Effects of the 5′-Triphosphate Metabolites of Ribavirin, Sofosbuvir, Vidarabine, and Molnupiravir on CTP Synthase Catalysis and Filament Formation: Implications for Repurposing Antiviral Agents against SARS-CoV-2

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Repurposing of antiviral drugs affords a rapid and effective strategy to develop therapies to counter pandemics such as COVID-19. SARS-CoV-2 replication is closely linked to the metabolism of cytosine-containing nucleotides, especially cytidine-5′-triphosphate (CTP), such that the integrity of the viral genome is highly sensitive to intracellular CTP levels. CTP synthase (CTPS) catalyzes the rate-limiting step for the de novo biosynthesis of CTP. Hence, it is of interest to know the effects of the 5′-triphosphate (TP) metabolites of repurposed antiviral agents on CTPS activity. Using E. coli CTPS as a model enzyme, we show that ribavirin-5′-TP is a weak allosteric activator of CTPS, while sofosbuvir-5′-TP and adenine–arabinofuranoside-5′-TP are both substrates. β-d-N4-Hydroxyctydine-5′-TP is a weak competitive inhibitor relative to CTP, but induces filament formation by CTPS. Alternatively, sofosbuvir-5′-TP prevented CTP-induced filament formation. These results reveal the underlying potential for repurposed antivirals to affect the activity of a critical pyrimidine nucleotide biosynthetic enzyme.

Introduction

With the advent of the pandemic of coronavirus disease in 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2),[1] it has become clear that there is an urgent need to develop broad spectrum therapeutic approaches to combat pandemics.[2] While vaccines offer an effective approach to prevent many viral infections, their development is laborious and requires long lead times. In the absence of a vaccine, prophylactic use of small molecule antiviral agents offers a promising alternative, especially to protect vulnerable and at-risk populations. Drug repurposing affords a strategy for identifying new uses for approved or investigational drugs in a cost-effective and timely manner when initially faced with no treatment options for a disease, as was the case with COVID-19.[10] Efforts have focused on assessing the efficacy of known broad spectrum antiviral compounds,[4] particularly remdesivir (GS-5734)[5] and molnupiravir (EIDD-2801 or MK-4482).[6] These nucleoside analogues are delivered as prodrugs and subsequently metabolized to their corresponding active 5′-triphosphate (5′-TP) by the action of kinases. Remdesivir-5′-TP directly inhibits the coronavirus RNA-dependent RNA polymerase (RdRp).[7] Competition with endogenous nucleotide substrates for the viral RdRp, which incorporates the analogue into the nascent viral RNA, leads to chain-termination. On the other hand, the 5′-TP metabolite of molnupiravir acts as a mutagen through RNA mutagenesis mediated by the template strand.[8]

Other synergistic approaches to antiviral therapies beyond the direct inhibition of the RdRp of SARS-CoV-2, may also be considered. One such approach is to limit the nucleotide pools to starve the viral replication machinery. Recently, suppression of pyrimidine biosynthesis by inhibition of dihydroorotate dehydrogenase has been shown to restore the antiviral inflammatory response and reduce viral yield upon SARS-CoV-2 infection.[9] Danchin and co-workers postulated that SARS-CoV-2 replication is closely linked to the metabolism of cytosine-containing nucleotides, especially cytidine-5′-TP (CTP).[11] Consequently, the integrity of the viral genome would be highly sensitive to intracellular CTP pool levels. CTP plays an integral role in crucial metabolic steps contributing to the manufacture of functional SARS-CoV-2 viral particles.[12] As well as being one of the four nucleotide precursors required for biosynthesis of the viral genome,[12] it is required for synthesis of the liponucleotide precursors of the viral envelope,[13] for the biosynthesis of the 3′-OH–CCA terminal end of human tRNAs via a CTP-dependent nucleotidytranferase (CCase),[14] and for post-translational glycosylation of viral proteins (e.g., the spike protein) via the endoplasmic reticulum, which requires dolichylphosphate formed through the action of a CTP-dependent dolichol kinase.[15] Furthermore, CTP is converted into the antiviral agent 3-deoxy-3,4-didehydro-CTP (ddhCTP) by the enzyme viperin as part of the innate immune response.[16]

The sole route for the de novo biosynthesis of cytosine in human host cells is through the adenosine-5′-TP (ATP)-dependent conversion of uridine-5′-TP (UTP) to CTP catalyzed by CTP synthase (CTPS),[17] utilizing glutamine (Gln, in vivo substrate) or ammonia (NH3) as the source of nitrogen (Scheme 1). The enzyme requires Mg2+ ion[18] and is regulated in a complex...
Results and Discussion

