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Viperin, through its radical-SAM activity, depletes cellular nucleotide pools and interferes with mitochondrial metabolism to inhibit viral replication

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Abstract

Viperin (RSAD2) is an antiviral radical S-adenosylmethionine (SAM) enzyme highly expressed in different cell types upon viral infection. Recently, it has been reported that the radical-SAM activity of viperin transforms cytidine triphosphate (CTP) to its analogue 3'-deoxy-3',4'-didehydro-CTP (ddhCTP). Based on biochemical studies and cell biological experiments, it was concluded that ddhCTP and its nucleoside form ddhC do not affect the cellular concentration of nucleotide triphosphates (NTPs) but act as replication chain terminators. However, our re-evaluation of the reported data and our data indicate that ddhCTP is not an effective viral chain terminator but depletes cellular nucleotide pools and interferes with mitochondrial activity to inhibit viral replication. Our analysis is consistent with a unifying view of the antiviral and radical-SAM activities of viperin.

Introduction

Expression of the radical S-adenosylmethionine (SAM) enzyme viperin (also known as RSAD2) is highly induced via interferon-dependent or -independent pathways in different cells [1,2]. It is reported that the enzyme is highly overexpressed in some types of cancers [2] and in a subset of microglia with neurotoxic properties [3]. The enzyme is found to be localized to the cytosolic face of the endoplasmic reticulum [4] or to lipid droplets [5]. It has been established that the expression of the enzyme restricts replication of different RNA or DNA viruses [1,2,6,7]. While in some types of viruses the antiviral activity is proposed to be due to the interaction of the enzyme with cellular or viral proteins, in most cases the antiviral activity is reported to be dependent on the radical-SAM domain of the protein. However, different molecular mechanisms have been proposed for how the radical-SAM activity of RSAD2 interferes with viral replication. Studies using HIV-1 led to the conclusion that the radical-SAM activity of viperin interferes with the mevalonate pathway, affecting cellular lipid rafts, and blocking viral exit and production [8]. Studies with human cytomegalovirus (HCMV) led to the suggestion that the enzymatic activity of viperin can interfere with the activity of mitochondrial β -oxidation pathway [9]. The effect of the radical-SAM activity of viperin on β -oxidation pathway has been shown to regulate thermogenesis in adipose tissues [10]. More recently, it has been demonstrated that the radical-SAM activity of RSAD2 catalyses transformation of CTP to its nucleotide analogue 3'-deoxy-3',4'-didehydro-CTP (ddhCTP) [11]. This activity of the enzyme is proposed to be coupled to the activity of mitochondrial cytidylate monophosphate kinase 2 (CMPK2), which supports formation of CTP as the substrate of viperin [11]. Using biochemical and cell biological assays it was concluded that ddhCTP and its nucleoside form, i.e. ddhC, do not affect cellular concentrations of NTPs but act as an effective viral replication chain terminator [11].

Here, we have re-evaluated the data reported for the activity of ddhCTP as a viral chain terminator and the effect of ddhCTP and ddhC on cellular concentrations of NTPs [11]. We demonstrate that our re-evaluation does not support the proposal that ddhCTP acts as a viral replication chain terminator. We show that ddhCTP/ddhC abolish cellular concentrations of nucleotides, specifically UTP and CTP, which would cause a reduction in viral replication. We tested if this effect of ddhCTP/ddhC on nucleotide pools can be because of their interference with mitochondrial activity, which is linked to the *de novo* biosynthesis of UTP and CTP via the activity of dehydroorotic acid dehydrogenase (DHODH) and mitochondrial respiratory complex I [12]. We found that the cellular enzymatic activity of viperin interferes with mitochondrial activity and metabolism.

Material and Methods.

Chemicals and buffers. All chemicals were reagent grade and purchased from Sigma Aldrich.

