ddhCTP produced by the radical-SAM activity of RSAD2 (viperin) inhibits the NAD$^+$-dependent activity of enzymes to modulate metabolism

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Introduction

Radical S-adenosylmethionine (SAM) domain-containing protein 2 (RSAD2; viperin) is a key enzyme in innate immune responses that is highly expressed in response to viral infection and inflammatory stimuli in many cell types. Recently, it was found that RSAD2 catalyses transformation of cytidine triphosphate (CTP) to its analogue 3'-deoxy-3',4'-didehydro-CTP (ddhCTP). The cellular function of this metabolite is unknown. Here, we analysed the extra- and intracellular metabolite levels in human induced pluripotent stem cell (hiPSC)-derived macrophages using high-resolution LC-MS/MS. The results together with biochemical assays and molecular docking simulations revealed that ddhCTP inhibits the NAD$^+$-dependent activity of enzymes including that of the housekeeping enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH). We propose that ddhCTP regulates cellular metabolism in response to inflammatory stimuli such as viral infection, pointing to a broader function of RSAD2 than previously thought.

Abbreviations
CTP, cytidine triphosphate; ddhCTP, 3'-deoxy-3',4'-didehydro-CTP; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; hiPSC, human induced pluripotent stem cell; LDH, l-lactate dehydrogenase; MDH, malic dehydrogenase; PPP, pentose phosphate pathway; RdRps, RNA-dependent RNA polymerases; RSAD2, Radical S-adenosylmethionine domain-containing protein 2; SAM, S-adenosylmethionine.
it is suggested that ddhCTP is a chain terminator of the RNA-dependent RNA polymerases (RdRps) of a number of flaviviruses [20]. This conclusion, however, was challenged after a re-evaluation of the data [19]. It is demonstrated that the reported data are consistent with the ddhCTP product of the radical-SAM activity of RSAD2 abolishing the cellular level of nucleotides and inhibiting mitochondrial activity [19]. These data suggest that ddhCTP modulates metabolism to restrict viral replication. It is not known how ddhCTP affects metabolism (Fig. 1).

Here, using high-resolution mass spectrometry we analysed the extracellular metabolite levels of HEK293 cells overexpressing wild-type RSAD2 (WT-RSAD2) or an inactive variant of the enzyme (DM-RSAD2), and the extra- and intracellular metabolite levels of the hiPSC-derived RSAD2-KO or WT-RSAD2-expressing macrophages (WT macrophages). Based on analysis of metabolite levels and biochemical assays, we demonstrate that ddhCTP inhibits the NAD⁺-dependent activity of enzymes, including that of the housekeeping enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Molecular docking simulations predict that ddhCTP binds to the NAD⁺ binding pocket. We conclude that ddhCTP acts as a regulator of cellular metabolism upon inflammatory response. The results suggest a more general cellular role of the radical-SAM activity of RSAD2 than previously thought and provide a mechanistic explanation for many data reported regarding the antiviral activity of RSAD2.

Methods

Chemicals

All chemicals were reagent grade and were purchased from Sigma Aldrich. S-adenosylmethionine salt (~75% purity) was purchased from Sigma Aldrich. The salt was dissolved in buffer (300 mM MOPS, 100 mM NaCl, pH 7.0) to a final concentration of 75 mM SAM. The solution was divided into aliquots of 20 µL and stored at a −20 °C freezer. For each reaction, a fresh aliquot of SAM was used. l-lactate dehydrogenase (LLDH) from rabbit muscle (10 KU), malic dehydrogenase (MDH) from bovine heart (10 KU), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from rabbit muscle (1 KU), and phosphoglycerate kinase (PGK) from S. cerevisiae (2 KU) were purchased from Sigma Aldrich. The lyophilized powder of GAPDH was dissolved in 1 mL of phosphate buffer 50 mM, 300 mM NaCl, pH 7.6.

Expression of RSAD2 and GFP in HEK293 cells

The construct for expression of wild-type RSAD2 (WT-RSAD2) was provided by Professor Peter Cresswell (Yale University). To create an inactive variant of human RSAD2, the highly conserved Ser300 and Tyr301 were replaced by alanine and phenylalanine, respectively. Mutation of these residues in a fungal homologue of the enzyme fully abolishes the activity [21]. To create the double variant (DM-RSAD2) of human RSAD2, the forward primer was 5' CCAGAGATGAAAGACGCCTTCCTTATTCTGGATG 3' and the reverse primer was complementary to the forward primer. Quick-change site-direct mutagenesis kit was used, and the insert of interest carrying the desired mutations was confirmed using sequencing (GENEWIZ). Transfection of HEK293 cells with the construct for expression of GFP, WT-RSAD2 or DM-RSAD2 was based on Lipofectamine 2000 or TurboFect Transfection Reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer protocol.

