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Inorganic tripolyphosphate (PPP_i) as a phosphate donor for human deoxyribonucleoside kinases☆

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Abstract

Inorganic tripolyphosphate (PPP_i) and pyrophosphate (PP_i) were examined as potential phosphate donors for human deoxynucleoside kinase (dCK), deoxyguanosine kinase (dGK), cytosolic thymidine kinase (TK1), mitochondrial TK2, and the deoxynucleoside kinase (dNK) from *Drosophila melanogaster*. PPP_i proved to be a good phosphate donor for dGK, as well as for dCK with dCyd, but not dAdo, as acceptor substrate, illustrating also the dependence of donor properties on acceptor. Products of phosphorylation were shown to be 5′-phosphates. In striking contrast to ATP, the phosphorylation reaction follows strict Michaelis–Menten kinetics, with K_m values of 74 and 92 μM for dCK and dGK, respectively, and V_{max} values 40–50% that for ATP. With the other three enzymes, as well as for dCK with dAdo as acceptor, no, or only low levels ($\leqslant$1% of that for ATP) of activity were observed. PP_i was inactive (<0.1%) as a phosphate donor with all enzymes, but was a competitive inhibitor vs ATP, as was PPP_i in systems with no or low donor activity. This is the first report on inorganic tripolyphosphate as a phosphate donor for nucleoside kinases, in particular human deoxyribonucleoside kinases.

Keywords: Deoxyribonucleoside kinases; Tripolyphosphate; Pyrophosphate; Phosphate donors; Phosphorylation

Nucleoside kinases catalyse the following reaction:

ATP + nucleoside → nucleoside-5′-phosphate + ADP

A notable feature of these reactions is that, in vitro, the phosphate donor ATP may frequently be replaced by other nucleoside-5′-triphosphates (NTPs). In a number of instances such NTPs have been shown to be even more effective than ATP, e.g., in the case of human deoxycytidine kinase (dCK), UTP is by far superior to ATP, and available evidence indicates that it should effectively compete with ATP in vivo ([1] and references cited). With the deoxyguanosine kinase (dGK) from *Bacillus subtilis*, UTP has also been reported as a 50-fold more effective donor than ATP in vitro [2]. A rather striking example is bovine mitochondrial dGK, for which ATP is the best donor at its optimal pH, 5.5, whereas at physiological pH its activity drastically decreases, while CTP and dCTP are as active as ATP, and UTP and dTTP twice as active [3].

Furthermore, most nucleoside kinases also display a very broad specificity as regards nucleoside acceptors, a fact widely profited from the development of antiviral and antitumour nucleoside analogues [4,5], the activities of which are dependent on their prior intracellular phosphorylation by some nucleoside kinase(s), e.g., the activity of the antiherpes agent Acyclovir is dependent on its phosphorylation by the viral thymidine kinase (TK), and the activity of Ganciclovir vs human cytomegalovirus (HCMV) is due to its intracellular phosphorylation by a kinase coded for by the product of the *UL97* gene of HCMV [5–7]. The very broad specificities

☆ *Abbreviations:* NTP, nucleoside 5′-triphosphate; PPP_i, tripolyphosphate; PP_i, pyrophosphate; polyP, polyphosphate; dNK, deoxyribonucleoside kinase from *Drosophila melanogaster*; dCK, deoxycytidine kinase; dGK, deoxyguanosine kinase; TK1, cytosolic thymidine kinase (low-affinity form); TK2, mitochondrial thymidine kinase.

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of most of these kinases for both donors and acceptors led us to consider whether the NTP phosphate donor must be a 5′-triphosphate. In this context, our attention was drawn to a long overlooked report of Kornberg and coworkers [8], who found that, although nucleoside-3′-triphosphates were not substrates for *Escherichia coli* DNA polymerase, they are effectively bound by the enzyme at the triphosphate binding site in the absence of a template, as is tripolyphosphate (PPP_i) itself, suggesting an overwhelming affinity for the triphosphate moiety. This prompted us to synthesize the 2′- and 3′-triphosphates of 3′-deoxyadenosine and 2-deoxyadenosine, which proved to be good ATP-competitive phosphate donors for human dCK [9] and, subsequently, for other human deoxynucleoside kinases [10]. In some instances these 2′- and 3′-triphosphates were even more effective donors than ATP.