Measuring effect of viperin on extracellular metabolites. The construct for expression of wild-type human viperin was generously provided by Professor Peter Cresswell (Yale University). The construct for expression of a fungal homologue of viperin (RSAD2) from the thermophilic fungus *Thielavia terrestris* (TtRSAD2 or TtV) was prepared as explained previously [13]. For site-directed mutagenesis to replace the highly conserved Ser300 and Tyr301 in human viperin with alanine and phenylalanine the forward primer was 5' CCAGAAGATGAAAGACGCCTTCCTTATTCTGGATG 3' and the reverse primer was complementary to the forward primer. The highly conserved serine and tyrosine residues in the fungal viperin (RSAD2), Ser251 and Tyr252, were replaced by alanine and phenylalanine, respectively, as explained before [14]. Site directed mutagenesis was performed as explained before [15]. The insert of interest with the correct mutation was confirmed using sequencing (GENEWIZ). Expression and purification of fungal viperin in *E. Coli* were performed as explained previously [13]. To investigate the effect of human viperin on cell metabolism and respiration, HEK293 cells were grown in 6-well plates to a confluency of 70-90 %. Then cells were transferred to a 96-well Seahorse microplate (3xE4 cells per well) or to a 6-well plate (2xE6 cells per well). At a confluency of 60-70%, cells in each well were transfected with the same amount of plasmid encoding WT-viperin or DM-viperin using lipofectamine 2000 or Turbofect transfection reagent (ThermoFisher Scientific) according to manufacturer protocol. For analysis of extracellular metabolites, the medium was refreshed after 24 hours then, 24 or 96 hours later, 500 µl of medium was removed from each well of the 6-well plate and analysed using accurate LC-MS. The growth medium was DMEM containing 10% FBS, 2mM L-glutamine, 100units/ml penicillin-streptomycin, and 25 mM glucose. For the Seahorse experiment, the medium was refreshed 24 hours after transfection; 72 hours later, the growth medium was replaced with DMEM containing 10mM glucose, 1mM pyruvate and 2mM L-glutamine, but omitting

bicarbonate. Subsequently, the Seahorse™ plate was incubated in at 37 °C with no CO₂ for 1 hour before starting the measurements. Total protein content was determined using BCA assay and, within experimental error, was the same regardless of whether the cells expressed WT-viperin, DM-viperin, or GFP. Expression of WT-viperin and DM-viperin was confirmed by western blot using viperin rabbit polyAb (Proteintech Europe) and anti-rabbit IgG HRP-linked antibodies.

Liquid chromatography-mass spectrometry (LC-MS) for measuring activity of fungal viperin and extracellular metabolites. For measuring extracellular metabolites in the growth medium of HEK293 cells, high resolution LC-MS analysis was performed as explained previously[13]. For analysis of formation of 5'-dA and ddhCTP, the same instrument as that used for metabolomics was used except that the column was SeQuant® ZIC®-HILIC, 5 µm particle size, 200 Å pore size, 150 × 4.6 mm bed. Buffer A was 90 vol.% MeCN (Honeywell, CHROMASOLV® 99.9%): 10 vol.% MilliQ, 20 mM ammonium acetate, pH 7.5. Buffer B was 100 vol.% Milli-Q, 20 mM ammonium acetate, pH 7.5. Gradient: [0-1 min]: 100:0 (A:B); [1-21 min] 100:0 (A:B) linearly changed to 20:80 (A:B); [21-23 min] 20:80 (A:B); and [23-25 min] 100:0 (A:B). Flow rate was 0.3 ml/min. Analysis of the LC-MS data was performed using MestReNova software. Six samples were prepared for analysis of formation of ddhCTP using fungal WT-viperin or DM-viperin. For the samples containing all components of the reaction: 250 µL of fungal viperin (TtRSAD2) was mixed with 20 µL of 100 mM SAM, 10 µL of 20 mM of CTP, and 5 µL of 500 mM solution of sodium dithionite. Four control samples were prepared in each of which a component of the reaction, i.e. enzyme, SAM, CTP, or dithionite, was not added and was replaced by the same volume of buffer. Buffer was 50 mM phosphate (pH 7.6) containing 300 mM NaCl.