Measuring extracellular metabolite levels of HEK293 cells using high-resolution mass spectrometry

The abundance of the extracellular metabolites in the growth medium before addition to cells and at 24 or 96 h post-transfection of HEK293 cells with GFP, WT-RSAD2 or DM-RSAD2 was measured using high-resolution liquid chromatography-mass spectrometry as explained previously [19].

Generating RSAD2-KO hiPSCs and deriving macrophages

Human iPSCs cell line SFC841-03-01 was obtained from the James Martin Stem Cell Facility, Sir William Dunn...
School of Pathology. The parental hiPSC line SFC841-03-01 has been previously published [22]. It was originally derived from dermal fibroblasts from a disease-free donor recruited through the Oxford Parkinson’s Disease Centre (participants were recruited to this study having given signed informed consent, which included derivation of hiPSC lines from skin biopsies (Ethics Committee: National Health Service, Health Research Authority, NRES Committee South Central, Berkshire, UK (REC 10/H0505/71))). Alt-R CRISPR-Cas9 system [Integrated DNA Technologies (IDT)] was applied as explained by the manufacturer. Mutagenic primers to target exon 3 of viperin (RSAD2) were gRNA (positive strand): 5' UAUCCAAGGAC CAAGCCCU and gRNA (negative strand): 5'AAGCACUAACCCUGCCGC. This results in deletion of 269 base pairs from exon 3 of RSAD2 gene. Wild-type cells expressing RSAD2 were underwent the same procedure for CRISPR-Cas9 knockout except that the mutagenic primers were not added. This is to control for any unknown effect of the CRISPR-Cas9 procedure on cells. After picking more than 96 single colonies, PCR was used to identify wild-type cells and cell lines in which 269 base pairs from the exon 3 of RSAD2 were removed (Fig. S1). For PCR analysis, the forward primer was 5’AGAAGATGTCCAGAAGACGG and the reverse primer was 5’AGGAAATCAGCAAAAGCGACC. Single nucleotide polymorphisms genotypic analysis was performed, and the results did not show any specific variation between RSAD2-KO and wild-type cells (Fig. S2). Two colonies were prepared and stored for subsequent studies. The resulting hiPSC cells were used to generate embryoid bodies (EBs) using spinning method, which were then used to create factories from which macrophage precursors were harvested, and differentiated to tissue-type macrophages as explained previously [23]. The physical properties and cell viability of EBs and macrophages derived from wild-type or RSAD2-KO iPSC cells were the same (Fig. S3). The macrophages derived from wild-type cells are referred to as wild-type (WT) throughout the manuscript. Macrophages were used for analysis of the extracellular and intracellular metabolite levels. Western blot analysis was performed as before [19].

**Preparation of samples from macrophages for untargeted metabolite analysis by negative ion mass spectrometry (LC-MS/MS)**

