




Re-evaluating adenosine-nucleoside polyphosphate levels in human cells by mass spectrometry

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ABSTRACT

Dinucleoside polyphosphates (Np_nNs) are known as alarmones but their functions in cellular metabolism remain largely unexplored till to date. Here, we report new data concerning their cellular quantification using mass spectrometry-based methods. Key for this approach were ¹³C-isotope-labeled internal standards of eight different compounds (¹³C-Ap_nN, n = 3,4; N = Adenosine, Cytidine, Guanosine, Uridine) that were chemically synthesized from ¹³C₅-adenosine. For this, a novel synthesis strategy was developed. These compounds were used to account for losses during the extraction for the determination of intracellular Ap_{3/4}N-levels. Cell samples from two human cell lines, HEK293T and H1299, were measured using a triple quad mass spectrometer (TQ-MS). Additionally, menadione was added to the cell dishes to generate oxidative stress. We were able to reproduce previous findings that all Ap_{3/4}N levels increase in stressed cells. In addition, we showed that cells lacking the Np₃N hydrolase Fhit (fragile histidine triad) (H1299, FHIT-negative) exhibit hundred-fold increased levels of Ap₃Ns but also ten-fold increased levels of Ap₄Ns. This finding contradicts previous data, where no impact of the expression of Fhit on Ap₄N-levels was detected. For FHIT-negative cells, no significant increase in Ap_{3/4}N levels was observed when oxidative stress was applied, suggesting that a change in hydrolase activity could be the primary stress response rather than increased biosynthesis.

1. Introduction

In the complex network of cellular metabolism, still very poorly understood is the role of dinucleotide polyphosphates (Np_nNs, with N = either A, G, C, U, n = number of phosphates) despite the attention they received in the 1970s [1]. Due to the accumulation of dinucleotide polyphosphates during stress, such as oxidative stress and heat shock, these molecules are often referred to as signalling molecules or alarmones [2]. Yet, it remains unclear if this poses the primary stress response or if their overproduction is the by-product of another cellular process [3]. Their chemical structure is closely related to the most essential compounds in cells: adenosine triphosphate (ATP), the cofactors flavin adenine dinucleotide (FAD), and nicotinamide adenine dinucleotide (NAD⁺), the latter also being dinucleotides linked by a phosphate chain (Fig. 1).

The Biosynthesis of dinucleotide polyphosphates has been reported by tRNA synthetases and DNA/RNA ligases, but never as a primary enzymatic product [4,5]. Their degradation, however is catalysed by specific hydrolases, such as the tumour suppressing Fhit (fragile histidine triad) protein for Np₃Ns [6]. Fhit however, has not yet been reported to significantly hydrolyse Np₄Ns and longer phosphate chains *in vivo* [7].

Instead, other enzymes, e.g. the human NudT2 of the nudix-type hydrolases family, catalyse their hydrolysis.

Many findings in the field of Np_nNs rely heavily on data reported several decades ago using quantification methods like TLC and phosphodiesterase-luciferase assays [8]. In this study a mass spectrometry (MS)-based quantification method is applied with internal standards to compensate for losses during the extraction procedure of the Np_nNs from cells [9]. A highly sensitive triple-quadrupole mass spectrometer is used to quantify trace amounts of these compounds that were not accounted for during previous studies.

With this approach, we were able to perform a precise determination of intracellular Ap_{3/4}N levels in HEK293T and H1299 cells and observe a stress response upon oxidative menadione treatment, paving the way for future investigations in this field.

2. Results and discussion

To determine the intracellular levels of Ap_nNs under different conditions using the chosen metabolomic approach, isotope labeled internal standards of these compounds had to be synthesized first. For the synthesis of dinucleoside polyphosphates, several methods have been

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described in the literature. As ^{13}C -adenosine is the only commercially available ^{13}C -labeled nucleoside, an unsymmetrical synthesis was necessary to access all eight labeled $\text{Ap}_{3/4}\text{Ns}$. This is usually achieved by a preactivation of one phosphate group to increase the susceptibility to a nucleophilic attack by another [10]. For this activation, a variety of amines e.g. morpholidine or imidazole can be coupled to the nucleoside phosphate (NMP) using dicyclohexylcarbodiimide (DCC) or carbonyldiimidazole (CDI), giving the respective phosphoramidates of the nucleotides [11,12]. The coupling can be achieved using nucleoside di- or tri-phosphates (NDPs or NTPs) to obtain dinucleoside tri- or tetra-phosphates (Np_3Ns or Np_4Ns) [13]. For the synthesis of the $^{13}\text{C}_5\text{-Ap}_{3/4}\text{Ns}$ presented here, $^{13}\text{C}_5$ -adenosine was first phosphorylated following the protocol from Yoshikawa et al. to get ^{13}C -AMP in a yield of 48 % [14]. Then, the preactivation was carried out with triphenylphosphine, 2, 2'-dithiopyridine (DTDP) and imidazole, following the protocol from Mukayama and Hashimoto [15]. The activated ^{13}C -AMP imidazolite was converted further by a Mg^{2+} -mediated coupling to nucleoside diphosphates (ADP, CDP, GDP, UDP) and nucleoside triphosphates (ATP, CTP, GTP, UTP), resulting in all eight $^{13}\text{C}_5\text{-Ap}_{3/4}\text{Ns}$ in yields listed in Table 1. This procedure ensures that no symmetrical $^{13}\text{C}_{10}\text{-Ap}_3\text{A}$ can be formed, which would not only be a waste of expensive material but would also be hardly separable from $^{13}\text{C}_5\text{-Ap}_3\text{A}$. As high purity of the internal standards has been the main objective, all eight compounds were consecutively purified by anion-exchange medium pressure liquid chromatography (IEX-MPLC) and reverse-phase high performance liquid chromatography (RP-HPLC). Although this procedure reduced the yield, NMR spectra indicate that sufficient purity was achieved successfully.

With all isotope labeled Ap_nNs in hand, the intracellular quantification experiments were performed. For both chosen cell lines, HEK293T and H1299, an extraction protocol has already been established for the extraction of metabolites [16–18]. Of the chosen cell lines, HEK293T is FHIT positive while H1299 is FHIT negative. Thereby, indirect assumptions about the Ap_nN hydrolysing activity of this enzyme can be made by comparing the intracellular levels.

For both cell lines, the extraction protocol of Lorenz et al. was adapted and optimized to fit the behaviour of the metabolites under investigation, the Ap_nNs (Fig. 2) [18]. After growing to 90 % confluence some samples were incubated with menadione for 1 h to induce oxidative stress. A tolerable menadione concentration was chosen according to the literature to avoid cell death [19]. Menadione has been reported to increase Ap_3A and Ap_4A levels in *Salmonella typhimurium* [2]. Besides menadione, H_2O_2 was tested to induce oxidative stress with the same effect on Ap_nN levels (data not shown).