Calculation of the reduction in UTP and CTP due to expression of viperin. Extended data Fig. 6 in ref. [11] was used to calculate the reduction in UTP and CTP. Initially, for cells expressing viperin (+viperin) or not expressing viperin (-viperin) the values for the data points reported at 16 hr and 72 hr were extracted accurately. Microsoft Fullscreen snip application was used. For each condition, i.e. -viperin or +viperin, and for each time point three data points were reported. For each condition, i.e. -viperin or +viperin, a data points for 72 hr was subtracted from a data point at 16 hr. Five different combinations were tried. The average of the 5 numbers ± standard deviation was plotted.

Results and discussion

ddhCTP is not an effective viral chain terminator. We re-evaluated the reported biochemical studies regarding the function of ddhCTP as a viral chain terminator [11]. Inhibition of the activity of different RNA-dependent RNA polymerases (RdRps) of flaviviruses was measured by varying the concentration of ddhCTP in the presence of different amounts of CTP up to a concentration of 100 µM [11]. Accordingly, it

was concluded that ddhCTP effectively chain terminates RNA synthesis of dengue virus (DV) and West Nile virus (WNV) RdRps [11] but does not chain terminate that of Human rhinovirus C (HRV-C) and poliovirus RdRps [11]. Upon analysis of the data, we observed that at a ddhCTP concentration of 100 μ M and CTP concentration of 100 μ M (at least 5-fold lower than the physiological concentration of CTP), inhibition of the activity of the RdRps of all tested viruses by ddhCTP is close to zero. We noticed that only at a ddhCTP concentration of about 2.5 folds more than its maximum measured cellular concentration (340 μ M) and a CTP concentration of 5-fold less than its cellular concentration, ddhCTP had some chain terminating activity. Therefore, we compared the reported IC₅₀ values of ddhCTP as a chain terminator with those reported for few known antiviral chain terminators (Table 1). The IC₅₀ value of ddhCTP as a chain terminator of DV RnRp is at least 400 folds larger than those reported for known antiviral chain terminators. In other words, ddhCTP is at least 400-fold less efficient as a chain terminator compared to other antiviral nucleotide analogues. This huge difference is unlikely to be due to differences in assays and conditions. Therefore, we conclude that under physiologically relevant conditions, ddhCTP is not an effective viral chain terminator and it potentially restricts viral replication via other mechanisms.

ddhCTP and ddhC abolish intracellular concentrations of UTP and CTP. Conversion of CTP to ddhCTP by the radical-SAM activity of viperin or conversion of ddhC to ddhCTP, which requires consumption of three equivalents of NTPs, would reduce cellular concentration of CTP or other nucleotide triphosphates. This reduction would abolish viral replication. Our re-analysis of the reported data [11] does not rule out this possibility. On the contrary, we found four pieces of evidence showing that the cellular activity of viperin and treatment of cells with ddhC abolish cellular concentrations of UTP and CTP and that the cytosolic activity of viperin somehow affects mitochondrial activity. (i) Intracellular concentration of NTPs was measured from 16 hr to 72 hr post transfection of HEK293 cells with viperin or empty vector [11]. Using these data, we estimated that the average reduction in cellular concentration of CTP (Fig. 1a) or UTP (Fig. 1b) was significantly more for viperin expressing cells. (ii) The intracellular formation of ddhCTP appears to significantly increase when human viperin is co-expressed with human CMPK2 [11], which is reported to catalyse phosphorylation of nucleotide monophosphates and diphosphates like CMP, CDP, UMP, and UDP [11,16]. The UMPK2 gene is always immediately after the viperin (RSAD2) gene in vertebrates and it is shown to be co-translated with viperin upon stimulation of cells with IFNs [17]. Therefore, it appears that the activity of CMPK2 supports formation of the CTP substrate required for formation of ddhCTP by the radical-SAM activity of viperin [11]. CMPK2 is shown to be localized to mitochondria [16]. These data together suggest that CMPK2 uses the mitochondrial pool of NTPs, specifically UTP, to convert CMP/CDP to CTP for export to the cytosol and support the synthesis of ddhCTP via the radical-SAM activity of viperin. Therefore, it appears that the radical-SAM activity of