The foregoing, in turn, led us to suspect that the triphosphate moiety of nucleoside triphosphate donors may be a more important factor for some nucleoside kinases than the nature of the base or nucleoside moiety. We have, in fact, found that inorganic tripolyphosphate (PPP_i) is a good ATP-competitive donor for two of the four known human deoxynucleoside kinases, dCK and dGK, the subject of the present communication.

Materials and methods

Materials. [5-^{3}H]2′-deoxycytidine (18.2 Ci/mmol) and [6-^{3}H]thymidine (30 Ci/mmol) were from Amersham (UK), and [2,8-^{3}H]2′-deoxyadenosine (44 Ci/mmol) and [8-^{3}H]2′-deoxyguanosine were from Moravek Biochemicals (USA). Tripolyphosphate (pentasodium salt, hexahydrate), pyrophosphate (tetrasodium salt, decahydrate), nonlabelled nucleosides, ATP, DTT, BSA, and Tris buffer were from Sigma (USA).

Enzymes. All deoxyribonucleoside kinases were recombinants and procedures for cloning and purification have been previously described, as follows: dCK [11], dGK [12,13], TK1 [14], TK2 [15], and dNK [16]. Their activities of the individual enzymes with standard substrates, determined with ATP as donor, are listed in a footnote to Table 1.

The preparation of dGK contains some ATP (for stability purposes). Since this may interfere with donor activity assays, dGK was additionally purified as described elsewhere [10], to reduce background activity to a level well below activities measured with PPP_i (Table 1).

TK1 may exist in so-called low-(dimer) and high-(tetramer) thymidine-affinity forms, the latter obtained by incubation of the concentrated enzyme with ATP [17,18]. Since testing donor properties of inorganic phosphates requires the absence of ATP, this study is limited to the low-affinity form of TK1.

Enzyme assays. Activities of all kinases were followed at pH 7.5 and 37 °C by a radiochemical assay with the use of ^{3}H-labelled nucleosides and DE-81 cellulose discs for product separation, as described elsewhere [9,10]. The activity of a given donor is expressed as the ratio of the velocity of the reaction to that with ATP. For control (background) activity, the reaction medium contained all components, except the phosphate donor, and, usually, with the exception of dGK (see above), showed no detectable activity.

The two enzymes which exhibit marked preference for one phosphate acceptor, TK1 and dGK, were tested with their preferred substrates, dThd and dGuo, respectively. dCK and TK2, with broader acceptor specificities, were tested with their major substrates, dCyd and dThd, respectively, and one additional acceptor, dAdo and dCyd, respectively, because the latter, with ATP as donor, are known to differ significantly in kinetic parameters from the major ones [19]. Since dNK exhibits the broadest acceptor specificity, and phosphorylates all four natural 2′-deoxynucleosides, it was tested with one pyrimidine (dCyd) and one purine nucleoside (dAdo) acceptor. Footnote c to Table 1 lists the actual activities of ATP with the different enzymes for the acceptors employed, each at a concentration exceeding its K_m value (see "Results").

Kinetic parameters. These were determined by the initial velocity method, where the concentration of $Mg^{2+} \cdot PPP_i$ was varied, and resulting velocities were fitted to the Michaelis–Menten $v = V_{max} \times [S]/(K_m + [S])$ or Hill $v = V_{max} \times [S]^h/(K_m^h + [S]^h)$ equations. Inhibition constants were determined by Dixon plots, using three or four concentrations of the substrate (ATP or PPP_i, usually in the range $K_m/2$ to $2K_m$), and four of the inhibitor (PPP_i or PP_i), where the median of the abscissa values of the intersection points of all fitted straight lines were taken as K_i. Concentrations of Mg^{2+} were constant and equimolar to the largest concentration of inhibitor employed. This procedure was preferred to varying the concentration of Mg^{2+} equimolarly with inhibitor, because in some cases an excess of Mg^{2+} with relatively low concentrations of ATP had a marked activating effect, which interfered with measurements of inhibition. This was particularly pronounced when the K_m of ATP (and thus the concentration of ATP used for inhibition measurements) was much lower than the concentration of inhibitor required for effective inhibition, e.g., for dNK ($K_m = 1.4\,\mu M$ for ATP) by both PP_i and PPP_i, which detectably inhibit in the range of several 100 μM (Fig. 1).