50 µL of M-CSF (100 µg·mL⁻¹), 45 µL of LPS (1 mg·mL⁻¹) and 30 µL of IFN-γ (100 µg·mL⁻¹) were added to 50 mL growth medium to prepare a stock solution of LPS and IFN-γ. When macrophages were ready, approximately 7 days after collection of macrophage precursors from EBs factories as described previously [23], fresh growth medium was added. Two hours later, 6 mL of growth medium was removed and 6 mL of the solution of LPS and IFN-γ was added. 72 h after stimulation, 500 µL of growth medium was removed for analysis of the extracellular metabolites and cells were collected for extraction of the intracellular metabolites. The aliquots of growth medium were filtered using 3 kDa Amicon Ultracentrifugal filters (Merck KGaA, Darmstadt, Germany), and the flow-through was used for analysis of the extracellular metabolite levels. To extract the intracellular metabolites, briefly, 50 mL of liquid nitrogen was added to fully cover the cells. Next, 500 µL cold methanol was added to extract the metabolites. Cells were removed with a cell scraper and mixed in methanol. Subsequently, the mixture was centrifuged at 16 000 g for 5 min to remove cells debris. Concentration of DNA was measured in the supernatant using NanoDrop™. The sample with the lowest concentration of DNA was used as reference, and all other samples were diluted to this concentration. The extra- and intracellular metabolite levels were analysed using untargeted metabolite analysis by high-resolution negative ion mass spectrometry (LC-MS/MS). Measurements and data analysis were performed using a Q Exactive hybrid quadrupole-Orbitrap mass spectrometer equipped with a heated electrospray ionization II probe in negative ion mode as explained in details previously [24]. Briefly, samples were analysed using Dionex ICS-5000+ capillary HPIC (Thermo Scientific, San Jose, CA, USA) (IC-MS/MS) or Dionex Ultimate 3000 UPLC system (HILIC-MS/MS) coupled to the Q Exactive mass spectrometer (Thermo Scientific). A Dionex IonPac AS11-HC column (2 x 250 mm, 4 µm) (IC-MS/MS) or a BEH Amide column (2 x 1000 mm, 1.7 µm) (HILIC-MS/MS) was used. Column temperature was 30 °C or 40 °C, respectively. For the IC-MS/MS method, the aqueous hydroxide ion gradient was: 0.0 min, 0.0 mM; 1.0 min, 0.0 mM; 15.0 min, 60.0 mM; 25.0 min, 100.0 mM; 30.0 min, 100.0 mM; 30.1 min, 0.0 mM; and 37.0 min, 0.0 mM, and Dionex AERS 500e 2 µm (Thermo Scientific) in external water mode was used for on-line conductive ion suppression. The flow rate was 0.25 mL·min⁻¹. For The HILIC-MS/MS method, mobile phases were: (a) 95% acetonitrile containing 10 mM ammonium acetate and (b) 50% acetonitrile containing 10 mM ammonium acetate, and the linear gradient was: 0.0 min, 1.0 % B; 1.0 min, 1.0 % B; 6.0 min 45.0 % B; 10.0 min, 95.0 % B; 12.0 min, 99.0 % B; 12.1 min, 1.0 % B; and 15.0 min, 1.0 % B. The flow rate was 0.4 mL·min⁻¹. Setting were: (i) source: sheath gas flow rate, 70 (IC-MS/MS) or 25 (HILIC-MS/MS); auxiliary gas flow rate, 20 (IC-MS/MS) or 8 (HILIC-MS/MS); sweep gas flow rate, 20 (IC-MS/MS) or 8 (HILIC-MS/MS); 3.6 kV; capillary temperature, 320 °C (IC-MS/MS) or 300 °C (HILIC-MS/MS); S-lens RF level, 70 (IC-MS/MS) or 55 (HILIC-MS/MS); and heater temperature, 350 °C (IC-MS/MS) or 300 °C (HILIC-MS/MS). (ii) MS scan parameters: microscans, 2; resolution, 70000; AGC target, 1-10⁶ ions (IC-MS/MS) or 5-10⁶ (HILIC-MS/MS); maximum IT, 120 ms; and scan range, 60-900 m/z. (iii) MS/MS scan parameters: microscans, 2; resolution, 1.75×10⁵; AGC target, 1-10⁵ ions; maximum IT, 250 ms (IC-MS/MS) or 80
Preparation of a mixture of ddhCTP and 5'-deoxyadenosine (5'-dA)

We have confirmed that the thermostable fungal RSAD2 from Thielavia terrestris (TtRSAD2) can efficiently catalyse transformation of CTP to ddhCTP [21]. Thus, to prepare a mixture of ddhCTP and 5'-dA we used TtRSAD2. Wild-type TtRSAD2 was overexpressed in E. coli and purified as described before [25]. The buffer was exchanged to 50 mM phosphate buffer, 300 mM NaCl, pH 7.6 for performing enzymatic transformation of CTP to ddhCTP. Two reactions were prepared: (R1) 500 µL TtRSAD2 (150 µm) + 20 µL SAM (75 µm) + 50 µL CTP (20 µm) + 5 µL phosphate buffer; (R2) 2500 µL TtRSAD2 (150 µm) + 100 µL SAM (75 µm) + 250 µL CTP (20 µm) + 25 µL sodium dithionite (500 µm). CTP and sodium dithionite were prepared in 50 mM phosphate, 300 mM NaCl, pH 7.6. In reaction (i), the fungal enzyme will not convert CTP to ddhCTP because the reducing agent sodium dithionite was not added. Both reactions were incubated in an anaerobic glove box with N₂ atmosphere (Coy Laboratories, O₂ < 5 ppm) overnight (approximately 16 h). Subsequently, samples were subjected to centrifugation using 10 kDa Amicon Ultracentrifugal filters (Merck) to separate the enzyme. The flow-through was diluted to a final concentration of approximately 1.5 mM ddhCTP and was used in further experiments to test the effect of ddhCTP and 5'-dA on the NAD⁺/NADH-dependent activity of different enzymes.

Colorimetric assays for measuring activity of glyceraldehyde 3-phosphate dehydrogenase