viperin and formation of ddhCTP can lead to the depletion of the mitochondrial pool of CTP and UTP. (iii) To confirm that the ddhCTP generated by viperin can inhibit viral replication, the effect of ddhC, the synthetic nucleoside form of ddhCTP, on cell survival and viral replication was measured using Vero cells [11]. Only after treatment of cells with 3-4 sequential additions of 1 mM ddhC, some antiviral effects on Zika virus could be observed. The use of ddhC does not mimic the enzymatic activity of viperin because (1) three molecules of cellular NTPs are consumed for the conversion of one molecule of exogenous ddhC to ddhCTP, and (2) enzymatic activity of viperin generates the toxic nucleoside analogue 5'-deoxyadenosine (5'-dA). Therefore, it is necessary to at least exclude the possibility that 3-4 sequential additions of 1 mM ddhC to the cells could perturb the intracellular concentration of NTPs, and thereby inhibit viral replication indirectly. However, the change in the intracellular concentration of NTPs before and after treatment with ddhC was not measured [11], leaving this an open question. Therefore, we cannot conclude that ddhC acts as a viral chain terminator and does not affect the cytosolic or mitochondrial concentration of NTPs. (iv) Consistent with the previously predicted cytotoxicity of ddhC [18], we found that treatment of Vero cells with ddhC caused some cytotoxicity. When we plotted the data reported for the effect of ddhC on cell viability [11] in a linear scale instead of logarithmic scale (Fig. 1c), we observed that in the absence of ddhC the number of live Vero cells increased 2-fold from 24 hr to 48 hr (Fig. 1c). However, in the presence of 1 mM ddhC the number of live cells increased 1.5-fold in the same period (Fig. 1c). Therefore, addition of ddhC caused a reduction of approximately 25% in the number of cells. Consequently, we suggest that the observed reduction in viral replication due to treatment with ddhC [11] is largely due to a reduction in cellular concentrations of UTP and CTP and interference with mitochondrial activity or nucleotide pools.

Enzymatic activity of RSAD2 interferes with metabolism and mitochondrial respiration. Our analysis of previous data suggested that cellular enzymatic activity of viperin could abolish cellular concentrations of UTP and CTP. Additionally, because the radical-SAM activity of viperin appears to be coupled to the activity of mitochondrial enzyme CMPK2, it is possible that formation of ddhCTP by viperin in the cytoplasm leads to depletion of UTP and CTP in mitochondria. A reduction in mitochondrial concentration of UTP and CTP would interfere with the activity of mitochondrial respiratory chain complex I [19]. Therefore, we investigated if the radical-SAM activity of viperin (Fig. 2a), affects mitochondrial respiration and metabolism. We used two independent methods: (i) Seahorse methodology to measure oxygen consumption rate (OCR), and (ii) analysis of the extracellular metabolites under low glucose conditions using high-resolution mass spectrometry to follow consumption of amino acids, whose catabolism requires mitochondrial enzymes. First, we created an inactive variant of human viperin (DM-viperin) (Methods), based on our biochemical studies with a fungal homologue. We have demonstrated that mutation of two