In some instances, only IC_{50} values were evaluated for inhibitory activities of PPP_i or PP_i with ATP as substrate and appropriate K_i

Table 1
Phosphate donor activities of inorganic tripolyphosphate (PPP_i) and pyrophosphate (PP_i) for deoxyribonucleoside kinases,[a] with their major phosphate acceptors at concentrations indicated[b]

	dCK		TK1	TK2		dNK		dGK
	dCyd (2.0 μM)	dAdo (50 μM)	dThd (30 μM)	dThd (2.5 μM)	dCyd (50 μM)	dCyd (10 μM)	dAdo (100 μM)	dGuo (10 μM)
ATP[c]	100	100	100	100	100	100	100	100
PPP_i	40	0.5	0	1.0	1.0	0	0	50
PP_i	0	0	0	0	0	0	0	0

[a] Shaded areas refer to activities tentatively classified as a group with relaxed donor specificity, and unshaded areas as a group with restricted donor specificity, as described previously [10]. Note the correlation between this classification and activities vs PPP_i.

[b] All activities measured at 1 mM concentration of donors, and expressed relative to that with ATP, taken as 100%. Zero denotes activity <0.1%.

[c] Activities of enzymes with ATP are as follows: dCK/dCyd, 7.0 nmol/min/mg; TK1/dThd, 615 nmol/min/mg; dCK/dAdo, 430 nmol/min/mg; dNK/dAdo, 3.1 μmol/min/mg; TK2/dThd, 180 nmol/min/mg; dGK/dGuo, 7.0 nmol/min/mg; TK2/dCyd, 540 nmol/min/mg; dNK/dCyd, 1.9 μmol/min/mg.

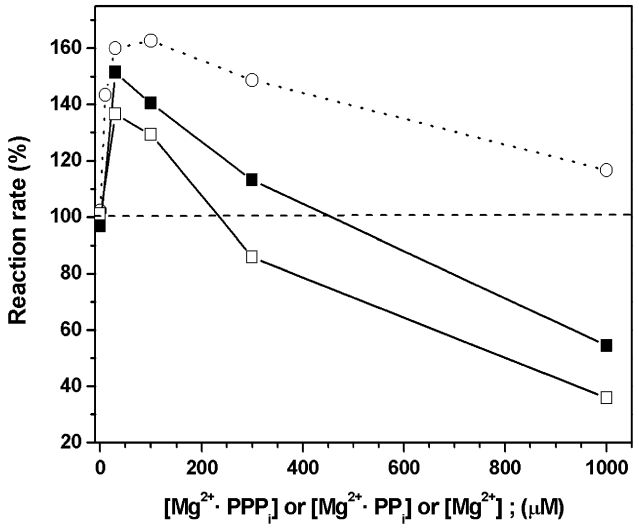

Fig. 1. Effect of increasing concentrations of $Mg^{2+} \cdot PPP_i$ (—■—) and $Mg^{2+} \cdot PP_i$ (—□—) on the rate of dCyd phosphorylation by dNK, with 3 μM $Mg^{2+} \cdot$ ATP as phosphate donor. The marked increase in rate in the 3–200 μM range, despite increasing concentrations of inhibitor, is due to the activating effect of magnesium ions, as shown by the effect of magnesium alone (···○···). The rate at 3 μM Mg^{2+} in the absence of inhibitor is taken as 100%. A similar effect was observed for TK2 with dThd (data not shown).

values were evaluated on the assumption that inhibition is competitive, i.e., $K_i = (K_m/[S] + K_m) \times IC_{50}$, where $[S]$ is the concentration and K_m is the Michaelis constant of the substrate with which the inhibitor competes [20].

Site(s) of phosphorylation were determined by treatment of the phosphorylated product with a rich source of 5′-nucleotidase (Russell's Viper venom), with no detectable activity vs nucleoside 2′(3′)-phosphates, as described elsewhere [10].

Results

The phosphate donor activities of PPP_i and PP_i with all five deoxyribonucleoside kinases are listed in Table 1. PPP_i proved to be a good donor for deoxyguanosine kinase (with dGuo as acceptor) as well as for deoxycytidine kinase with dCyd, but not dAdo, as acceptor. In both cases PPP_i exhibits very high activity, at levels of 50% and 40%, respectively, that for ATP. Products of phosphorylation were shown to be 5′-phosphates.