Buffer was either phosphate 50 mM, 300 mM NaCl, pH 7.6 or Mops 50 mM, 20 mM NaCl, 10 mM phosphate, pH 7.6. 50 µL of GAPDH stock solution (as explained in chemical section) was added to 500 µL of buffer to prepare a working solution of GAPDH, and 50 µL of phosphoglycerate kinase (PGK) was mixed with 10 mL of buffer to prepare a working solution of PGK for different assays. To measure activity of GAPDH using glyceraldehyde 3-phosphate (G3P) as substrate (forward reaction), two assays were used to measure steady-state kinetics of formation of NADH: (a) direct measurement of conversion of G3P by GAPDH, or (b) coupling the activity of GAPDH, which converts G3P to 1,3-bisphosphoglycerate (1,3BPG), to the activity of PGK, which converts 1,3BPG to 3-phosphoglycerate. In the latter assay, activity of PGK allows near complete consumption of G3P by GAPDH. In assay (a), the following components were added to a quartz cuvette in order: 440 µL of working solution of GAPDH, 5 µL of stock NAD⁺ (40 mM), and 10 or 50 µL of a solution containing ddhCTP and 5'-dA. To prepare the control reaction, buffer was added instead of the solution containing ddhCTP and 5'-dA. To test if inhibition of the enzyme activity is because of ddhCTP or 5'-dA, 5–10 µL solution of 5'-dA (10 mM) was added instead of the solution containing ddhCTP and 5'-dA. In each assay, the total volume of reaction was set to 500 µL by addition of an aliquot of buffer if required. Each solution was added to a quartz cuvette (1 mL total volume and 1 cm path length), and the cuvette was placed in a UV-visible spectrometer. The absorbance was recorded at 340 nm for 1 min to establish a stable baseline. Subsequently, 2 or 5 µL of stock solution G3P (60 mM) was added to the solution and mixed rapidly (circa 3 s). Absorbance was followed at 340 nm for formation of NADH. In the second assay, first 5 µL of NAD⁺ (40 mM) was mixed with 390 µL working solution of GAPDH. The mixture was added to a quartz cuvette, the cuvette was placed in a UV-visible spectrometer, and the absorbance was recorded for 1–2 min to establish a stable baseline. Then, 5 µL G3P (60 mM) was added to the solution, mixed rapidly, and formation of NADH was followed until absorbance plateaued approximately after 3 min. Subsequently, a mixture of (Rc) 50 µL working solution of PGK + 50 µL phosphate buffer + 5 µL ADP (40 mM), or (Rc) a mixture of 50 µL working solution of PGK + 50 µL solution of ddhCTP and 5'-dA + 5 µL ADP (40 mM), or (Rc) a mixture of 50 µL working solution of PGK + 50 µL solution of CTP and SAM + 5 µL ADP (40 mM) was added. In each case, the solution was mixed rapidly, and absorbance was recorded at 340 nm for formation of NADH. To determine the inhibitory constant (IC_{50} value) of ddhCTP and to measure activity of the reverse reaction, the NADH-dependent conversion of 1,3-bisphosphoglycerate to G3P by GAPDH, the enzyme-coupled assay was used. To measure the activity of the reverse reaction with enzyme coupled assay, first PGK was mixed with ATP and 3-phosphoglycerate to generate 1,3BPG. Subsequently, a mixture of GAPDH and NADH in the presence or absence of ddhCTP was added. For measuring the IC_{50} value of ddhCTP, the volume of the mixture of ddhCTP + 5'-dA added in Rc was varied and the final volume was set to 500 µL by adding an aliquot of buffer, when required. All experiments were repeated three times. All measurements were performed at room temperature, approximately 22 °C. Rates of NADH formation or consumption were obtained from a linear fit to the data points recorded for the first 30 s and using a millimolar extinction coefficient (ε_{340}) of 6.22 mm.
Colorimetric assay for measuring activity of L-lactate dehydrogenase or malic enzyme

The total volume of each reaction was 500 µL. To measure formation of NADH by the catalytic activity of LLDH or MDH, the following components were added to a 1 mL quartz cuvette (1 cm path length) in order: 470 or 490 µL of buffer, 5 µL of enzyme (LLDH or MDH), 5 µL of NAD⁺ (40 mM stock), and 0 or 20 µL of solution of ddhCTP and 5'-dA. Next, the cuvette was placed in a UV-visible spectrometer, and the absorbance was recorded at 340 nm to establish a stable baseline. After circa 1 min, 2 µL of a solution of 20 mM substrate (L-lactate or malate) was added to the cuvette. The solution was rapidly mixed, and formation of NADH was followed at 340 nm. To measure the reverse reaction using LLDH, 20 mM stock solution of pyruvate was used as substrate and NAD⁺ was replaced by NADH. Buffer was either phosphate 50 mM, 300 mM NaCl, pH 7.6 or Mops 50 mM, 20 mM NaCl, 10 mM phosphate, pH 7.6. All measurements were performed at room temperature, approximately 22 °C. Rates of NADH formation or consumption were obtained as explained above.

Molecular docking simulations

The 3D model of ddhCTP was obtained using Chem3D software and was saved as a mol2 file. We used the X-ray crystal structure of GAPDH (PDB Code: 5C70) and LLDH (PDB Code: 1T2F) solved in the presence of NAD⁺. To perform the molecular docking, first the NAD⁺ ligand was removed from the structure of each enzyme. Subsequently, the ligand-free structure and the 3D model of a ligand (ddhCTP, CTP or ADP) were loaded into the SwissDock server [26]. Using this approach, global molecular docking without bias towards a defined site is done. The structures with the highest Fullfitness rank and clustering numbers for a specific binding site were used for analysis of a ligand binding.