highly conserved residues, namely Ser251 and Tyr252 in the fungal RSAD2, fully abolishes catalytic activity of the enzyme[14] (Supplementary Figure 1). Our mechanistic studies revealed that tyrosine is involved in a proton-coupled electron transfer step for reductive dehydration of CTP to ddhCTP [14]. Therefore, we created the inactive double variant of human viperin by substituting the highly conserved serine and tyrosine, i.e. Ser300 and Tyr301 in human viperin, with alanine and phenylalanine respectively (Methods). HEK293 cells, which do not express viperin, were used to express WT-viperin or DM-viperin. As compared to cells expressing inactive DM-viperin, cells expressing WT-viperin had a lower OCR but the same extracellular acidification rate (ECAR) (Fig. 2b). Thus, enzymatic activity of viperin reduced the rate of mitochondrial respiration. Then, we analysed extracellular metabolites at 24 and 96 hours post transfection with WT-viperin, DM-viperin, or green fluorescence protein (GFP) (Fig. 2 and Supplementary Fig. 2), which like DM-viperin does not generate ddhCTP and 5'-dA. These time points were chosen because between 24-96 hours maximum intracellular concentration of ddhCTP was reported in HEK293 cells[11] and glucose concentration was low (Supplementary Fig. 2). We noticed that at 96 hr the levels of amino acids, e.g. arginine (Fig. 2c), in the growth medium of cells expressing WT-viperin were higher. Thus, WT-viperin expressing cells had a lower rate of catabolism of amino acids from 24 to 96 hr. Additionally, at 96 hr the extracellular level of xanthine (Fig. 2d), which is a measure of mitochondrial oxidative stress, was significantly higher in the growth medium of cells expressing WT-viperin. However, synthesis of phenylethylamine, which does not require mitochondrial-related activity, was not affected by expression of WT-viperin (Fig. 2e). These data together strongly suggest that the cellular enzymatic activity of viperin affects metabolism and mitochondrial respiration.

Concluding remarks

In conclusion, we demonstrate that ddhCTP is not an effective chain terminator of RnRps. Our analysis of previous data showed that ddhC/ddhCTP have cytotoxicity and reduce intracellular concentration of NTPs potentially by interfering with mitochondrial activity. Consistently, we found that the cellular activity of viperin decreases the rate of mitochondrial respiration and reduces the rate of consumption of amino acids as carbon source, which requires activity of different mitochondrial enzymes and metabolic pathways. This observation is consistent with a recent report showing that the cellular activity of viperin regulates thermogenesis in adipose tissue via interfering with mitochondrial β -oxidation pathway [10]. Therefore, in contrast to the proposal of different mechanisms of the antiviral and cellular functions for the radical-SAM activity of viperin, our analysis and data point to a potentially unifying mechanism of functions as we hypothesized previously [2]. Based on our analysis we propose that the radical-SAM activity of viperin alters cellular metabolism, which would either directly or indirectly interfere with mitochondrial respiration and can inhibit viral replication in different ways (Figure 3). Future studies should shed molecular insight into this mechanism.

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Author contribution. KHE conceived the study, designed and performed the experiments, and analysed the data; DH assisted in using Seahorse; JR developed HPLC method for measuring nucleotides; WSJ discussed the data; WSJ, JM, and FAA provided space and fund for consumables; KHE wrote the manuscript with contribution from all the authors.

Competing financial interest. None.

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

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Table 1. Comparison of the IC₅₀ values of ddhCTP as viral chain terminator with those reported for some

Compound	Virus	IC ₅₀ values (μM)	ref
ddhCTP	DV	20000	[11]
	WNV	30000	[11]
Sofosbuvir (β-D-2'-deoxy-2'-α-fluoro-2'-β-C-methyluridine)	DV	4.9	[20]
3-dUTP	ZIKA	0.67	[21]
	DV	0.68	[21]
2-F-2-C-Me-UTP	ZIKA	90.76	[21]
	DV	55.13	[21]
2-C-ME-UTP	ZIKA	5.78	[21]
	DV	4.94	[21]
2-C-ethylanyl-UTP	ZIKA	0.46	[21]
	DV	0.33	[21]

known chain terminator nucleotide analogues.