The metabolism of the cells was rapidly stopped by placing the dishes on ice and washing with cold isotonic sodium chloride solution. Then, the internal standards were added and the cells were lysed with methanol/chloroform (9:1) cooled on dry ice. The collected lysate was then purified using a SepPak tC18 column to remove macromolecules. Afterwards the extracted metabolites were analysed by LC-TQMS, determining the intracellular levels of all eight Ap_nNs in biological triplicates and technical duplicates. LC-TQMS consists of a HPLC and three

Table 1

Obtained yields for the synthesized ^{13}C -labeled internal standards following coupling with ^{13}C -AMP imidazolite (2).

Compound	Yield
$^{13}\text{C}_5\text{-Ap}_3\text{A}$ (3) ²	15 %, 23.3 μmol
$^{13}\text{C}_5\text{-Ap}_3\text{C}$ (4)	9 %, 13.6 μmol
$^{13}\text{C}_5\text{-Ap}_3\text{G}$ (5)	10 %, 15.4 μmol
$^{13}\text{C}_5\text{-Ap}_3\text{U}$ (6)	15 %, 21.7 μmol
$^{13}\text{C}_5\text{-Ap}_4\text{A}$ (7)	16 %, 24.2 μmol
$^{13}\text{C}_5\text{-Ap}_4\text{C}$ (8)	16 %, 24.2 μmol
$^{13}\text{C}_5\text{-Ap}_4\text{G}$ (9)	6 %, 8.4 μmol
$^{13}\text{C}_5\text{-Ap}_4\text{U}$ (10)	13 %, 20.1 μmol

quadruples. The first quadrupole (Q1) functions as a mass filter, selecting ions based on their mass-to-charge ratio (m/z). For this analysis, the exact mass of each compound was used. Only ions with the specified m/z values can pass into the second quadrupole (Q2), where fragmentation occurs. Each compound was fragmented using specific collision energies and the most prominent fragments were identified. The third quadrupole (Q3) also acts as a mass filter, analysing the final fragments [20,21].

In order to assess the extent to which the oxidative damage induced by the menadione treatment damages the Np_nNs themselves, the necessary control experiments were carried out. For this, the concentration of the internal standards in cell free medium was determined before and after menadione treatment. In Fig. 3 the percentage of loss of compound during this procedure is shown. A deviation above 10 % in comparison to the untreated control is only evident for $\text{Ap}_{3/4}\text{A}$ or $\text{Ap}_{3/4}\text{G}$, probably due to the potential formation of 8-oxo-guanosine or 8-oxo-adenosine [22].

The intracellular levels of Ap_nNs were then calculated using a calibration curve. It was constructed prior to every experiment using solutions of the internal standards with seven different concentrations and fitting to a linear model. During the cell workup, a variable quantity of metabolites was lost. To assess the extent of this loss, a specific amount of each internal standard was added to each sample. Following the measurement of the calibration curve and the sample, the loss incurred during the extraction and workup was calculated.

To ensure statistical robustness, all experiments were conducted using biological triplicates ($n = 3$), where each replicate consisted of a separate petri dish treated identically within a given experiment. Technical duplicates were measured for each petri dish to account for instrumental variability. The complete experimental procedure including biological triplicates and technical duplicates was itself repeated eight times for HEK293T cells. For H1299 cells, three repetitions of this experimental procedure were performed.

The well-documented effect, that cellular stress increases Ap_nN levels in cells, among others HEK293T, was successfully reproduced for all Ap_nNs (Fig. 4) (see Fig. 5).

The most significant increase was observed for Ap_3C , with almost no detectable baseline signal in cells without oxidative stress. The observed stress response is potentially even more pronounced considering the oxidative degradation of the compounds and the resulting loss of signal.

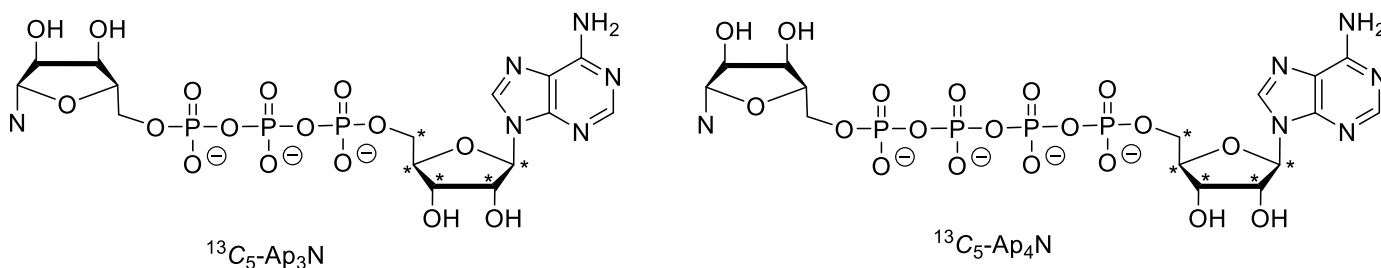


Fig. 1. Chemical structure of the Ap_3Ns and Ap_4Ns under investigation in this study ($N = \text{A, C, G, U}$). Depicted are the ^{13}C -labeled homologues (carbon isotopes are marked by *), which were used as internal standards for the quantification of the intracellular $\text{Ap}_{3/4}\text{N}$ levels.