Results and discussion

Activity of RSAD2 in HEK293 cells inhibits NAD⁺/NADH-dependent enzymatic reactions

Previously, we overexpressed wild-type RSAD2 (WT-RSAD2), an inactive double variant of RSAD2 (DM-RSAD2), or green fluorescence protein (GFP) as a negative control in HEK293 cells. We analysed the extracellular metabolite levels 24 or 96 h post-transfection of HEK293 cells with a construct for expression of each protein [19]. We chose these time points because between 24 and 96 h maximum intracellular amount of ddhCTP generated by the radical-SAM activity of WT-RSAD2 was reported [20]. The results confirmed that the cellular activity of RSAD2 abolished catabolism of different amino acids and led to an increase in the extracellular level of xanthine [19]. Further analysis of data revealed that the cellular activity of RSAD2 abolished the extracellular levels of products of phenylalanine and tyrosine metabolism, namely phenylactic acid and 4-hydroxyphenyllactic acid (Fig. 2). A common step in metabolic pathways of amino acids, xanthine, phenylactic acid and 4-hydroxyphenyllactic acid is their dependency on the catalytic activity of NAD⁺/NADH-dependent enzymes. Specifically, conversion of xanthine to uric acid requires the NAD⁺-dependent

![Fig. 2. Expression of RSAD2 in HEK293 cells abolished the extracellular levels of metabolites whose formation requires activity of the NAD⁺/NADH-dependent enzymes. The extracellular levels of (A) phenylactic acid and (B) 4-hydroxyphenyllactic acid were lower for HEK293 cells expressing WT-RSAD2 as compared to those for cells expressing inactive enzymes, that is GFP or an inactive variant of RSAD2 (DM-RSAD2). The extracellular metabolite levels were measured 24 or 96 h post-transfection of HEK293 cells with GFP, WT-RSAD2, or the inactive DM-RSAD2. The results are plotted relative to the amount of metabolite in the growth medium before addition to the cells. Data are average of three independent biological replicates ± standard deviation.](image-url)
activity of xanthine dehydrogenase and formation of phenylacetate requires the NAD$^+$-dependent activity of aldehyde dehydrogenases. Therefore, it is possible that the radical-SAM activity of RSAD2 inhibited the NAD$^+$/NADH-dependent enzymatic reactions leading to an elevated extracellular level of xanthine and abolishing formation of phenylacetic acid and 4-hydroxyphenyllactic acid.

**Macrophages expressing RSAD2 have a lower activity of NAD$^+$-dependent enzymes**

To further test if the enzymatic activity of RSAD2 can inhibit NAD$^+$-dependent enzymatic reactions, we shifted to more physiologically relevant conditions. We knocked out RSAD2 gene in hiPSCs cells and derived macrophages as we have described and characterized before [23,27] (Methods). First, we confirmed that the RSAD2-KO cells were fully deficient in expressing the enzyme. To this end, we used western blot analysis and measured expression of RSAD2 before and after stimulation with a mixture of lipopolysaccharides (LPS) and IFN-γ. Before and after stimulation, only wild-type (WT) cells showed expression of RSAD2 (Fig. 3A). The expression of protein in WT cells was significantly induced after stimulation with the mixture of LPS and IFN-γ (Fig. 3A). The results confirmed that RSAD2-KO cells could not express the enzyme. Next, we investigated the difference between the extracellular metabolite levels of RSAD2-KO cells and those of WT cells, after stimulation with a mixture of LPS and IFN-γ (Methods). For several metabolites, we noticed a significant difference ($P$ value < 0.05) between WT and RSAD2-KO cells (Table S1). Among different metabolites, the most significant change was observed in the extracellular level of taurine (Fig. 3B). It was 3.5-fold higher in RSAD2-KO cells as compared to WT cells. Formation of taurine directly requires the NAD$^+$-dependent activity of hypotaurine dehydrogenase (HDH) (Fig. 3B). Another metabolite whose extracellular level was 3-fold more in WT cells as compared to RSAD2-KO cells was 2-oxoglutarate (2-OG) (Fig. 3C), which is converted by the NAD$^+$-dependent activity of 2-oxoglutarate dehydrogenase (2-OGDH) to succinate. One explanation for these observations is that the enzymatic activity of RSAD2 can...
inhibit the NAD$^+$-dependent enzymatic reactions in macrophages to block formation of taurine and consumption of 2-OG. Besides taurine that has anti-inflammatory properties\cite{28,29}, we noticed that the level of another anti-inflammatory metabolite, namely guanosine\cite{30,31}, was higher in RSAD2-KO cells as compared to WT cells (Fig. 3D). These data suggest to us that the cellular activity of RSAD2 in macrophages induces a pro-inflammatory metabolic state.