Figure 1

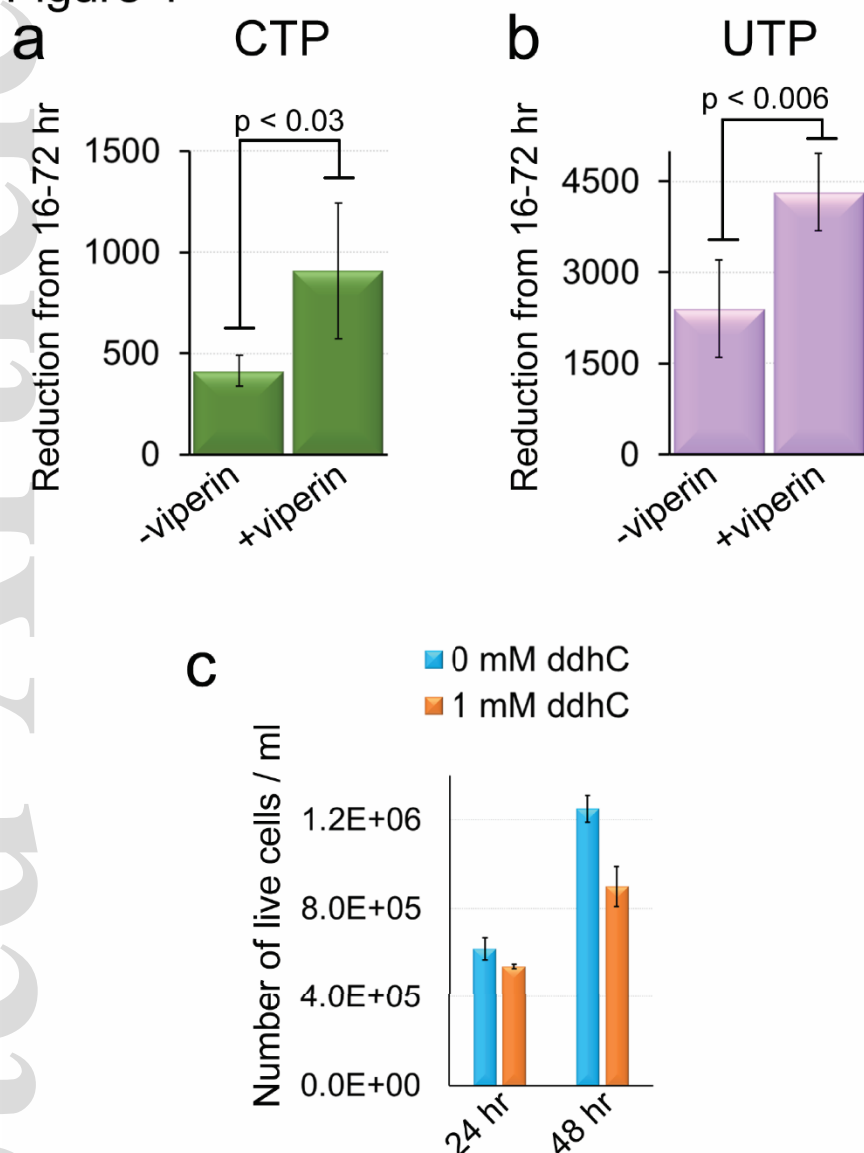


Figure 1. ddhC/ddhCTP are cytotoxic and abolish intracellular concentration of nucleotides. (a-b) Expression of viperin in HEK293 cells abolished cellular concentration of (a) CTP and (b) UTP. Values were obtained as explained in the methods. (c) Addition of ddhC to cultured Vero cells resulted in approximately 25% reduction in the number of live cells. Data are extracted from (Fig. 5b ref.[11]).