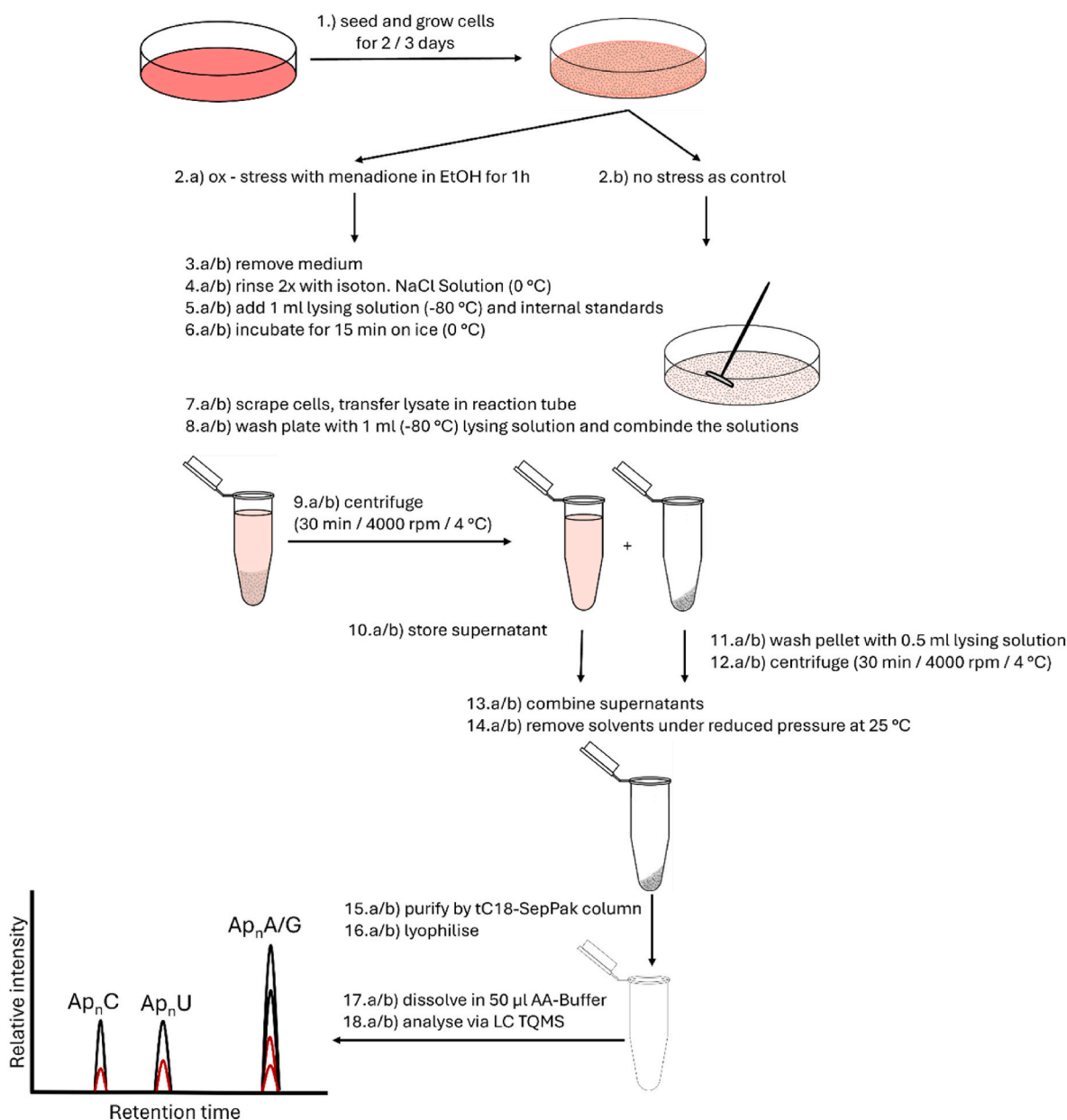


Fig. 2. The extraction protocol for Ap_nNs from HEK293T and H1299 cells established in this study. Menadione as a source of reactive oxygen species (ROS) is applied in step 2. The internal standards are added after removal of the medium and undergo the extraction process as well to account for losses.

Generally, the detected levels of Ap₃Ns were much lower than those of Ap₄Ns, with Ap₄A being the overall most abundant.

In contrast, the cell line H1299 lacking the hydrolase Fhit showed no increase in the intracellular Ap_nN levels after menadione treatment (Fig. 4). The observed reduction in concentration levels, especially for Ap₃A and Ap₄A is most likely attributed to the oxidative damage to these compounds caused by menadione that is visible in Fig. 3. Unlike in HEK293T cells, the concentration of Ap₃Ns was tenfold higher than for the Ap₄Ns, and almost 1000-fold higher than in HEK293T cells. This demonstrates the accumulation of these compounds in the absence of the hydrolase Fhit. Not only were the Ap₃N levels increased, but also Ap₄N concentrations. This could indicate that Fhit is able to hydrolyse Np₄Ns *in vivo*, albeit not as efficient. Maybe this effect is diminished by other hydrolases compensating the absence of Fhit, like NudT2 that specifically degrade Np₄Ns.

As no stress response in form of increased Ap_nN levels could be detected in the FHIT negative cell line H1299, it seems reasonable that a change in hydrolase activity might be the primary stress response.

To further evaluate the quality of the data reported in this study, a comparison with previously reported dinucleoside polyphosphate levels is necessary (Table 3). Due to the limited data published for human cell lines, only Ap₃A and Ap₄A levels are considered (see Table 4).

The overall higher concentrations reported in this study suggest that the developed combination of internal standards together with an optimized extraction protocol can be successfully applied for the determination of intracellular Ap_nN levels. While A. G. McLennan could only detect very low levels of 0.079 picomol of Ap₃A per million HEK293T cells in the presence of the Fhit hydrolase, 0.38 picomol per million cells are reported in this study. In FHIT negative cells McLennan reports increased levels of up to 0.91 picomol per million cells compared to 214.6 picomol per million cells detected for H1299 cells in this study. The fact that McLennan could not observe a significant increase in Ap₄A levels in cell lines lacking the FHIT hydrolase compared to this study could result from the overall lower recovery of dinucleoside polyphosphates from the lysate.

Although this study provides new intracellular Ap_nN quantification

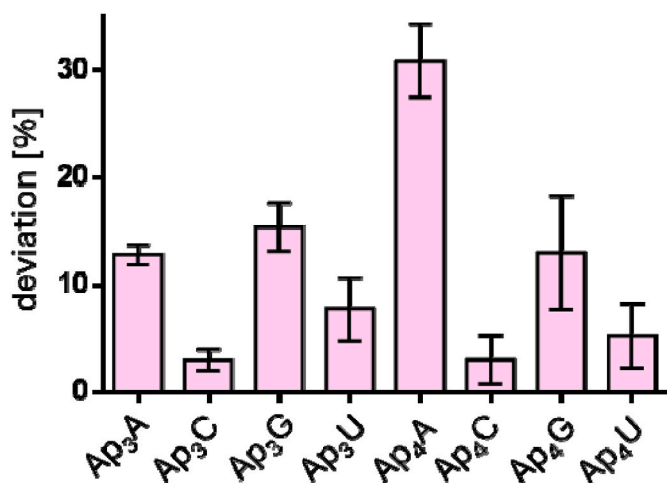


Fig. 3. Loss of Ap_nNs through oxidation by menadione in a cell free medium. Solutions of the internal standards in the same concentration that were not exposed to menadione are used as reference. The deviation between the two samples is the highest for Ap_{3/4}A and Ap_{3/4}G, with over 10 % up to almost 30 % for Ap₄A.

Table 2

The intracellular Ap_{3/4}N levels determined by MS/MS using two cell lines and menadione to induce oxidative stress. The concentration is given in picomol per million cells.

	Ap ₃ A	Ap ₃ C	Ap ₃ G	Ap ₃ U
HEK293T	0.38 ± 0.04	0.04 ± 0.01	0.04 ± 0.01	0.16 ± 0.01
HEK293T stressed	0.74 ± 0.03	0.22 ± 0.03	0.22 ± 0.03	0.35 ± 0.03
H1299	214.6 ± 23.25	23.31 ± 11.86	7.79 ± 0.49	49.69 ± 1.87
H1299 stressed	116.4 ± 10.11	8.11 ± 0.67	3.47 ± 0.37	24.571 ± 3.68
	Ap ₄ A	Ap ₄ C	Ap ₄ G	Ap ₄ U
HEK293T	1.91 ± 0.19	0.08 ± 0.05	0.49 ± 0.09	1.05 ± 0.12
HEK293T stressed	2.75 ± 0.14	0.35 ± 0.07	0.82 ± 0.11	1.29 ± 0.13
H1299	12.94 ± 0.42	3.33 ± 0.37	4.44 ± 0.26	12.26 ± 0.57
H1299 stressed	8.62 ± 0.99	2.14 ± 0.17	2.49 ± 0.14	6.80 ± 0.50

HEK 293: Human embryonic kidney cell line; H1299: human non-small cell lung carcinoma cell line.