Effects of ribavirin-5'-TP on EcCTPS activity

Ribavirin is a broad-spectrum antiviral drug wherein the carboxamide moiety of the pseudobase serves as a structural mimic of guanosine and inosine. In silico docking studies suggested that RBV–TP would be bound by the SARS-CoV-2 RdRp. Ribavirin appears to be somewhat efficacious as a treatment for COVID-19 in combination with other antiviral agents or with interferon. Ribavirin’s antiviral effects can arise through several mechanisms, including inhibition of RNA capping activity, immunomodulatory effects, inhibition of viral polymerases, and increased mutational frequency due its incorporation into the RNA genome during virus replication. In vivo, phosphorylation leads to the 5'-monophosphate, 5'-diphosphate, and 5'-TP, with the latter often being the major metabolite. Ribavirin-5'-monophosphate acts as a competitive inhibitor of human inosine-5'-monophosphate dehydrogenase (IMPDH) with respect to GMP, leading to depletion of the intracellular pools of GTP, which contributes indirectly to ribavirin’s antiviral activity. For example, cultured Madin Darby Canine Kidney (MDCK) cells infected with A/WSN-strain influenza virus reduce the GTP concentration ~50%, in the presence of RBV (100 μM). Interestingly, the intracellular CTP and UTP pools typically increase with ribavirin treatment (See Table 1 for typical intracellular concentrations of ribonucleoside-5'-TPs.) The concomitant elevation of the CTP pools is unexpected considering that GTP is an allosteric activator of CTPS-catalyzed Gln-dependent CTP formation. Consequently, we assessed the direct effect of RBV–TP on EcCTPS activity.

Interestingly, RBV–TP served as an allosteric activator similar to GTP (Figure 1). The \( k_{\text{act}} \) values accompanying activation by GTP and RBV–TP were 9.2 s\(^{-1}\) and 13.8 s\(^{-1}\), respectively (Table 1). While the \( k_{\text{act}} \) values were similar, there was a marked increase in the \( K_i \) value for RBV–TP (2.44 mM) relative to the \( K_i \) value observed for GTP (0.068 mM). Our observation that RBV–TP activates EcCTPS-catalyzed Gln-dependent CTP formation is surprising because the structural requirements for activation are quite stringent, with O6 of GTP being required for the CTPS-catalyzed Gln-dependent CTP formation.

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Previously, we demonstrated that inosine-5'-TP (ITP) also acts as an allosteric activator, but was bound weakly by the enzyme with a \( K_i \) value of 2.9 mM, indicating that the 2'-NH\(_2\) group contributes significantly to binding. Consequently, the carboxamide group on the pseudobase of RBV–TP is able to mimic the interaction of O6 with EcCTPS to afford activation, but the missing part of the purine structure diminished the binding affinity similar to ITP.

Because of the ability of nucleotides to induce or reverse filament formation by CTPS\(^{21c,22–23,39}\), we examined the effect of RBV–TP on filament formation. Neither GTP nor RBV–TP were capable of inducing EcCTPS filaments alone (Figure S1). Additionally, no distinct differences in filament abundance or length were observed with EcCTPS that had been incubated with CTP, CTP and GTP, or CTP and RBV–TP, indicating that RBV–TP has no apparent effect on filament formation by EcCTPS.

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ChemMedChem 2022, 17, e202200399 (2 of 10)
Table 1. Kinetic parameters for EcCTPS-catalyzed Gln-dependent and NH₃-dependent CTP formation.