RSAD2-expressing macrophages produce ddhCTP and have a lower activity of GAPDH

Next, we investigated the effect of RSAD2 expression on the intracellular metabolite levels. We studied the difference between the intracellular metabolite levels in RSAD2-KO cells and those of WT cells (Methods). A list of metabolites whose levels were significantly different between WT and RSAD2-KO cells is given in Table S2. The results revealed that in the RSAD2-KO cells catalytic transformation of CTP to ddhCTP did not occur (Fig. 4A). No ddhCTP was detected in RSAD2-KO cells (Fig. 4A), and the deoxyadenosine level, which is the sum of 5'-deoxyadenosine and 5'-deoxyadenosine (5'-dA) as measure by LC-MS/MS, was higher in WT cells as compared to RSAD2-KO cells (Fig. 4A). Among the different metabolites detected, we noticed that the intracellular level of glyceraldehyde 3-phosphate (G3P), which is consumed by the NAD$^+$-dependent activity of the housekeeping enzyme GAPDH, was higher in WT cells as compared to RSAD2-KO cells (Fig. 4B). Additionally, we found that the levels of sedoheptulose 7-phosphate and ribose 5-phosphate (Fig. 4C), which are intermediates of the pentose phosphate pathway (PPP), were higher in WT cells as compared to RSAD2-KO cells. This is consistent with inhibition of the glycolytic enzyme GAPDH and the increase in flux through the PPP. Therefore, we conclude that the enzymatic activity of RSAD2 inhibited the NAD$^+$-dependent conversion of G3P to 1,3-biphosphoglycerate (1,3BPG) by the catalytic activity of GAPDH, which led to an increase in the intracellular level of G3P and consequently, the flux through the PPP. Consistent with the results of the extracellular levels of taurine and 2-OG, which suggested to us that the activity of RSAD2 inhibits the NAD$^+$-dependent activity of HDH and 2-OGDH (Fig. 3), the intracellular level of taurine was lower (Fig. 4D) and that of 2-OG was higher in WT cells as compared to RSAD2-KO cells (Table S2). Therefore, the results of the extra- and intracellular metabolite levels together strongly suggest that the radical-SAM activity of RSAD2 inhibits the NAD$^+$-dependent activity of different enzymes.
ddhCTP inhibits the NAD⁺-dependent activity of GAPDH

Guided by the results of metabolomics analysis, using biochemical assays we tested if the ddhCTP product of the radical-SAM activity of RSAD2 can inhibit the NAD⁺-dependent activity of GAPDH (Fig. 5A). First, we measured whether a mixture of ddhCTP and 5′-dA produced by the enzymatic activity of RSAD2 can inhibit the activity of GAPDH. To this end, we produced the mixture of ddhCTP and 5′-dA using RSAD2 [21] (Methods). Then, the NAD⁺-dependent conversion of G3P to 1,3BPG by GAPDH was

Fig. 5. Steady-state kinetics of inhibition of the NAD⁺-dependent activity of GAPDH, LLDH and MDH by ddhCTP. (A) Steady-state progress curves and initial rates of formation of NADH due to conversion of G3P by GAPDH were measured using the enzyme-coupled assay as explained in the methods. (i) Addition of G3P (final concentration of 600 µM) to a solution containing GAPDH and NAD⁺ (final concentration of 400 µM). (ii) Addition of a mixture of PGK and ADP (final concentration of 400 µM) (control), PGK, ADP, and ddhCTP + 5′-dA (orange), or PGK, ADP and CTP + SAM (grey). Addition of PGK and ADP in the second solution (ii) pushes the catalytic conversion of G3P by GAPDH to near completion, because PGK catalyses conversion of the 1,3-biphosphoglycerate (1,3BPG) produced by GAPDH to 3-phosphoglycerate. (B) Steady-state initial rate of formation of NADH by the catalytic activity of GAPDH in the presence of ddhCTP and 5′-dA or 5′-dA alone as compared to that of control. (C) Steady-state progress curve and initial rate of formation of NADH by the catalytic activity of LLDH in the presence of a mixture of ddhCTP and 5′-dA as compared to those of control. (D) The initial rate of formation of NADH by the catalytic activity of MDH in the presence of a mixture of ddhCTP and 5′-dA as compared to that of control. Concentration of ddhCTP was approximately (A) 180 µM, (B) 36 µM and (C,D) 72 µM. The control reaction contains only the enzymes and substrates.
measured either directly or alternatively, using an enzyme-coupled assay (Methods). The results revealed that the mixture of ddhCTP and 5'-dA reduces the NAD^+-dependent activity of GAPDH by approximately 3-fold (Fig. 5A). To test whether this inhibition was due to the presence of ddhCTP or 5'-dA, we measured activity of GAPDH in the presence of synthetic 5'-dA. We observed that 5'-dA alone could not inhibit the activity of GAPDH (Fig. 5B). Therefore, we conclude that the ddhCTP generated by the radical-SAM activity of RSAD2 is necessary for the inhibition of GAPDH activity, either alone or in cooperation with the 5'-dA. Next, we tested if the reverse reaction catalysed by GAPDH, namely the NADH-dependent conversion of 1,3BPG to G3P, was inhibited by ddhCTP (Fig. S4A). The results showed that ddhCTP could not inhibit the NADH-dependent conversion of 1,3BPG to G3P.