Kinetic measurements showed that PPP_i follows strict Michaelis–Menten kinetics, with K_m values of 74 μM for dCK and 92 μM for dGK (Table 2), in marked contrast to ATP, which exhibits negative cooperativity, and lower values of K_m, 7 μM for dCK (with dCyd) and 20 μM for dGK (with dGuo) [10]. Since all assays were performed at 1 mM concentration of donor (Table 1), which is ⩾10-fold higher than K_m values referred to above, donors were saturated in all cases, and initial rates of phosphorylation with PPP_i (Table 1), i.e., 40% for dCK with dCyd and 50% for dGK with dGuo, reflect, in fact, the V_{max} values for PPP_i relative to that for ATP. Consequently, with both enzymes, the V_{max}/K_m values for PPP_i are about an order of magnitude lower than that for ATP, due mainly to the higher K_m values for PPP_i.

With TK2, both pyrimidine substrates, the major one (dThd) and the second (dCyd), are phosphorylated at a very low level (~1%). Unexpectedly, in the case of dCK, replacement of dCyd by dAdo led to a decrease in donor activity of two orders of magnitude (~0.5%), pointing to interdependence between phosphate donor and acceptor, as also previously shown for ATP and its 2′- and 3′-triphosphate analogues [10]. Furthermore, with TK1 (dThd as acceptor) and dNK (dCyd and dAdo as acceptors) phosphorylating activity was below our limit of detection (<0.1%); but PPP_i behaved as an ATP-competitive inhibitor, with $K_i \sim 10$ μM for TK2 (dThd as acceptor) and ~140 μM for dNK (dCyd as acceptor) (Table 2).

The ability to utilize PPP_i as phosphate donor strictly correlates with a previous classification of the deoxyribonucleoside kinase activities into two groups with re-

Table 2
K_m values (μM)) for the phosphate donor PPP_i, and ATP for comparison, and inhibitory constants K_i (μM) for PP_i and PPP_i, with dCK (25 μM dCyd as acceptor), dCK (50 μM dAdo as acceptor), TK2 (1 μM Thd as acceptor), dNK (10 μM dCyd as acceptor), and dGK (20 μM dGuo as acceptor)

Donor	Inhibitor	dCK/dCyd		dCK/dAdo		TK2/dThyd		dNK/dCyd		dGK/dGuo	
		K_m	K_i	K_m	K_i	K_m	K_i	K_m	K_i	K_m	K_i
ATP	—	7(1)[a]		60(7)[a]		2[b]		1.4[c]		20(3)[a]	
PPP_i	—	74(5)		[d]		[d]		[e]		92(10)	
ATP	PPP_i				n.d.		10(2)		143[f]		
ATP	PP_i		310(20)		80[g]		1.4(3)		80[f]		n.d.
PPP_i	PP_i		310(50)		n.d.		n.d		n.d		n.d

Standard deviations (in brackets) refer to the last digits. n.d., Not determined.

[a] Data from [10].

[b] Data from [37].

[c] Data from [38].

[d] Substrate activity too weak (Table 1) to determine K_m value.

[e] Inactive as a substrate.

[f] Calculated from IC_{50} values (450 and 250 μM for PPP_i and PP_i, respectively), obtained for reaction with 3 μM ATP, assuming that inhibition is competitive.

[g] Calculated from IC_{50} value (100 μM), obtained for reaction with 2 μM ATP, assuming that inhibition is competitive.

laxed and restricted donor specificities, based on their activities with the 2′- and 3′-triphosphates of adenosine, and some sugar-fluorinated analogues [10]. The enzymes from the group with relaxed donor specificity (dGK, and dCK only with dCyd as acceptor, denoted by shaded areas in Table 1) efficiently utilize PPP_i as phosphate donor, while those belonging to the group with restricted donor specificity (TK1, TK2, dNK, and dCK with dAdo as acceptor, unshaded areas in Table 1) do so only minimally or below our limit of detection.