| varied ligand | Gln-dependent CTP formation | NH₃-dependent CTP formation |
|--------------|-----------------------------|-----------------------------|
|              | \( k_{cat} \) or \( k_{cat}^{\text{u}} \) [s⁻¹] | \( k_{cat} \) or \( k_{cat}^{\text{u}} \) [s⁻¹] | \( k_{cat}/K_{u}^{\text{u}} \) or \( k_{cat}/K_{u} \) [s⁻¹ mM⁻¹] | \( n \) | \( k_{cat} \) or \( k_{cat}^{\text{u}} \) [s⁻¹] | \( k_{cat} \) or \( k_{cat}^{\text{u}} \) [s⁻¹ mM⁻¹] | \( n \) |
| L-Gln[^a]   | 0.25 ± 0.04                 | 10.6 ± 1.1                  | 42.4 ± 8.1                    | 4.7 | 1.4 ± 0.3                  | 0.4 ± 0.5                  | 4.0 ± 0.5 |
| L-Gln[^b]   | 0.32 ± 0.03                 | 1.21 ± 0.03                 | 3.78 ± 0.37                   | 4.7 | 1.4 ± 0.3                  | 0.4 ± 0.5                  | 4.0 ± 0.5 |
| NH₃[^c]     | 0.21 ± 0.02[^d]             | 11.6 ± 0.6                  | 55.2 ± 6.0[^c]                | 1.18 ± 0.01 | 0.57 ± 0.03[^d] | 8.0 ± 0.1 | 14.0 ± 0.8[^d] | 3.0 ± 0.5 |
| ATP[^e]     | 0.20 ± 0.00[^e]             | 8.4 ± 0.1                   | 42.5 ± 1.0[^e]                | 2.17 ± 0.18 | 0.77 ± 0.06[^e] | 8.1 ± 0.3 | 10.56 ± 0.86[^e] | 1.6 ± 0.1 |
| UTP[^f]     | 0.068 ± 0.01[^f]            | 9.2 ± 1.3[^f]               | 135 ± 48[^f]                  | 3.1 ± 0.9 |  – – – – | – – – – | – – – – |
| GTP[^g]     | 0.35 ± 0.05[^g]             | 13.8 ± 2.6[^g]              | 5.6 ± 2.4[^g]                 | 2.6 ± 0.4 |  – – – – | – – – – | – – – – |
| RBV–TP[^h]  | 2.44 ± 0.92[^h]             | 1.35 ± 0.03                 | 6.75 ± 0.33[^h]              | 1.09 ± 0.14 |  – – – – | – – – – | – – – – |
| SFU–TP[^i]  | 0.20 ± 0.01[^i]             | 3.1 ± 0.5                   | 22.1 ± 4.8[^i]               | 1.2 ± 0.1 | 0.60 ± 0.19[^i] | 1.5 ± 0.3 | 2.49 ± 0.68[^i] | 1.4 ± 0.3 |
| ara–ATP[^j] | 0.14 ± 0.02[^j]             | 3.0 ± 0.5                   | 6.0 ± 0.5[^j]                | 1.2 ± 0.1 |  – – – – | – – – – | – – – – |

[^a] \( [\text{S}]_{\text{u}} \) is the nucleotide concentration that yields half-maximal velocity. For comparison, the intracellular pool sizes of ribonucleoside-5’-TPs in either uninfected or influenza A virus-infected MDCK cells are 0.28–0.59 mM CTP, 0.96–1.6 mM UTP, 6.5–7.8 mM ATP, and 1.1–1.4 mM GTP[^41] and average values in dividing and resting mammalian cells are 0.28 mM and 0.083 mM CTP, 0.57 mM and 0.23 mM UTP, 3.2 mM and 2.5 mM ATP, and 0.47 mM and 0.23 mM GTP, respectively[^42].[^43]

[^b] \( k_{cat} \) value; [c] \( k_{cat}^{\text{u}} \) value; [d] \( k_{cat}/[\text{S}]_{\text{u}} \) value; [e] \( k_{cat}/K_{u} \) value; [f] \( [\text{UTP}] = [\text{UTP}] = 1.0 \text{ mM} \); [g] \( [\text{ATP}] = [\text{SFU–TP}] = 1.0 \text{ mM} \).

Effects of sofosbuvir-5’-TP on EcCTPS activity

SARS-CoV-2 and hepatitis C virus (HCV) are both positive-sense single-stranded RNA viruses requiring an RdRp for genome replication and transcription. Since the amino acid sequence at the active site is highly conserved among such RdRps, it was hypothesized that nucleotide analogues used to treat HCV infections, such as sofosbuvir, might also be effective against COVID-19[^31,43]. In silico docking studies suggested that the SARS-CoV-2 RdRp would bind SFU–TP[^31]. Indeed, SFU–TP is incorporated into RNA by the highly error-prone SARS-CoV-2 RdRp, but not by a host-like high-fidelity DNA polymerase, terminating extension due to its bulky 2’-methyl group[^43b,44]. However, other studies have shown that SFU–TP does not inhibit SARS-CoV-2 RdRp[^43c,45] or markedly inhibit SARS-CoV-2-induced cytopathic effects[^46], which casts doubt on the utility of sofosbuvir as a treatment for COVID-19[^47].