Figure 2

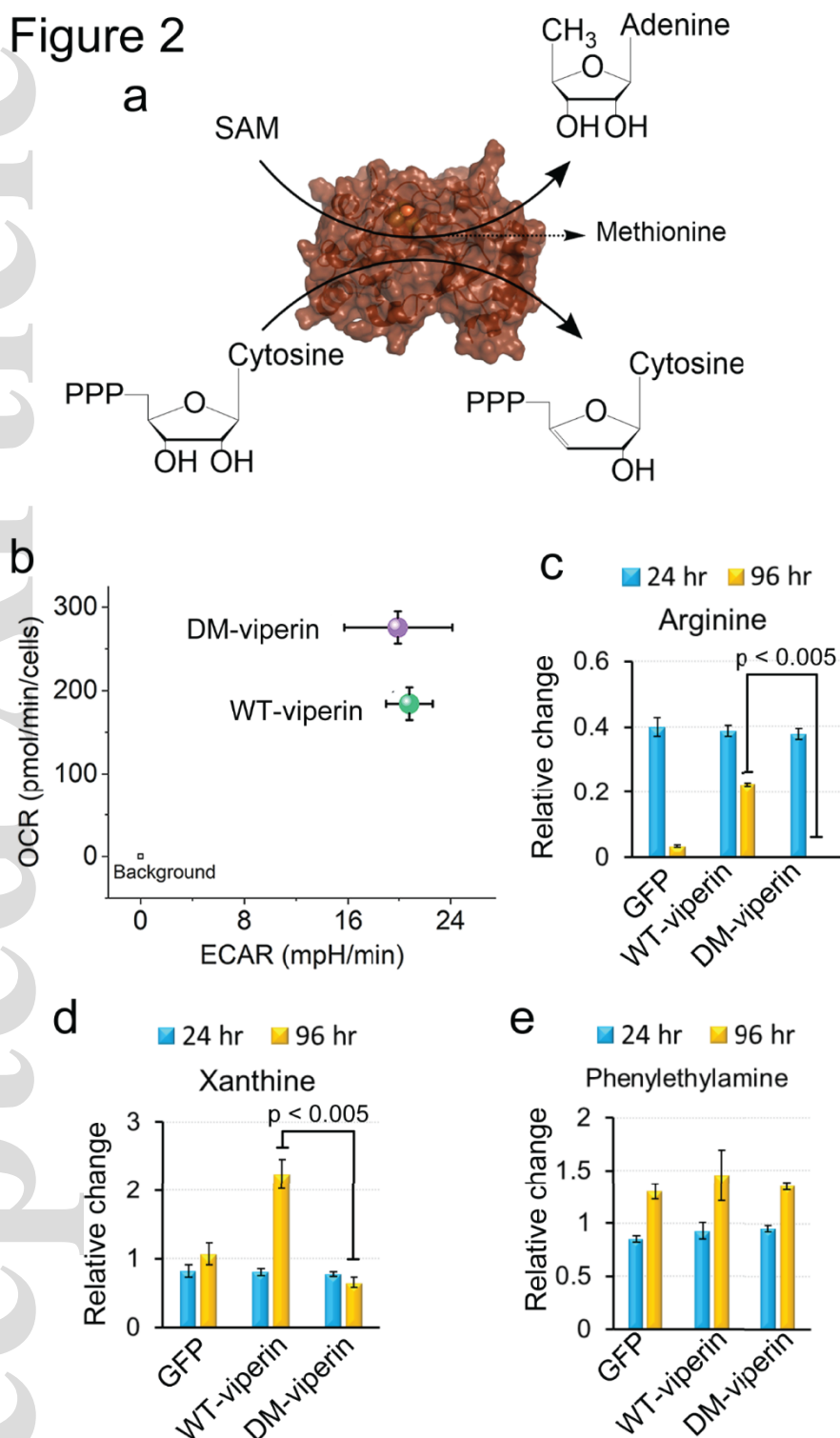


Figure 2. Catalytic activity of viperin reduces mitochondrial respiration. (a) Catalytic activity of viperin generates nucleoside/nucleoside analogues ddhCTP/5'-dA. (b) Cells expressing WT-viperin had a lower dioxygen consumption rate (OCR) as compared to cells expressing DM-viperin, while extracellular acidification rate (ECAR) was not affected. OCR and ECAR were measured using Seahorse™ assay. (c)

High-resolution mass spectrometry was used to measure extracellular levels of arginine $[M+H]^+$ ($m/z = 175.1189$), (d) xanthine $[M-H]^-$ ($m/z = 151.0256$), and (e) phenylethylamine $[M+H]^+$ ($m/z=122.0964$). Metabolites were analysed 24 or 96 hours after transfection of cells with WT-viperin, DM-viperin, or GFP. For each metabolite, the intensity of the LC-MS peak at each time point was plotted relative to that in the fresh growth medium. Data are the average of three independent biological replicates \pm standard deviation. The p-values were obtained from one-way ANOVA analysis.

