 8.3.1 at the Advanced Light Source at 100 K and wavelength 1.11583 Å using the Pilatus3 S 6 M detector and indexed, integrated, and scaled using XDS.³⁵ Pointless and Aimless³⁴ via automated beamline software ELVES.³⁵ Phasing was carried out by molecular replacement with the previously solved D9 structure in the post-catalytic conformation, 7SEZ.²⁸ The structure was then iteratively refined in PHENIX³² and manually adjusted in COOT.³³

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at [https://pubs.acs.org/doi/10.1021/acschembio.2c00049](https://pubs.acs.org/doi/10.1021/acschembio.2c00049).

Detailed results of inhibitory studies obtained from FLINT assay, kinetic and hydrolysis experiments, HTS assay optimization, susceptibility to D9 hydrolysis, in vitro decapping assay with PAGE gels and sequences of the used proteins, and HPLC profiles and HRMS spectra for newly synthesized compounds (PDF)

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