Because of the attention focused on sofosbuvir, as well as its current use as an anti-HCV drug[^46], we explored the effect of SFU–TP on CTPS activity and found that SFU–TP was a substrate for Gln-dependent EcCTPS-catalyzed amination (Figure 2). While EcCTPS exhibited the same binding affinities for UTP and EcCTPS-catalyzed NH₃-dependent amination (Figure 4), although not as efficiently as previously reported for UTP. Upon increasing the concentration of SFU–TP to ~5 mM, there was a marked decrease in filament...
Effects of adenine–arabinofuranoside-5'-TP on EcCTPS activity

In silico screening and molecular dynamics suggested that Ara–A (vidarabine) could inhibit the interaction between the SARS-CoV-2 spike protein S1 receptor binding domain and the ACE2 receptor. \cite{56} Similarly, ara–A was identified through deep learning-based screening as a potential drug to target the 3 C-like protease of SARS-CoV-2. \cite{51} Consequently, we examined the effect of the 5'-TP metabolite of ara–A (ara–ATP) on EcCTPS activity.

Ara–ATP served as a substrate for both Gln-dependent and NH₃-dependent EcCTPS-catalyzed CTP formation (Figure 5). The ability of ara–ATP to replace ATP as a substrate for EcCTPS has not previously been recognized. Interestingly, the kcat/|S|max value for ara–ATP was ~2.5-fold less than that of ATP for Gln-dependent CTP formation; however, ara–ATP was a much less efficient substrate for NH₃-dependent CTP formation by ~5.5-fold, relative to ATP. Structural studies by Baldwin and co-workers had revealed that the ribose 2'- and 3'-OH groups have no direct protein contacts in the EcCTPS complex with ADP (although they are near Lys 306 and Asp 303, respectively), which along with the observation that ATP and dATP were equally effective co-substrates with UTP, led them to conclude that 2'-OH recognition is not important. However, kinetic studies on the K306A variant suggested that Lys 306 plays a role in bringing about the conformational changes that mediate interactions between the ATP-binding site and the UTP-binding site, as well as the glutamine amide transfer domain. \cite{53} The weaker binding of ara–ATP, relative to ATP, reveals that EcCTPS is sensitive the stereochemistry at the 2'-position likely due to unfavorable steric interactions between the arabino-2'-OH group and the side chain of Ile 20. In varicella-zoster virus-infected human foreskin fibroblasts, the concentration of ara–ATP is about 4-fold lower than the concentration of ATP, which suggests that while our observations suggest that ara–ATP could assist in maintaining the intracellular CTP pools
when employed as an antiviral agent, the effect may be minimal.

Effects of β-d-N4-hydroxycytidine-5'-TP on EcCTPS activity

The cytidine analogue β-d-N4-hydroxycytidine (NHC, which as its isopropyl-ester prodrug is known as molnupiravir or EIDD-2801) exhibits antiviral activity against multiple coronaviruses, including SARS-CoV-2[64,65].

The active 5'-TP form of molnupiravir (i.e., N4-OH–CTP) acts as a mutagen[34,36,38] causing the RdRp-dependent incorporation of either G or A into RNA products that escape proofreading[9c,e,57]. Consequently, we tested the effect of N4-OH–CTP on EcCTPS activity and filament formation.

N4-OH–CTP was prepared by the reaction of CTP with hydroxylamine, followed by purification using weak anion-exchange chromatography (Figures S2–S7) similar to the protocol described by Painter et al.[34] Product formation was confirmed by NMR spectra (Figures S8 and S9) and MS data (Figure S10), and the product was shown to be pure by reversed-phase HPLC analysis (Figure S11). We determined the IC50 values for the inhibition of EcCTPS by CTP and N4-OH–CTP at two fixed concentrations of UTP (50 and 200 μM) with Gln as the nitrogen source (Figure S12). Comparison of the IC50 values revealed that N4-OH–CTP was a weak inhibitor of EcCTPS, relative to CTP (Table 2).

We then investigated the mode of inhibition and determined the value of the inhibition constant (K) of both CTP and N4-OH–CTP (Figure 6). In accord with previous results,[18] CTP was a competitive inhibitor of EcCTPS with respect to UTP. Similarly, N4-OH–CTP was also a competitive inhibitor of EcCTPS with respect to UTP, albeit binding with an affinity that was 7.7-fold weaker than CTP (Table 2). Given the ability of CTP to induce filament formation by EcCTPS,[21b,39c] we investigated the ability of N4-OH–CTP to induce filament formation. Indeed, N4-OH–CTP (1.0 mM) was able to induce the formation of EcCTPS filaments that were similar in size and abundance to those induced in the presence of CTP (Figure 4, cf. panels B and E).

Thus, the active metabolite of NHC weakly inhibits EcCTPS activity and effects filament assembly. The ability of N4-OH–CTP to inhibit EcCTPS has not previously been recognized. Overall, these observations suggest that N4-OH–CTP, in addition to causing mutation in the viral genome,[60] could also reduce the intracellular CTP pools. However, the latter effect may be weak.