HEK293

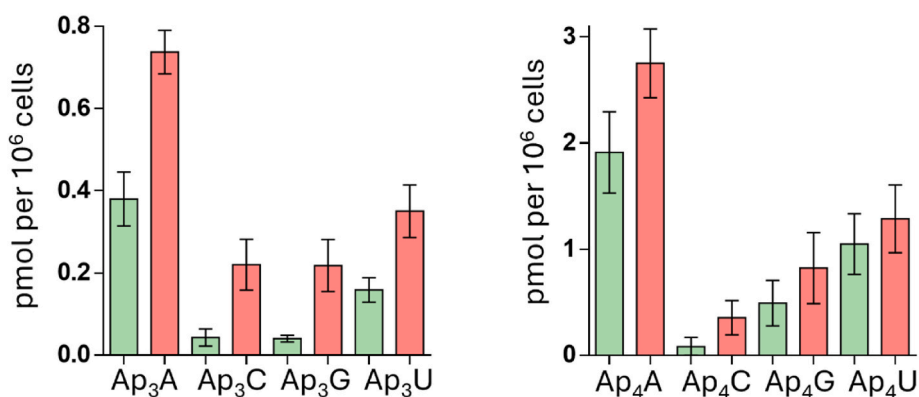


Fig. 4. Determined Ap_nN levels in HEK293T cells in unstressed (green) and stressed conditions (red). Oxidative stress was induced by incubation with menadione. For all compounds, the intracellular concentration increased with stress. The determined concentrations are given in picomol per million cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

H1299

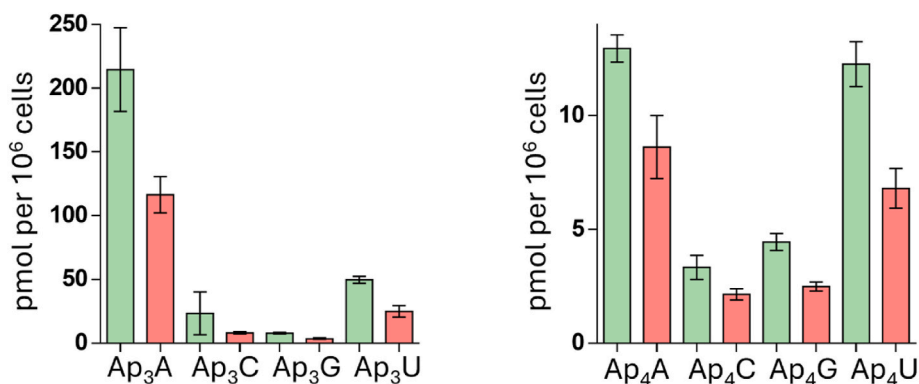


Fig. 5. Determined Ap_nN levels in FHIT negative H1299 cells in unstressed (green) and stressed conditions (red). Oxidative stress was induced by incubation with menadione. For all compounds, the intracellular concentration was increased compared to the FHIT positive cell line HEK293T supporting hydrolase activity of Fhit for Ap₃Ns as well as – to some extent – for Ap₄Ns. The determined concentrations are given in picomol per million cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 3

Comparison of intracellular Ap_{3/4}A levels reported in the literature. The cell line and the method by which the data was measured is listed as well. The concentration is given in picomol per million cells.

Reference	Cell line	Ap ₃ A	Ap ₄ A
A.G. McLennan, 2000 [7] (Luciferin/Luciferase assay, Luminometry)	HEK293	Not detected	0.16 ± 0.01
	HRT18	0.071 ± 0.006	0.082 ± 0.012
	RT112 (FHIT negative)	0.23 ± 0.08	0.095 ± 0.016
	2780 (FHIT negative)	0.91 ± 0.14	1.12 ± 0.02
	HEK293	0.079 ± 0.012	0.500 ± 0.037
A.G. McLennan, 2008 [23] (Luminometry)	(apoptotic stress)	0.510 ± 0.044	0.776 ± 0.134
	HL-60	4.8	0.7
L. Kisselev, 1997 [24] (Scintillation)	(induced apoptosis)	1.6	2.7
	EU1 leukemic cells	–	4.0
A. Ogilvie, 1981 [25], 1983 [26] (Luminometry)	Human platelets	0.66 ± 0.28	0.46 ± 0.1
	HTC	–	0.5 ± 0.075
P. Remy, 1987 [27] (Luminometry)	A549 lung carcinoma	–	0.4 ± 0.08
	Fibroblasts	–	0.32 ± 0.024
B. Ames, 1988 [28] (Luminometry)	(induced DNA damage)	–	2.89 ± 0.015
	HL-60	–	0.39 ± 0.024
H. Bergstrand, 1990 [29] (Luminometry)	(PMA treatment)	–	0.67 ± 0.015
	HEK293T	0.38 ± 0.04	1.91 ± 0.19
A. Marx, 2025 (LC-MS/MS)	(stressed menadione)	0.74 ± 0.03	2.75 ± 0.14
	H1299 (FHIT negative)	214.6 ± 23.25	12.94 ± 0.42

Table 4

Amount of seeded cells in 10 mL medium for the respective days of grow time.

	H1299	HEK293T
2 days	1.2 × 10 ⁶	2.4 × 10 ⁶
3 days	5.5 × 10 ⁶	1.2 × 10 ⁶

HEK 293T: Human embryonic kidney cell line; H1299: human non-small cell lung carcinoma cell line.

data with a high potential for improved accuracy due to the use of mass spectrometry and higher analyte recovery compared to previous publications, there are still some limitations regarding the scope of these findings. The analysed human cell lines HEK293T and H1299 are not representative of human tissue in general and a variation in the Ap_nN levels can be expected for other cell lines. However, previous studies report relatively small deviations in these levels for a broad range of cell lines [7]. In Addition, the significantly higher Ap_nN levels in H1299 cells compared to HEK293T cells could result from various factors apart from the lack of the Fhit hydrolase.

While many different dinucleoside polyphosphates are present in human cells, Ap_{3/4}Ns represent the most common subgroup with Ap₄A being the most abundant. The Ap_{3/4}Ns levels precisely determined in this study are therefore a reasonable basis for an overall re-evaluation of dinucleoside polyphosphate levels using the developed protocol.

3. Summary

In this work, the synthesis of eight isotope-labeled standards for the

quantification of intracellular Ap_{3/4}N levels is reported. A novel synthesis strategy towards asymmetric ¹³C-isotope labeled Ap_{3/4}Ns was developed. The synthesis involved three steps starting with phosphorylation of ¹³C-adenosine (48 %), preactivation of the resulting ¹³C-AMP (43 %) and coupling with the respective di- or tri-nucleotides, resulting in the successful preparation of all eight compounds in yields between 6 and 16 %, demonstrating the robustness and flexibility of the synthesis strategy (Table 1).

Adding to this, a sensitive method for the quantification of intracellular Ap_{3/4}N levels using the synthesized ¹³C-labeled internal standards was developed. Therefore, a comprehensive protocol for cell extraction and LC-TQMS analysis was established, allowing precise quantification of Ap_{3/4}Ns even in the presence of higher concentrations of mononucleotides.