PP_i did not exhibit detectable phosphate donor activity with any of the enzymes studied here (Table 1), but is an ATP-competitive inhibitor with K_i values in the range 1.5–300 μM (Table 2). With TK2 and ATP as donors, PPP_i and PP_i are potent ATP-competitive inhibitors, with K_i values of 10 and 1.4 μM, respectively. Since PP_i should be a product released during phosphorylation with PPP_i, its inhibitory properties are especially interesting in the reaction with PPP_i as donor, e.g., dCK with dCyd as acceptor. The K_i for PP_i in this instance is ~300 μM; hence, bearing in mind that typical concentrations of the acceptor do not exceed 50 μM, feedback inhibition should be negligible.

Discussion

There is presently mounting evidence for key roles of inorganic polyphosphates, ranging from PP_i, through PPP_i to longer phosphate chains (PolyP), both in microorganisms and higher organisms, leading to the proposal that, because of their high energy and phosphate content, they may be plausible precursors to RNA, DNA, and proteins [21]. PP_i-dependent phosphofructokinases [22] have long been known. Other recent examples include polyphosphate kinase, which has been shown to act as a nucleoside diphosphate kinase, using polyP as phosphate donor in place of ATP [23]. PPP_i has been shown to be a weak phosphate donor for adenylate kinase [24]. NAD^+ kinases from actinomycetes exhibit $NADP^+$-synthesizing activities equally well when ATP is replaced by PPP_i [25].

In the realm of protein kinases, ATP-competitive PP_i-dependent phosphorylation of spinach thylakoid proteins has been reported by Pramanik et al. [26], while Mijakovic et al. [27] found that the *B. subtilis* HPr kinase/phosphorylase (HPrK/P), a bifunctional sensor enzyme for catabolite repression, is capable of utilizing PP_i, as well as PPP_i, for phosphorylation of the HPr protein.

Insofar as we are aware, this is the first demonstration of the existence of PPP_i-dependent nucleoside kinases, in this instance human deoxyribonucleoside kinases. This aspect has hitherto been overlooked, largely because of emphasis on nucleoside, and nucleoside analogue, acceptors, many of which are potential antitumour and antiviral agents [5,28].

The existence of PP_i-dependent phosphorylation of purine *ribo*nucleosides in extracts of *Acholeplasma laidlawii* was long ago demonstrated by Tryon and Pollack [29,30], followed by the finding that some *Acholeplasma* species contain PP_i-dependent purine deoxynucleoside kinase activity [31]. It should be noted, in this context, that human dCK readily phosphorylates ribocytidine [32] and *Drosophila* dNK phosphorylates ribonucleosides [33].

The presence of PP_i-dependent deoxyribonucleoside, but not thymidine, kinase activities in extracts of *Acholeplasma laidlawii* has been confirmed by Wang et al. [34], who simultaneously demonstrated the existence in this, and related organisms, of all four natural ATP-dependent deoxyribonucleoside kinases. Furthermore, one of the latter enzymes from a strain of *Mycoplasma mucoides*, coded for by an ORF for a dGK-like enzyme, was cloned, purified to homogeneity, and shown to be devoid of PP_i-dependent activity. It therefore appears that the as yet unidentified PP_i-dependent (deoxy)ribonucleoside kinase activities in the extracts of *M. mucoides* do not reside on the ATP-dependent enzymes. It would have been instructive if the authors had tested for possible competition between PP_i and ATP.

The crystal structures of the complexes of dNK with dCyd, and of dGK with ATP, have been recently reported [35]. Note that each of these enzymes is classified in a different group as regards donor specificity (Table 1, footnote a). In the case of dNK, dCyd is located, as expected, in the acceptor site. In striking contrast, in the absence of a competing deoxyribonucleoside acceptor, dGK was found to bind ATP in a direction opposite to that anticipated for a donor; so that, whereas the ATP phosphate groups are correctly positioned in the phosphate donor site, the location of its adenosine moiety is similar to that of the dCyd acceptor in dNK and close to the way the substrate dAdo would be expected to bind.