Table 2. Inhibition of EcCTPS-catalyzed Gln-dependent CTP formation by CTP and N4-OH–CTP.

| Inhibitor | [UTP] = 50 μM | [UTP] = 200 μM | IC50 μM |
|-----------|---------------|----------------|---------|
| CTP       | 107 ± 4       | 1.6 ± 0.1      | 234 ± 21|
| N4-OH–CTP | 1112 ± 48     | 1.7 ± 0.2      | 1461 ± 44| 1.5 ± 0.4 |

(a) The intersection of the lines on the Lineweaver-Burk plot appears slightly to the left of the y-axis (Figure 6E). Treating the inhibition as linear mixed-type yields an additional weak binding constant of \(K_{i_{\text{int}}}\) of \(> 28 \text{ mM}\) for the interaction of N4-OH–CTP with the enzyme-substrate complex.
Conclusions

Since repurposing of small molecule antiviral drugs can afford a rapid and effective strategy to develop therapies to counter pandemics such as COVID-19,[2] it is important to assess potential synergistic or detrimental effects that such drugs might have against alternative enzyme targets. Indeed, the close link between SARS-CoV-2 replication and the metabolism of cytosine-containing nucleotides, especially CTP, and the sensitivity of the viral genome to intracellular CTP levels,[11] necessitates exploration of the effect of the active metabolites of antiviral drugs on the activity and regulation of CTPS. Surprisingly, the effect of the 5′-TP metabolites of many antivirals on CTPS activity have not been examined. Consequently, we employed EcCTPS as a model enzyme to explore the effects of RBV–TP, SFU–TP, ara–ATP, and N′-OH–CTP on the activity of EcCTPS and its ability to form filaments in vitro. Ara–ATP was able to replace ATP as a substrate for EcCTPS. Despite ara–ATP being less efficient (i.e., $k_{\text{cat}}/K_m$) than ATP at supporting Gln- and NH$_4$-dependent CTP formation by ~2.5- and ~5.5-fold, respectively, these observations suggest that ara–ATP could assist in maintaining the intracellular CTP pools when employed as an antiviral agent. Similarly, SFU–TP replaced UTP as a substrate for EcCTPS, albeit ~6-fold less efficient than UTP. The UTP-binding site exhibits exquisite specificity for UTP;[25] however, it appears that the enzyme can tolerate substitution at the 2′-position since 2′,2′-difluoro-dUTP is also a substrate for EcCTPS.[54] Interestingly, the amination product, 4-NH$_2$–SFU–TP, is also a known antiviral agent.[61] Additionally, SFU–TP prevented CTP-dependent filament formation by the enzyme. RBV–TP, like GTP, served as an allosteric activator of Gln-dependent CTP formation, indicating that the carboxamide group on the pseudobase of RBV–TP is able to mimic the interaction of O6 with EcCTPS to afford activation. However, the missing part of the purine structure diminished the binding affinity ~36-fold relative to GTP, resulting in the overall efficiency of activation ($k_{\text{cat}}/K_m$) by RBV–TP being ~24-fold less than that of GTP. Possibly, the allosteric activation of CTPS by RBV–TP may account, in part, for the continued production of CTP despite the depletion of the GTP pools arising from the inhibition of IMPDH by ribavirin-5′-monophosphate. Finally, EcCTPS was inhibited by the active metabolite of molnupiravir, N′-OH–CTP, although the competitive inhibition constant was ~7.7-fold weaker than that accompanying feedback inhibition by CTP. Like, CTP, N′-OH–CTP induced filament formation by the enzyme. Overall, these observations demonstrate that the 5′-TP metabolites of several antiviral drugs can affect the activity of CTP synthase and, consequently, may impact the level of the intracellular CTP pools. Such an impact may also have an effect on the host immune response that relies on the activation and proliferation of T cells and B cells for adaptive immunity.[106,64] These results underscore the need to explore such effects by these and other antiviral metabolites on the human isoforms of CTPS.

Experimental Section

General

All chemicals, unless stated otherwise, were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). GTP, ara–ATP, and RBV–TP were purchased from Jena Bioscience (Jena, Germany). SFU–TP was purchased from Toronto Research Chemicals (Toronto, ON, Canada) and SFU Bioresearch (Tucson, AZ). β-D-Deoxy-2′-α-F-2′-β-C-methyluridine was obtained from Synmova (Edmonton, AB, Canada) and β-D-deoxy-2′-α-F-2′-β-C-methylcytidine was obtained from Toronto Research Chemicals (Toronto, ON, Canada).