The impact of oxidative stress on intracellular Ap_{3/4}N levels was measured in two different cell lines. A significant increase on intracellular concentrations was observed for all Ap_{3/4}Ns in FHIT-negative cells compared to FHIT-positive cells. The implication that Fhit is able to accept and hydrolyse Np₄Ns *in vivo* needs to be further investigated by directly measuring the enzyme activity. Oxidative stress treatment in FHIT negative cells (H1299) had no significant influence on Ap_{3/4}N levels, while in FHIT positive cells (HEK293T) an increase of all Ap_{3/4}N levels was observed. This could indicate, that a decrease in Fhit hydrolyse activity is the main stress response, and not an increased biosynthesis. All measured concentrations are summarized in Table 2.

4. Materials and methods

General procedures for chemical synthesis and chromatography are given in the supporting information.

4.1. Synthesis of ¹³C₅-adenosine-5'-monophosphate (1)

To a solution of ¹³C₅-adenosine (100 mg, 0.37 mmol, 1 eq., obtained from Eurisotop, Saarbrücken, Germany) in trimethylphosphate (2.0 mL) freshly distilled POCl₃ (0.04 mL, 86 mg, 0.44 mmol, 1.2 eq.) was added. The reaction was stirred for 2 h at 0 °C. By adding TEAB buffer (0.1 M, 5 mL) the reaction was stopped. The solution was washed with ethyl acetate (2 × 20 mL). The aqueous phase was evaporated under reduced pressure. The crude product was freeze dried and purified via IEX-MPLC. After freeze drying the desired ¹³C₅-Adenosine monophosphate (¹³C₅-AMP, 1) was obtained as triethyl ammonium salt in a yield of 48 % (0.18 mmol).

¹H NMR (400 MHz, D₂O) δ = 8.48 (s, 1H), 8.14 (s, 1H), 6.07 (d, *J* = 165.9 Hz, 1H), 4.79 (d, *J* = 101.4 Hz, 1H), 4.55 (s, 1H), 4.25 (s, 1H), 4.24 (d, *J* = 51.25 Hz, 1H), 3.88 (s, 1H), 3.23–3.10 (m, 6H), 1.28–1.18 (m, 9H).

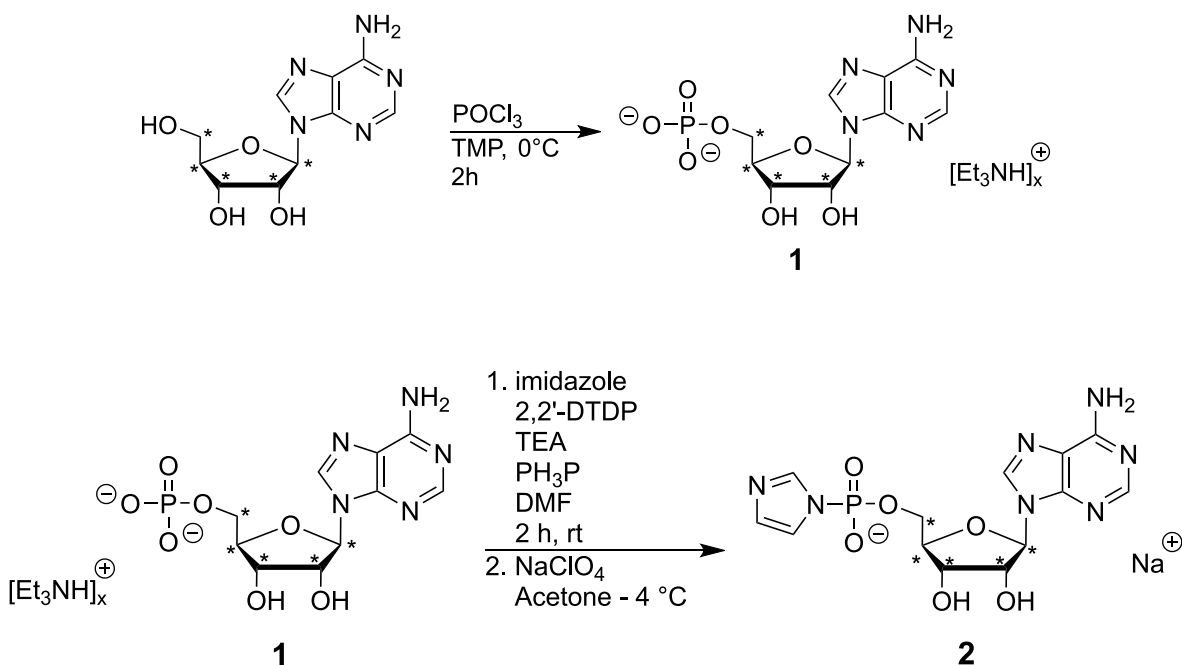
¹³C NMR (100 MHz, D₂O) δ = 155.3, 152.6, 148.8, 139.9, 118.3, 86.9 (dd, *J* = 42.7, 3.6 Hz), 84.3 (ddd, *J* = 42.7, 38.4, 8.7 Hz), 74.4 (dd, *J* = 42.7, 37.7 Hz), 70.5 (td, *J* = 38.1, 3.5 Hz), 64.0 (dd, *J* = 42.7, 4.8 Hz), 46.6, 8.2.

³¹P NMR (162 MHz, D₂O) δ = 1.80 (s).

HR-ESI-MS *m/z* calc. for ¹²C₅¹³C₅H₁₃N₅O₇P [M – H] 351.0723, found 351.0724.

4.2. Synthesis of ¹³C₅-adenosine-5'-phosphorimidazolide (2)

¹³C₅-AMP (1) (63 mg, 0.18 mmol, 1 eq.) was dissolved in dry dimethylformamide (DMF) (1.7 mL) followed by addition of dry triethylamine (TEA) (75.0 μL, 55 mg, 0.54 mmol, 3 eq.), imidazole (123 mg, 1.8 mmol, 10 eq.), 2,2'-dipyridyldisulfide (DTDP) (119 mg, 0.54 mmol, 3 eq.) and triphenylphosphine (PPh₃) (141.6 mg, 0.54 mmol, 3 eq.). The reaction mixture was stirred for 2 h at room temperature. A solution of NaClO₄ monohydrate (3 eq., 75 mg) in cold acetone (15 mL) was added to the reaction mixture. The suspension was centrifuged for 5 min with



5000 rpm. The precipitate was washed with cold acetone (3×15 mL) until the solution was clear. The yellow precipitate was dried under reduced pressure to obtain a yellow powder. Yield: 43 % (77.6 μmol).

^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ = 8.40 (s, 1H), 8.13 (s, 1H), 7.61 (d, J = 1.2 Hz, 1H), 7.27 (s, 2H), 7.07 (q, J = 1.3 Hz, 1H), 6.84 (t, J = 1.3 Hz, 1H), 6.92 (d, J = 162.0 Hz, 1H), 5.35 (d, J = 70.4 Hz, 1H), 4.57 (d, J = 146.5 Hz, 1H), 4.16 (d, J = 34.9 Hz, 1H), 2.08 (s, 2H).