Figure 3

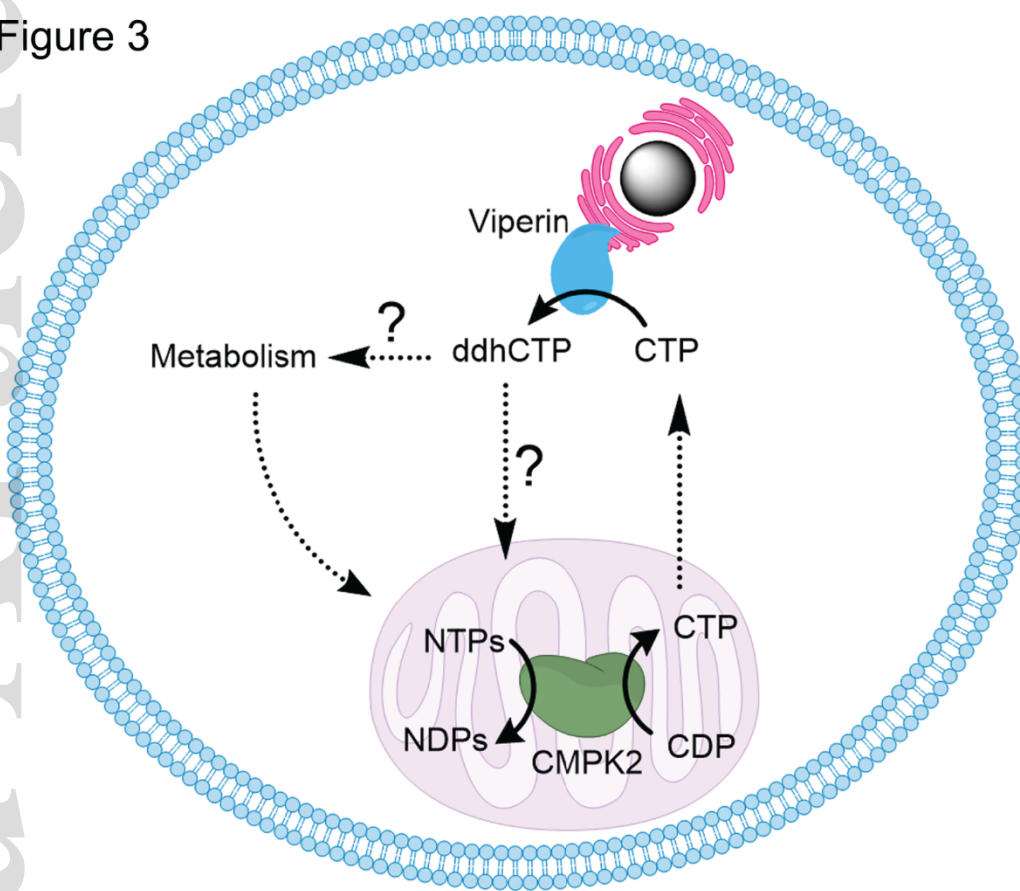


Figure 3. The proposed unifying mechanism of functioning for viperin (RSAD2). The proposed coupled activity of viperin and mitochondrial CMPK2 [11] leads to synthesis of ddhCTP by the radical-SAM activity of viperin. This activity either affects metabolism and thereby somehow mitochondrial activity or it directly interferes with mitochondrial function and activity. Future studies should reveal molecular details of this activity.