Conclusions

Inhibition constants ($K_m$) are shown. The inhibition constants ($K_m$) were 5.0 μM and 3.9 μM when CTP and UTP were assayed as substrates for EcCTPS and its ability to form filaments, respectively. Representative replots of the apparent $V_{\text{max}}/K_m$ values (obtained from direct fits of eqn. 2 with $n = 1$ to the initial velocity data) as a function of the concentrations of CTP (C) and N′-OH–CTP (F) are shown. The inhibition constants ($K_m$) are given in Table 2.

Considering that the concentration of N′-OH–CTP in peripheral blood mononuclear cells has been shown to reach concentrations of 60–85 μM in patients receiving a single oral dose of 1600 mg,[81] i.e., ~20-fold lower than the $K_m$ values reported in Table 2.
His-Bind resin (Novagen) was purchased from EMD Millipore (San Diego, CA, USA). For HPLC experiments, a Waters 510 pump and automated gradient controller were used for solvent delivery. Injections were made using a Rheodyne 7725i sample injector fitted with a 20-μL injection loop, and a Waters 486 absorbance detector was used to detect nucleotides. Kinetic studies were conducted using a Agilent 8453 UV-vis diode array spectrophotometer. Low resolution (LR) and high resolution (HR) electrospray ionization (ESI) mass spectra (MS) were collected using a Bruker microTOF Focus orthogonal ESI-TOF mass spectrometer instrument operating in negative ion mode. H and 31P NMR spectra were obtained using a Bruker AV 500 MHz spectrometer at the Dalhousie University Nuclear Magnetic Resonance Research Resource Centre (NMR3). Chemical shifts (δ) in ppm for H and 31P NMR spectra are reported relative to the residual solvent signal for D2O (δ 4.79) and an external standard of 85% phosphoric acid, respectively[56].

Nº-OH–CTP

Nº-OH–CTP was obtained by reacting CTP with hydroxylamine following a protocol similar to that described by Painter et al.[57] CTP (0.137 g, 0.260 mmol) was dissolved in a solution of hydroxylamine (2.0 mL, 2.0 M, pH 5) and the pH was adjusted to 5.0 by addition of NaOH. The reaction mixture was placed in a sealed 5-mL reaction vial and heated with stirring at 55°C for 5 h. The mixture was then cooled to room temperature and a solution of triethylammonium bicarbonate (TEAB, 100 mM, 2 mL) was added. TEAB buffer (1.0 M, pH 8.0) was prepared by bubbling CO2 through a solution of triethylamine (1.0 M) for 5 h. The reaction mixture was then subjected to anion-exchange chromatography on a DEAE-Sephadex A-25 matrix (2.5 cm i.d. × 42 cm) and eluted at a flow rate of 0.7 mL/min using a 1.5-L gradient of TEAB (0.1–0.5 M), followed by a 0.2-L gradient of TEAB (0.5–1.0 M) and 0.5 L of TEAB (1.0 M). Fractions (12 mL) were collected and the absorbance of those fractions was measured at 260 nm. The mixture was then pooled to yield three combined fractions (I–III, Figure S2). Production of the hydroxamic acid was verified visually by production of a purple colour upon treatment with 1% FeCl3 in 1 N HCl.[67] The solvent was removed from each of these combined fractions using rotary evaporation (≤37°C). The residue was then dissolved in water and lyophilized several (∼4) times until a consistent mass was obtained. The various phosphorylated derivatives of NHC in the combined fractions were identified using ESI-MS. Fraction III contained Nº-OH–CTP and the purity of the nucleotide (≥99%) was verified using reversed-phase HPLC on a Kinetics 5 μ C18 100A column (250×4.6 mm, Phenomenex) using a flow rate of 1.0 mL/min and NH4HCO3 solution (50 mM) containing acetonitrile (10%) and (n-Bu)4NH (2.0 mM), adjusted to pH 7.0 with acetic acid, as the eluent and UV detection at 260 nm. Nº-OH–CTP was converted to its sodium salt by treatment with 1 M Na2SO4. The concentration of Nº-OH–CTP was determined by comparison of the integration of the H6 proton (δ 7.15) in the 1H NMR spectrum with the signal arising from a pyrazine internal standard (0.80 mM, δ 8.59). Yield = 18%. Nº-HMR (500 MHz, D2O) δ 7.18 (d, J = 8.3 Hz, 1H), 5.93 (d, J = 6.5 Hz, 1H), 5.81 (d, J = 8.3 Hz, 1H), 4.46–4.31 (m, 2H), 4.23–4.08 (m, 3H); 31P NMR (202 MHz, D2O) δ –10.10 (d, J = 19.4 Hz), –11.44 (d, J = 20.2 Hz), –23.13 (t, J = 19.8 Hz); HR-ESIMS m/z calcld for C35H46N6O13P2 [M–H]+: 497.9721, found 497.9725. The 31P NMR spectral data were in agreement with published data.[57]