No further characterisation was performed as the compound shows limited stability.

4.3. Synthesis of $^{13}\text{C}_5$ -dinucleosid triphosphates (3–6)

All Ap_3N 's were synthesized using the following protocol. Commercially available nucleosidediphosphates (A, C, G, U) as disodiumsalts were converted to triethyl ammonium salts prior to the reaction. This was done using IEX-MPLC and triethylammonium bicarbonate buffer (1 M). Then, the triethylammonium salts of the corresponding diphosphate nucleosides (4 eq.) were dissolved in formamide (1 mL) and MgCl_2 (8 eq.) was added. This mixture was stirred for 10 min at room temperature. Afterwards, $^{13}\text{C}_5$ -adenosine-5'-(imidazole) phosphate (2) (25 mg, 0.038 mmol, 1 eq.) was added and the mixture was stirred for 2 days. During this time reaction monitoring via HPLC was performed. When the reaction showed full conversion, the reaction was stopped by adding water (5 mL). The crude product was purified via IEX-MPLC and RP-HPLC. Fractions containing the product were pooled concentrated under reduced pressure and freeze-dried resulting in the

desired $^{13}\text{C}_5$ -dinucleoside triphosphates as white powders.

4.3.1. Data for $^{13}\text{C}_5$ - Ap_3A (3)

^1H NMR (400 MHz, D_2O) δ = 8.30 (s, 2H), 8.11 (s, 2H), 6.00 (d, J = 4.7 Hz, 1H), 6.02 (d, J = 167.6 Hz, 1H), 4.60 (t, J = 4.8 Hz, 1H), 4.58 (d, J = 58.62 Hz, 1H), 4.48 (t, J = 4.7 Hz, 2H), 4.34 (s, 1H), 4.29 (s, 2H), 4.26 (d, J = 483.6 Hz, 1H), 4.29 (s, 2H), 1.96 (s, 4H).

^{13}C NMR (100 MHz, D_2O) δ = 87.31 (d, J = 42.4 Hz), 83.25 (d, J = 41.8 Hz), 74.90 (d, J = 41.8 Hz), 69.75 (t, J = 38.3 Hz), 64.77 (d, J = 40.4 Hz).

^{31}P NMR (162 MHz, D_2O) δ = -11.58 (s, 2P), -23.11 (s, 1P).

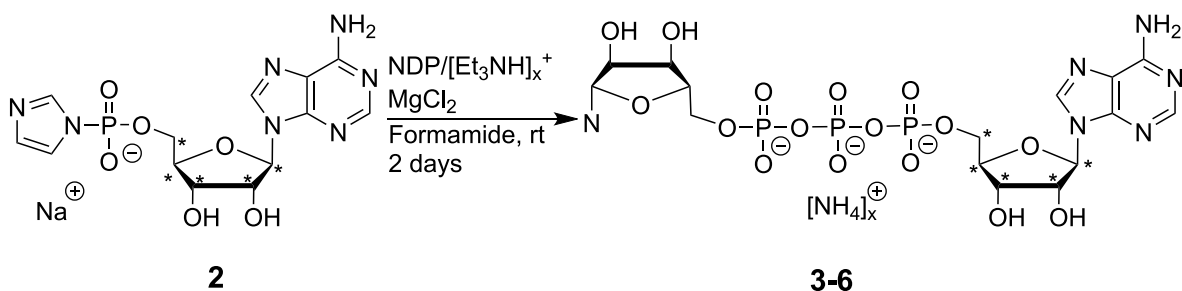
HR-ESI-MS m/z calc. for $^{12}\text{C}_{15}^{13}\text{C}_5\text{H}_{26}\text{N}_{10}\text{O}_{16}\text{P}_3$ [$\text{M} - \text{H}$] 760.0912, found 760.0905.

4.3.2. Data for $^{13}\text{C}_5$ - Ap_3C (4)

^1H NMR (500 MHz, D_2O) δ = 8.52 (s, 1H), 8.26 (s, 1H), 7.96 (d, J = 7.8 Hz, 1H), 6.13 (d, J = 165.8 Hz, 1H), 6.05 (d, J = 7.8 Hz, 1H), 5.83 (d, J = 3.7 Hz, 1H), 4.89 (s, 1H), 4.82 (s, 1H), 4.64 (d, J = 52.3 Hz, 1H), 4.48 (d, J = 76.6 Hz, 1H), 4.42 (s, 1H), 4.27–4.21 (m, 4H), 4.13 (s, 1H), 2.03 (s, 8H).

^{13}C NMR (126 MHz, D_2O) δ = 178.51, 161.77, 153.90, 152.06, 150.70, 148.81, 142.60, 140.50, 118.96, 95.55, 89.45, 87.06 (dd, J = 42.6, 3.3 Hz), 84.01 (ddd, J = 42.4, 38.4, 9.0 Hz), 82.76 (d, J = 9.3 Hz), 74.67 (dd, J = 37.0, 5.9 Hz), 70.48 (td, J = 38.1, 3.4 Hz), 68.88, 65.34 (dd, J = 42.6, 5.4 Hz), 64.60 (d, J = 5.0 Hz).

^{31}P NMR (202 MHz, D_2O) δ = -10.86 (s, 2P), -22.27 (s, 1P).



HR-ESI-MS m/z calc. for $^{12}\text{C}_{14}^{13}\text{C}_5\text{H}_{26}\text{N}_8\text{O}_{17}\text{P}_3$ [M - H] 736.0799, found 736.0800.

4.3.3. Data for $^{13}\text{C}_5\text{-Ap}_3\text{G}$ (5)

^1H NMR (500 MHz, D_2O) δ = 8.37 (s, 1H), 8.16 (s, 1H), 7.98 (s, 1H), 5.96 (d, J = 165.8 Hz, 1H), 5.79 (d, J = 5.2 Hz, 1H), 4.82 (s, 1H), 4.76 (s, 1H), 4.63 (t, J = 5.1 Hz, 1H), 4.56 (d, J = 75.4 Hz, 1H), 4.47 (t, J = 4.1 Hz, 2H), 4.30 (s, 1H), 4.27 (d, J = 75.3 Hz, 1H), 4.28 (d, J = 5.0 Hz, 2H), 4.16 (s, 1H), 3.53 (q, J = 7.2 Hz, 3H), 3.20 (q, J = 7.3 Hz, 3H), 2.01 (s, 2H), 1.33 (t, J = 7.2 Hz, 4H), 1.28 (t, J = 7.3 Hz, 4H).

^{13}C NMR (126 MHz, D_2O) δ = 158.15, 153.81, 153.63, 151.11, 150.88, 148.26, 139.95, 137.02, 118.01, 115.52, 87.64 (dd, J = 42.4, 3.4 Hz), 87.11, 83.22 (ddd, J = 42.9, 38.8, 9.0 Hz), 74.78 (dd, J = 42.4, 38.1 Hz), 74.19, 69.63 (td, J = 38.5, 3.4 Hz), 64.78 (dd, J = 42.9, 5.4 Hz), 58.65, 46.71, 21.96.