It has been proposed that ddhCTP acts as an effective chain terminator of viral RdRps [20]. Therefore, we sought to compare the efficiency of ddhCTP for inhibiting the activity of GAPDH with its reported efficiency as a viral RdRp chain terminator. We determined the half inhibitory concentration (IC_{50} value) of ddhCTP as an inhibitor of GAPDH. We measured the NAD^+-dependent conversion of G3P by GAPDH in the presence of physiologically relevant concentration of NAD^+, approximately 200–500 µM [32,33], by varying the concentration of ddhCTP (Fig. S4B). The resulting IC_{50} value was 55.8 ± 0.2 µM. We compared this with the IC_{50} values reported for ddhCTP as a viral RdRp chain terminator [20] (Table 1). The comparison suggested that ddhCTP is 400–600 000 folds more effective at inhibiting GAPDH compared to its proposed inhibitory activity as a viral RdRp chain terminator (Table 1).

Table 1. Comparison of the IC_{50} value of ddhCTP for inhibition of GAPDH with those of ddhCTP as chain terminator of RdRps. DV, dengue virus; WNV, West Nile virus; HRV-C, human rhinovirus C; PV, poliovirus. The IC_{50} value for inhibition of GAPDH by ddhCTP was obtained in the presence of a physiologically relevant concentration of NAD^+, that is 400 µM. The IC_{50} values for RdRp of different flaviviruses were estimated.

| Enzyme     | IC_{50} values (µM) | Reference |
|------------|---------------------|-----------|
| GAPDH      | 55                  | This work |
| DV RdRp    | 30 000              | [20]      |
| WNV RdRp   | 20 000              | [20]      |
| HRV-C RdRp | 3 000 000           | [20]      |
| PV RdRp    | >30 000 000         | [20]      |

ddhCTP inhibits the NAD^+-dependent activity of LLDH and MDH

Our metabolomics data suggested that ddhCTP could potentially inhibit the activity of some other NAD^+-dependent reactions. Thus, we tested inhibition of the NAD^+-dependent activity of LLDH (Fig. 5C) and malic enzyme (MDH) (Fig. 5D and Fig. S5A). We observed that the mixture of ddhCTP and 5'-dA could efficiently block activity of both enzymes. Consistent with our observation that the 5'-dA alone did not inhibit the NAD^+-dependent activity of GAPDH, we found that the 5'-dA alone could not inhibit the NAD^+-dependent activity of LLDH (Fig. S5B). Next, we tested whether the reverse reaction catalysed by LLDH (Fig. S5C), namely the NADH-dependent conversion of pyruvate, was inhibited by ddhCTP. The results confirmed that ddhCTP could not inhibit the reverse reaction (Fig. S5C), consistent with our observation for GAPDH. Using biochemical assays, we found that ddhCTP in the presence of physiologically relevant amount of NAD^+, that is 200–500 µM [32,33], could inhibit the activity of NAD^+-dependent enzymes more than 2 folds (Fig. 5). This level of inhibition measured by biochemical assays is consistent with metabolomics data. The extra- or intracellular levels of taurine, 2-OG, or G3P (Figs 3–4), whose formation or consumption requires the NAD^+-dependent activity of enzymes, were 1.5–3.5 folds different between WT and RSAD2-KO cells.

ddhCTP binds to the NAD^+ binding pocket

Next, we performed molecular docking simulations (Methods) to predict the binding pocket of ddhCTP in GAPDH and LLDH. First, we compared the NAD^+ binding pocket in the X-ray crystal structure of GAPDH (Fig. 6A) or LLDH (Fig. S6A) with the predicted binding pocket of ddhCTP (Fig. 6B and Fig. S6B). The results revealed that ddhCTP binds to the NAD^+ binding pocket in GAPDH and LLDH. Then, we predicted binding pocket of ADP (Fig. 6C and Fig. S6C), which is a known weak inhibitor of GAPDH (apparent K_{i} value of 2.1 mM) [34] and LLDH [35], or CTP (Fig. 6D and Fig. S6D), which does not inhibit activity of the enzymes. We found that ADP binds to the binding pocket of NAD^+, while CTP binds elsewhere. The predicted change in the Gibbs free energy (ΔG) was used to calculate the dissociation constant (K_{d}) of ddhCTP, CTP or ADP (Table 2). It can be observed that the dissociation constants of ddhCTP are significantly less than those of CTP. These data strongly suggest that ddhCTP
competes with NAD⁺, and it can effectively inhibit the NAD⁺-dependent activity of GAPDH and LLDH. The predicted dissociation constant of ddhCTP for LLDH is 4-fold more than that for GAPDH. Therefore, ddhCTP has a higher affinity for the NAD⁺ binding pocket in LLDH and should reduce the activity of LLDH more than that of GAPDH. This prediction is consistent with our biochemical data. In the presence of approximately 180 µM ddhCTP, the activity of GAPDH decreased circa 3-fold (Fig. 5A), while in the presence of approximately 72 µM ddhCTP, the activity of LLDH dropped approximately 10-fold (Fig. 5C).