Expression and purification of recombinant EcCTPS

Wild-type EcCTPS was purified from E. coli BL21(DE3) cells transformed with the pET-15b-CTPS plasmid as previously described.[58] Soluble EcCTPS bearing an N-terminal His6-tag was purified by metal ion affinity chromatography using established protocols (Novagen)[66] and dialyzed into assay buffer (HEPES (70 mM, pH 8.0) containing EGTA (0.5 mM) and MgCl2 (10 mM)). Recombinant enzyme preparations were ≥97% pure as determined using SDS-PAGE (10%) analysis and the purity was determined using Bradford assays conducted according to the manufacturer’s directions (Bio-Rad Laboratories, Mississauga, ON) with bovine serum albumin standards. The N-terminal His6-tag was not removed from the protein.

Enzyme assays

EcCTPS activity was determined at 37°C using a continuous spectrophotometric assay as previously described.[59] In brief, the rate of EcCTPS-catalyzed conversion of UTP to CTP was measured by following the change in absorbance at 291 nm (ε291 = 1331 M–1 cm–1) for 60 s. When using SUF-TP as the substrate, the change in absorbance at 282 nm was monitored (ε282 = 4036 M–1 cm–1, vide infra). Reactions were conducted in HEPES buffer (70 mM, pH 8.0) and typically contained EcCTPS (4.0–20.0 µg/ ml), EGTA (0.5 mM), MgCl2 (10 µM), UTP (1.0 mM), and ATP (1.0 mM) in a total volume of 0.3 mL in a 0.2-cm quartz cuvette, unless mentioned otherwise. Enzyme and nucleotides were pre-incubated at 37°C for 2 min followed by the addition of the ammonium source (NH4Cl or l-Gln) to initiate the reaction. For reactions using NH4Cl as the substrate (5.0–150.0 mM), KC1 was used to maintain ionic strength at 0.15 M. The (NH4)2 present at pH 8.0 was calculated using a pk(NH3+ ·H+) of 9.24 (i.e., [NH4+] = 0.0575 · [NH4Cl]initial).[68] For reactions using Gln as the substrate (0.05–60.0 mM), the concentration of ATP was 0.25 mM. GTP-dependent activation assays were conducted using Gln (60 mM) as the substrate and varying the concentration of GTP (0.05–1.00 mM) with the concentrations of UTP (1.0 mM), ATP (1.0 mM), and EcCTPS (4.7 µg/mL) as indicated. The kinetic parameters for UTP (0–3.0 mM) were obtained by following Gln-dependent CTP formation with the concentrations of GTP (0.25 mM), ATP (1.0 mM), and EcCTPS (7.6 µg/mL) as indicated. The values of kcat and Km were determined by fitting eqn. 1 to initial velocity data using non-linear regression analysis with Kaleidagraph v. 4.02 from Synergy Software (Reading, PA).[69] Similarly, kcat/[S]0 and (Hill coefficient) values for ATP, UTP, ara–ATP, and SUF–TP as substrates were determined by fitting eqn. 2 to the corresponding initial velocity data. For the activation and inhibition of EcCTPS by GTP, the values of kcat, kcat/km, kcat/km, and n were obtained by fitting eqn. 3 to the initial velocity data. For the activation of EcCTPS by RBV–TP, the values of kcat, kcat/km, and kcat were obtained by fitting eqn. 4 to the corresponding initial velocity data. All kinetic parameters were determined in triplicate and average values are reported. The reported errors are the standard deviations.

\[
\begin{align*}
V_i &= \frac{k_{cat}[S]}{K_m + [S]} \\
V_i &= \frac{k_{cat}[S]^n}{[S]^n + [S]^n} \\
V_i &= \frac{k_+ + \frac{k_{cat}K_m}{K_m}}{1 + \frac{k_{cat}K_m}{K_m}} \\
V_i &= \frac{k_+ + \frac{k_{cat}K_m}{K_m}}{1 + \frac{k_{cat}K_m}{K_m}}
\end{align*}
\]

IC50 values for the inhibition of EcCTPS by CTP and Nº–OH–CTP were determined by following the Gln-dependent formation of CTP from UTP (either 50 µM or 200 µM) in the presence of Gln (60 mM), ATP