^{31}P NMR (202 MHz, D_2O) δ = -10.96 (s, 2P), -22.35 (s, 1P).

HR-ESI-MS m/z calc. for $^{12}\text{C}_{15}^{13}\text{C}_5\text{H}_{26}\text{N}_{10}\text{O}_{17}\text{P}_3$ [M - H] 776.0861, found 776.0863.

4.3.4. Data for $^{13}\text{C}_5\text{-Ap}_3\text{U}$ (6)

^1H NMR (500 MHz, D_2O) δ = 8.52 (s, 1H), 8.23 (s, 1H), 7.70 (d, J = 7.8 Hz, 1H), 6.10 (d, J = 164.7 Hz, 1H), 5.93 (d, J = 5.8 Hz, 1H), 5.73 (d, J = 8.4 Hz, 1H), 4.79 (d, J = 18.9 Hz, 1H), 4.78 (d, J = 102.3 Hz, 1H), 4.56 (d, J = 35.3 Hz, 1H), 4.39 (s, 1H), 4.31 (t, J = 5.0 Hz, 1H), 4.26 (t, J = 5.0 Hz, 1H), 4.22 (ddd, J = 4.7, 2.9, 1.2 Hz, 2H), 4.19 (dq, J = 5.4, 2.8 Hz, 1H), 4.10 (s, 1H).

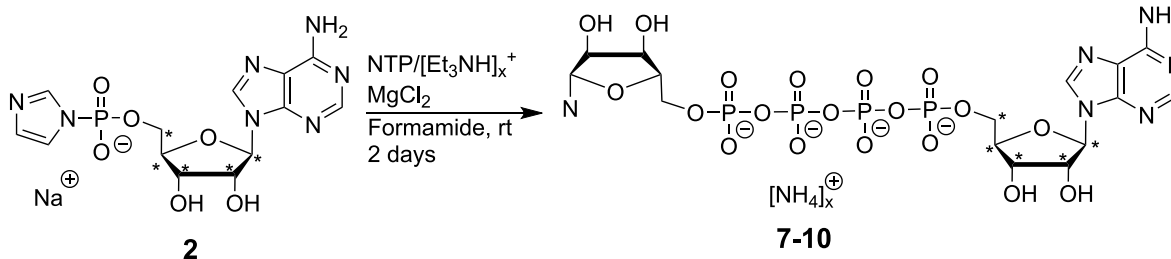
^{13}C NMR (126 MHz, D_2O) δ = 173.56, 157.15, 155.43, 152.90, 149.14, 140.26, 139.78, 118.66, 102.92, 88.44, 86.88 (d, J = 3.3 Hz), 84.04 (ddd, J = 42.6, 38.4, 9.1 Hz), 82.64, 74.58 (dd, J = 43.0, 37.7 Hz), 70.51 (td, J = 38.1, 3.4 Hz), 69.46, 65.29 (dd, J = 42.6, 5.5 Hz), 59.03, 46.53, 8.45.

^{31}P NMR (202 MHz, D_2O) δ = -10.98 (s, 2P), -22.61 (s, 1P).

HR-ESI-MS m/z calc. for $^{12}\text{C}_{14}^{13}\text{C}_5\text{H}_{25}\text{N}_7\text{O}_{18}\text{P}_3$ [M - H] 737.0639, found 737.0636.

4.4. Synthesis of $^{13}\text{C}_5$ -dinucleoside tetraphosphates (7–10)

All of the Ap_4N 's were synthesized using the described procedure. Commercially available triphosphates of the nucleosides (A, C, G, U) as sodium salts were resalted as triethyl ammonium salts using IEX-MPLC. The triethylammonium salts of the corresponding triphosphate nucleoside (4 eq.) were then dissolved in formamide (1 mL) with MgCl_2 (8 eq.) and the mixture was stirred for 10 min at room temperature. Afterwards, $^{13}\text{C}_5$ -adenosine-5'-phosphorimidazolide (**2**) (25 mg, 0.038 mmol, 1 eq.) was added and the mixture was stirred for 2 days. During this time reaction monitoring via HPLC was performed. When the reaction showed full conversion, the reaction was stopped by adding water (5 mL). The crude product was purified via IEX-MPLC and RP-HPLC. Fractions containing the product were pooled, concentrated under reduced pressure and freeze-dried resulting in the desired $^{13}\text{C}_5$ -dinucleoside tetraphosphates as a white powder.



4.4.1. Data for $^{13}\text{C}_5\text{-Ap}_4\text{A}$ (7)

^1H NMR (500 MHz, D_2O) δ = 8.45 (s, 2H), 8.14 (s, 1H), 5.98 (d, J = 5.3 Hz, 1H), 5.96 (d, J = 165.4 Hz, 1H), 4.78 (d, J = 31.6 Hz, 1H), 4.62 (t, J = 5.2 Hz, 1H), 4.52 (t, J = 4.5 Hz, 1H), 4.46 (s, 1H), 4.45 (d, J = 216.3, 1H), 4.40–4.26 (m, 3H), 4.16 (s, 1H), 3.51 (q, J = 7.2 Hz, 1H), 3.18 (q, J = 7.5 Hz, 1H), 1.98 (s, 8H).

^{13}C NMR (126 MHz, D_2O) δ = 153.15, 150.31, 148.10, 140.41, 87.22 (dd, J = 42.5, 3.3 Hz), 83.74 (ddd, J = 42.5, 38.4, 9.0 Hz), 74.82 (dd, J = 42.5, 37.8 Hz), 70.14 (td, J = 38.2, 3.3 Hz), 65.28 (dd, J = 42.7, 5.3 Hz), 22.15.

^{31}P NMR (202 MHz, D_2O) δ = -10.62 (s, 2P), -22.11 (s, 2P).

HR-ESI-MS m/z calc. for $^{12}\text{C}_{15}^{13}\text{C}_5\text{H}_{27}\text{N}_{10}\text{O}_{19}\text{P}_4$ [M - H] 840.0575, found 840.0590.

4.4.2. Data for $^{13}\text{C}_5\text{-Ap}_4\text{C}$ (8)

^1H NMR (500 MHz, D_2O) δ = 8.40 (s, 1H), 8.11 (s, 1H), 7.79 (d, J = 7.7 Hz, 1H), 6.00 (d, J = 168.4 Hz, 1H), 5.93 (d, J = 7.6 Hz, 1H), 5.77 (d, J = 4.2 Hz, 1H), 4.79 (s, 1H), 4.66 (d, J = 40.5 Hz, 1H), 4.46 (d, J = 34.9 Hz, 1H), 4.30 (s, 1H), 4.22 (t, J = 5.1 Hz, 1H), 4.20–4.09 (m, 4H), 4.00 (s, 1H), 3.07 (q, J = 7.3 Hz, 2H), 1.88 (s, 8H), 1.15 (t, J = 7.3 Hz, 3H).