Conclusions

In summary, our analysis of the extra- and intracellular metabolite levels, biochemical studies and molecular docking simulations revealed that ddhCTP binds to the NAD⁺ binding pocket and inhibits the NAD⁺-dependent enzymatic reactions. Inhibition studies using the housekeeping enzyme GAPDH showed that unlike the previously reported inefficient role of ddhCTP as a viral chain terminator (IC₅₀ values of >20–30 mM) (Table 1), inhibition of GAPDH by ddhCTP is much more efficient (IC₅₀ value of 0.055 mM) and occurs at physiologically relevant concentrations of ddhCTP and NAD⁺. The difference in the IC₅₀ value of ddhCTP as an inhibitor of GAPDH and those reported for ddhCTP as a viral RdRps chain terminator is very large. Therefore, we contend that this difference is not linked to variations in assays and conditions.

Inhibition of the NAD⁺-dependent activity of enzymes will affect a range of downstream and/or upstream metabolic and signalling pathways. NAD⁺ is a ubiquitous cofactor central to metabolism in all life forms, it is the source of ADP in ADP-ribosylation reactions [36], and it is the precursor for the cyclic ADP-ribose, a second messenger involved in Ca²⁺ release and signalling [37,38]. Consequently, the radical-SAM activity of RSAD2 will have a wide range of downstream effects, which would impact many cellular processes. On the other hand, inhibition of the activity of GAPDH by the ddhCTP product of the radical-SAM activity of RSAD2 can affect upstream metabolic pathways. Specifically, it can increase the flux through the pentose phosphate pathway (PPP), which will increase the rate of regeneration of NADPH.

Table 2. Comparison of the predicted ΔG and dissociation constants (Kᵣ) of ddhCTP, CTP and ADP.

| Enzyme | Ligand | ΔG (kcal·mol⁻¹) | Kᵣ (µM) |
|--------|--------|----------------|---------|
| GAPDH  | ddhCTP | −9.79          | 65.9    |
|        | CTP    | −7.54          | 2949.8  |
|        | ADP    | −8.99          | 254.85  |
| LLDH   | ddhCTP | −10.59         | 17.0    |
|        | CTP    | −9.12          | 204.6   |
|        | ADP    | −9.26          | 161     |

ΔG (kcal·mol⁻¹) was obtained from molecular docking simulation. Kᵣ was calculated from the Gibbs free energy: ΔG = RTlnKᵣ, in which R is the universal gas constant and equal to 1.987 (kcal·mol⁻¹·K⁻¹) and T is temperature (K) and was assumed to be 298 K.
In conclusion, inhibition of the NAD⁺-dependent enzymatic reactions can have a broad cellular impact leading to the restriction of viral replication and other physiological or pathophysiological conditions. These impacts will potentially depend on the variation in the dissociation constants of ddhCTP for the NAD⁺-binding pockets in proteins, the expression level of different enzymes, and the activity levels of various metabolic pathways.

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**Author contributions**

KHE conceived the study. KHE designed and performed the experiments, and analysed data. JV and CB assisted in creating RSAD2-KO cells. WSJ suggested experiments. WSJ and JM provided support and facilities for experiments and discussed data, and KHE wrote the manuscript with contribution from all the authors.

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**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. PCR analysis of exon 3 of RSAD2 gene for the single colonies obtained after performing CRISPR-Cas9 method.

Fig. S2. SNP genotypic analysis does not show any specific difference between WT-RSAD2 expressing and RSAD2-KO cells.

Fig. S3. Comparison of the physical properties of WT and RSAD2-KO EBs and macrophages.

Fig. S4. ddhCTP inhibits the NAD+-dependent activity of GAPDH but not its NADH-dependent activity.

Fig. S5. ddhCTP inhibits the NAD+-dependent activity of enzymes but not the NADH-dependent reactions.

Fig. S6. ddhCTP binds to the NAD+ binding pocket in LLDH.

Fig. S7. ddhCTP does not chain terminate RNA-dependent RNApolymerase (RdRp) activity of Dengue virus (DV) or West Nile virus (WNV).

Table S1. A list of extracellular metabolites identified with a statistically significant change between WT-RSAD2 and RSAD2-KO cells.

Table S2. A list of intracellular metabolites identified with a statistically significant change between WT-RSAD2 and RSAD2-KO cells.