ChemMedChem 2022, 17, e20020399 (7 of 10) © 2022 Wiley-VCH GmbH
(1.0 mM), and increasing concentrations of CTP or N°-OH–CTP. IC_{50} values were determined by fitting initial velocities to eqn. S, where \( v_i \) and \( v_o \) correspond to the velocities observed in the absence and presence of inhibitor, respectively. Furthermore, initial velocities were measured in the presence of fixed concentrations of CTP (0, 0.06, 0.12, and 0.18 mM) and N°-OH–CTP (0, 0.60, 1.20, and 1.80 mM) and apparent \( S_{0.5}/V_{max} \) values, obtained from non-linear regression analysis of the Michaelis-Menten plots, were replotted against the concentration of the inhibitor to estimate the value of the competitive inhibition constant \( K_i \) in accord with eqn. 6. \( K_i \) determinations with N°-OH–CTP were conducted using a 0.1-cm quartz cuvette.

\[
\frac{v_i}{v_o} = \frac{IC_{50}}{IC_{50} + [I]}^n
\]

(5)

\[
\frac{v_i}{[E]_T} = \frac{k_{cat}[S]}{S_{0.5} \left(1 + \frac{K_m}{S} \right) + [S]}
\]

(6)

**SFU–TP-dependent UV assay**

Although \( \Delta A_{343} \) could be used to monitor EcCTPS activity with SFU–TP, the difference in extinction coefficients between SFU–TP and 4-NH₂–SFU–TP was greater at 282 nm. To estimate the \( \Delta A_{343} \) value for the triphosphates, the extinction coefficients for TP-dependent UV assay (1.0 mM), and increasing concentrations of CTP or \( \Delta A_{343} \) of SFU were determined using the absorbance at 282 nm of solutions of the nucleosides (50.0, 75.0, 100.0, and 150.0 μM) in assay buffer and using the Beer-Lambert law (Figure S13). Kinetic parameters for EcCTPS-catalyzed turnover of SFU–TP were therefore determined using UV spectrophotometry by following the change in absorbance at 282 nm (\( \Delta A_{343} = 4036 \pm 494 \text{ M}^{-1} \text{ cm}^{-1} \)).

Gln-dependent amination of SFU–TP was measured using saturating conditions of Gln (6.0 mM) and ATP (1.0 mM), and varying amounts of SFU–TP (0.025–1.000 mM). GTP was maintained at a fixed saturating concentration of 0.25 mM in all assays with varying concentrations of SFU–TP. Additionally, Gln-dependent amination of SFU–TP was measured at fixed concentrations of SFU–TP (1.0 mM) and ATP (1.0 mM), and varying amounts of Gln (0.05–10.00 mM). Eqn. 1 was fitted to the initial velocity data when Gln was the variable substrate and eqn. 2 was fitted to the initial velocity data when SFU–TP was the variable substrate. Non-linear regression analysis was used to determine values of \( k_{cat}, K_m, S_{0.5}, n \), and \( n \). Unfortunately, the cost of SFU–TP precluded detailed examination of the less physiologically relevant NH₂-dependent amination.

**Product analysis of enzymatically prepared 4-NH₂–SFU–TP**

To confirm the EcCTPS-catalyzed conversion of SFU–TP to 4-NH₂–SFU–TP, SFU–TP (2.0 mM) was incubated at 37 °C in assay buffer containing EcCTPS (0.5 μM) in the presence of ATP (1.0 mM) and NH₂Cl (150 mM) in a total volume of 1.0 mL. NH₂Cl was used as the substrate rather than Gln to avoid the presence of an added nucleotide (i.e., GTP) in the analysis. At 0, 5, 15, 30, 60, 120, and 180 min, aliquots (100 μL) were removed, and the enzyme was removed by centrifugation through a 10-kDa MWCO spin-filter (Millipore). The flow-through samples were analyzed using LRESI-MS.

**Electron microscopy**

The ability of nucleotide analogues to induce or disrupt filament formation by EcCTPS was assessed by TEM using a protocol similar to those previously described[21b,39c]. EcCTPS (15 μM) was incubated in HEPES (assay) buffer (70 mM, pH 8.0) containing EGTA (0.5 mM), MgCl₂ (10 mM), and either GTP (1.0 mM), RBV–TP (1.0 mM), SFU–TP (1.0 mM or 5.0 mM) or N°-OH–CTP (1.0 mM) in the absence or presence of CTP (1.0 mM) for 30 min at 37 °C. Samples were diluted 10-fold using assay buffer containing 50% glycerol before being deposited on Formvar-coated carbon grids (TAAB Laboratories, Berkshire, UK) for uranyl acetate (0.7%) staining. Negative stain transmission electron micrographs were obtained using a JEOL 1230 transmission electron microscope.

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**Conflict of Interest**

The authors declare no conflict of interest.

**Data Availability Statement**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Keywords:** CTP synthase · antiviral · inhibition kinetics · activation · filaments

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