^{13}C NMR (126 MHz, D_2O) δ = 180.35, 164.01, 154.89, 152.00, 141.95, 140.15, 96.14, 89.22, 86.82 (dd, J = 42.8, 3.3 Hz), 82.86 (d, J = 9.1 Hz), 74.48 (dd, J = 42.9, 37.7 Hz), 70.49 (td, J = 38.0, 3.3 Hz), 69.21, 65.38 (dd, J = 42.6, 5.5 Hz), 46.71, 22.60, 8.28.

^{31}P NMR (202 MHz, D_2O) δ = -10.69 (s, 2P), -22.48 (s, 2P).

HR-ESI-MS m/z calc. for $^{12}\text{C}_{14}^{13}\text{C}_5\text{H}_{27}\text{N}_8\text{O}_{20}\text{P}_4$ [M - H] 816.0463, found 816.0467.

4.4.3. Data for $^{13}\text{C}_5\text{-Ap}_4\text{G}$ (9)

^1H NMR (500 MHz, D_2O) δ = 8.49 (s, 1H), 8.26 (s, 1H), 8.08 (s, 1H), 6.06 (d, J = 167.5 Hz, 1H), 5.79 (d, J = 5.7 Hz, 1H), 4.91 (s, 1H), 4.82 (s, 1H), 4.76 (s, 1H), 4.72 (d, J = 8.0 Hz, 1H), 4.68 (t, J = 5.4 Hz, 1H), 4.58–4.50 (m, 1H), 4.47 (s, 1H), 4.33 (dt, J = 5.0, 2.5 Hz, 1H), 4.30–4.22 (m, 2H), 4.17 (s, 1H), 2.04 (s, 2H).

^{13}C NMR (126 MHz, D_2O) δ = 179.60, 158.17, 154.00, 153.60, 151.21, 148.26, 139.85, 137.01, 118.07, 87.62 (dd, J = 42.3, 3.3 Hz), 87.13, 83.35 (dd, J = 37.9, 8.1 Hz), 74.76 (dd, J = 42.4, 38.0 Hz), 74.17, 69.63 (td, J = 38.4, 3.4 Hz), 64.79 (dd, J = 42.7, 5.3 Hz), 58.67, 46.71, 22.15.

^{31}P NMR (202 MHz, D_2O) δ = -10.78 (s, 2P), -22.09 (s, 2P).

HR-ESI-MS m/z calc. for $^{12}\text{C}_{15}^{13}\text{C}_5\text{H}_{27}\text{N}_{10}\text{O}_{20}\text{P}_4$ [M - H] 856.0524, found 865.0524.

4.4.4. Data for $^{13}\text{C}_5\text{-Ap}_4\text{U}$ (10)

^1H NMR (500 MHz, D_2O) δ = 8.56 (s, 1H), 8.28 (s, 1H), 7.88 (d, J = 8.1 Hz, 1H), 6.11 (dt, J = 166.9, 4.6 Hz, 1H), 5.92 (d, J = 5.2 Hz, 1H), 5.86 (d, J = 8.1 Hz, 1H), 4.93 (s, 1H), 4.79 (d, J = 30.4 Hz), 4.58 (d, J = 29.6 Hz, 1H), 4.43 (s, 1H), 4.39 (dd, J = 5.3, 3.8 Hz, 1H), 4.34 (t, J = 5.2 Hz, 1H), 4.31–4.20 (m, 3H), 4.10 (s, 1H), 3.20 (q, J = 7.3 Hz, 3H), 2.01 (s, 8H), 1.37–1.24 (m, 9H).

^{13}C NMR (126 MHz, D_2O) δ = 179.15, 166.02, 154.09, 151.74, 150.90, 148.93, 141.51, 140.54, 102.58, 88.19, 86.98 (dd, J = 42.8, 3.3 Hz), 84.16 (ddd, J = 42.5, 38.3, 9.0 Hz), 83.36 (d, J = 9.0 Hz), 74.55

(dd, $J = 42.8, 37.7$ Hz), 73.84, 70.43 (td, $J = 38.1, 3.3$ Hz), 69.67, 65.34 (dd, $J = 42.6, 5.5$ Hz), 46.71, 8.28.

^{31}P NMR (202 MHz, D_2O) $\delta = -10.67$ (s, 2P), -22.43 (s, 2P).

HR-ESI-MS m/z calc. for $^{12}\text{C}_{14}^{13}\text{C}_5\text{H}_{26}\text{N}_7\text{O}_{21}\text{P}_4$ [M - H] 816.0470, found 816.0473.

4.5. Cell culture

HEK293T and H1299 cells were grown in 10 mL Dulbeccos Modified Eagle Medium (DMEM) containing 10 % fetal bovine serum (FBS) on 10 cm polystyrene cell dishes (Tissue culture (TC) dish 100, standard, Sarstedt). During the growing time the atmosphere contained permanent 5 % CO_2 at 37 °C.

Cells were split to ensure 90 % confluence for subsequent experiments. The exact number of cells seeded was counted using trypan blue staining and an automated cell counter (Countess™ Automated Cell Counter). For the intracellular nucleoside concentrations, only living cells were considered.

4.6. Cell stressing

Prior to harvesting, the cells were treated with 10 μL of a 40 mM solution of menadione in ethanol. This resulted in a menadione concentration of 40 μM in the cell dish (10 mL medium). The treatment was carried out for 1 h at 37 °C in a humidified atmosphere containing 5 % CO_2 . After the treatment, the cells were handled following the same protocol as the unstressed samples.

4.7. Cell harvesting and processing

When the cells reached a confluence of 90 % or after the stress treatments, the plate was placed directly on ice. The medium was removed and the cell dish was washed twice with 2 mL of ice-cold isotonic NaCl solution. Then 1 mL of -80 °C lysis solution (90 % aq. MeOH/ CHCl_3 9:1) was added to the dish and the solution was evenly distributed over the plate. The internal standard solution (2 μL of an 50 μM aqueous solution) was then added to the plate and evenly distributed. The plate was incubated for 15 min on ice. The lysed cells were then removed from the surface of the cell dish by thorough scraping with a cell lifter and the cell suspension was transferred to a 2 mL Eppendorf tube. The plate and cell lifter were washed thoroughly with 1 mL of the lysis solution and the suspension was added to the Eppendorf tube. The cell suspension was centrifuged at 15,000 rpm, 4 °C for 30 min (Heraeus Biofuge Primo R centrifuge) and the supernatant was transferred to a new 2 mL Eppendorf tube. The lysed cell pellet was resuspended and washed with 500 μL of lysis solution in an ultrasonic bath for 2 min. This suspension was centrifuged again (15'000 rpm, 4 °C, 30 min) and the supernatants were combined. The resulting clear solution was evaporated in a SpeedVac vacuum concentrator (ThermoFisher) and the colourless pellet was stored at -20 °C.

CRedit authorship contribution statement

Eirike R. Reinalter: Writing – original draft, Methodology, Investigation. **Jakob Arnold:** Writing – review & editing, Writing – original

draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Jakob Zwicker:** Investigation, Formal analysis. **Andreas Marx:** Writing – review & editing, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.carres.2025.109518>.

Data availability

Data will be made available on request.

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