Our data show that interaction of the base moieties of 5′-NTPs with dCK and dGK is not essential for donor activity and the phosphate transfer reaction. This is in line with previous observations [19] showing that, for human deoxyribonucleoside kinases, all 5′-NTPs, excluding feedback inhibitors, e.g., dCTP vs dCK and dGTP vs dGK, may serve as donors. This prompted us to compare the appropriate P-loop sequences, which may possibly reflect the differences in donor specificity between these enzymes vs PPP_i. Such a comparison (Fig. 2) shows that the enzymes with relaxed donor specificity (dCK and dGK), and those with restricted donor specificity (TK1, TK2, and dNK), differ from each other by replacement of several amino acid residues. Ser42 in dGK (Ser25 in dCK) is replaced by Cys21 (TK2), Leu24 (dNK) or Val23 (TK1); Val49 (dGK) and Ala32 (dCK) by Ser28 (TK2), Ser31 (dNK) or Ser30 (TK1); Phe54 (dGK) and Phe37 (dCK) by Cys33 (TK2), Tyr36 (dNK) or Glu35 (TK1); and Val55 (dGK) and

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dGK   (40) RLSIEGNIAVGKSTFV
dCK   (23) KISIEGNIAAGKSTFV
TK2   (19) VICVEGNIASGKTTCL
dNK   (22) TVLIEGNIGSGKTTYL
TK1   (21) IQVILGPMFSGKSTEL
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Fig. 2. Structure-based multiple alignment of amino acid sequences of the P-loop regions of human dGK (SwissProt Q16854), dCK (P27707), TK2 (O00142), and TK1 (P04183), and *Drosophila melanogaster* dNK (EMBL Y18048). The numbers in brackets refer to the first amino acid residue at the start of each line. The sequences of dGK and dCK (~45% identity) classified as a group with relaxed phosphate donor specificity (see text) are essentially the same in this region. Bold letters represent residues identical in most of the sequences and shaded ones residues identical (or very similar) only in dGK and dCK.

Val38 (dCK) by Leu34 (TK2), Leu37 (dNK) or Leu36 (TK1). Their importance may be evaluated by site-directed mutagenesis and/or molecular modelling studies.

Intriguing is the interdependence between phosphate acceptor and donor, which, in the case of dCK, led to a dramatic decrease in donor activity of PPP_i on replacement of dCyd by dAdo, as with the 2′- and 3′-triphosphate analogues of ATP [10]. It appears unlikely that this is due to steric hindrance, resulting from the difference in size of the base moieties of pyrimidine and purine nucleosides. Crystallographic structures of dCK–PPP_i and/or dNK–PPP_i complexes would clearly be helpful to interpret the effect of the acceptor on phosphate donor properties. Molecular modelling studies on simultaneous binding of phosphate donor and acceptor are presently being attempted. The same strategy may be useful in the case of other nucleoside kinases, as well as other kinases, e.g., protein kinases.

The efficiency with which PPP_i replaces ATP as phosphate donor for human dCK and dGK raises the question as to which was the original donor in primitive evolution. Relevant to this is a proposal of Sanders et al. [24], who found that $Mg^{2+} \cdot PPP_i$ is a very weak donor for adenylate kinase, 10^{-4}–10^{-5} that of $Mg^{2+} \cdot ATP$. Combining 5′-deoxyadenosine (which cannot be phosphorylated) with $Mg^{2+} \cdot PPP_i$ indicated that optimal catalysis requires the adenosine moiety to be linked to the triphosphate. Thus, the adenine ring of ATP plays the role, mediated through the linking ribose, of an "anchor" for one end of the triphosphate chain. Together with the finding that there is a modest increase in expression of the binding energy for the adenine ring of ATP as it passes through the transition state, the adenine ring would provide specificity for the transition state. With this scenario, it is conceivable that the primitive donor for human deoxyribonucleoside kinases was PPP_i and that donor specificity evolved by its conversion to 5′-NTPs. But this does not explain why the 2′(3′)-triphosphates of 3′(2′)-deoxyadenosines are excellent donors for human dCK and dGK [9,10].

Kumble and Kornberg [36] have adduced compelling evidence for the ubiquity of polyphosphates, including tripolyphosphates, in mammalian cells, and for their involvement in a variety of as yet undefined functions. However, bearing in mind the relatively high intracellular concentrations of 5′-NTPs, especially ATP (~5 mM), hence at least two orders of magnitude higher than those of polyP [36], it is problematic whether PPP_i can effectively compete in vivo with ATP and other NTPs as a donor for nucleoside kinases.

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